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(54) **Title**: MODULATION OF LIPID HOMEOSTATIS, METHODS AND COMPOSITIONS RELATED THERETO

(57) **Abstract**: Methods and compositions for the modulation of lipid homeostasis and/or the treatment of metabolic diseases is provided herein. More particularly, methods and compositions for the modulation of histone deacetylases, such as Class IIa histone deacetylases is provided herein.

Modulation of Lipid Homeostasis, Methods and Compositions Related Thereto

RELATED APPLICATION

This application claims priority from U.S. Provisional Application Serial No. 61/485,506 filed May 12, 2011, which application is herein incorporated by reference.

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with the assistance of government support under United States Grant Nos. R01 DK049777 and R01 DK083834 from the National Institutes of Health. The government may have certain rights in the invention.

FIELD OF THE INVENTION

The invention relates generally to methods and compositions for the modulation of lipid homeostasis and/or the treatment of metabolic diseases. More particularly, the invention relates to methods and compositions for the modulation of histone deacetylases, such as Class IIa histone deacetylases.

BACKGROUND OF THE INVENTION

Obesity is a major risk factor in the development of insulin resistance, which is characterized by an inability for insulin to promote glucose uptake into muscle and to inhibit glucose production by the liver. Obesity-dependent increases in circulating free fatty acids have been associated with ectopic deposition of lipid in liver and muscle, where they interfere with insulin signaling (Kim et al., 2004).

Because of its short life cycle and ease of genetic manipulation, Drosophila has emerged as an important model organism for the study of obesity and diabetes (Baker and Thummel, 2007). A considerable percentage of all fly genes have clear mammalian orthologs, and over 75% of known human disease genes have functional orthologs in flies (Reiter and Bier, 2002). Indeed virtually all of the known components of the insulin signaling pathway are also present in the fly.

Glucose and lipid homeostasis in Drosophila is maintained by a group of neurosecretory cells in the brain that produce insulin-like peptides (Ikeya et al., 2002). Fasting metabolism is coordinated by a distinct group of cells in the ring gland that elaborate adipokinetic hormone (AKH) (Kim and Rulifson, 2004). Both hormones maintain energy balance through their actions on the fat body, the fly counterpart of mammalian liver and adipose tissue. Disruption of insulin-producing cells in Drosophila leads to increases in circulating glucose levels, mimicking certain features of type II diabetes.

Insulin has been shown to regulate glucose and lipid metabolism by triggering a cascade of lipid kinases that culminate in the activation of the Ser/Thr kinase AKT (Brazil and Hemmings, 2001). In turn, AKT regulates the expression of metabolic programs in part through the phosphorylation and cytoplasmic sequestration of the forkhead transcription factor FOXO (Barthel et al., 2005).

Superimposed on effects of AKT, FOXO activity is also inhibited through acetylation by the histone acetyl transferase paralogs P300 and CBP (Fukuoka et al., 2003; Matsuzaki et al., 2005). Acetylation has been shown to disrupt FOXO activity by reducing its DNA binding affinity, leading to increases in AKT-mediated phosphorylation (Matsuzaki et al., 2005). Conversely, FOXO is activated in part through deacetylation by the NAD+ dependent deacetylase SIRT1 (Brunet et al., 2004; Daitoku et al., 2004; Frescas et al., 2005) in response to nutrient deprivation, although the regulatory effects of feeding and fasting hormones on SIRT1 or other deacetylases have not been addressed.

SUMMARY OF THE INVENTION

It is demonstrated herein that Drosophila SIK3, a member of the AMPK family of Ser/Thr kinases, plays a role in energy balance. It was determined that SIK3 is activated by AKT during feeding, and that upon activation it promotes lipid storage by blocking FOXO activity. It is further shown that SIK3 regulates FOXO activity by modulating its deacetylation by a class IIa HDAC. Taken together, these studies show that SIK3 links insulin signaling to FOXO-dependent changes in triglyceride storage.

In certain embodiments, the instant invention provides a method of treating obesity, comprising administering to a subject in need thereof an agonist of a Class IIa histone deacetylase (HDAC). Examples of Class Ila HDAC include HDAC 4, 5, 7, and 9.

In some embodiments, the Class IIa HDAC agonist enhances the activity of at least one Class IIa HDAC selected from the group consisting of HDAC 4, 5, 7, and 9. In a particular embodiment, the Class IIa HDAC agonist enhances the activity of HDAC 4.

In certain embodiments, the Class Ila HDAC agonist lowers lipid levels in the subject. In other embodiments, the Class IIa HDAC agonist results in an increase in lipolysis in the subject. In yet other embodiments, administration of the Class IIa HDAC agonist results in a reduction of one or more triglyceride stores in the subject.

In some embodiments, the Class IIa HDAC agonist enhances expression of a Class IIa HDAC. In other embodiments, the Class IIa HDAC agonist enhances nuclear localization of a Class IIa HDAC. In yet other embodiments, the Class IIa HDAC agonist enhances dephosphorylation of a Class IIa HDAC. In some embodiments, the Class IIa HDAC agonist enhances Class IIa HDAC mediated deacetylation of a

transcription factor. In certain embodiments, the transcription factor is a FOXO transcription factor.

In some embodiments, the Class IIa agonist enhances the interaction of one or more Class IIa HDACs with an interacting protein. Examples of Class IIa HDAC interacting proteins include HDAC3, SMRTER, EBI, and/or a homolog thereof.

In some embodiments, the instant invention relates to a method of screening for a compound for treating obesity, comprising expressing a Class IIa HDAC in the fat body of a fly, wherein the fly is *Drosophila,* in the absence and presence of a test compound, and evaluating the activity of the Class IIa HDAC in the absence and presence of the test compound.

In certain embodiments, the activity of the Class IIa HDAC is selected from the group consisting of: expression, localization, phosphorylation state, and FOXO transcription factor deacetylation. In further embodiments, a compound for treating obesity is identified where the compound increases the activity of the Class IIa HDAC compared to the activity of the Class IIa HDAC in the absence of the compound. In some embodiments, the compound lowers lipid levels in the subject. In certain embodiments, the compound increases lipolysis. In some embodiments, the compound decreases one or more triglyceride stores.

In yet other embodiments, the instant invention relates to a method of treating a metabolic disease, comprising administering to a subject in need thereof a modulator of a Class IIa histone deacetylase (HDAC), wherein the modulator modulates the activity of a Class IIa HDAC interacting protein. In certain embodiments, the Class IIa HDAC interacting protein is SIK3 or a homolog thereof.

In some embodiments, the Class IIa HDAC modulator modulates the activity of HDAC 4. In some embodiments, the Class IIa HDAC modulator is an inhibitor of SIK3 or a homolog thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Reduced fat stores in SIK3 mutant flies, also see Figure 8.

(A) Diagram ofSIK3 (CG15072) genomic locus is depicted. Location of P element EY06260 shown, along with SIK348 and SIK372 deletions generated by imprecise excision. SIK3+51 is a precise excision control line. SIK348 lacks the first (non-coding) exon, resulting in a significant reduction of SIK3 mRNA. The SIK3 72 deletion extends through the first two exons, removing sequences that encode part of the SIK3 kinase domain, and acts as a null allele.

(B) Q-PCR analysis ofSIK3 mRNA levels in SIK3+ (SIK3+51) and SIK3^{-} (SIK348) adult male and female flies under feeding conditions.

(C) Immunohistochemical analysis of SIK3 expression in fat bodies of WT (yw) and SIK3^{-} (yw;SIK348/Df(2R)P34) L3 larvae. SIK3 protein shown in green, DNA shown in red. Scale bar; 10 μm.

(D, E) Relative fasting (24 hours) or ad libitum feeding lipid levels (D) and starvation sensitivities (E) of SIK3+ (SIK3+51) and SIK3^{-} (SIK348) flies. (n=10 per genotype for lipid measurement and n=50 for starvation assays).

(F) Effect of fat body (FB) specific expression of SIK3 on lipid levels in SIK3^{-} flies. FB>(FB-GAL4/+), SIK3^{-},FB>(FB-GAL4,SIK348/SIK348), SIK3^{-}, FB>SIK3 (FB-GAL4, SIK348 / SIK348;UAS-SIK3/+),SIK3^{-},UAS-SIK3(SIK348,UAS-SIK3/+) and FB>SIK3 (FB-GAL4/+; UAS-SIK3/+) flies are shown. (n=16 per genotype).

(G) Fat body specific expression of SIK3 rescues starvation sensitivity in SIK3- flies. Shown are survival curves for SIK3^{-}, FB>SIK3 (FB-GAL4, SIK348 / SIK348;UAS-SIK3/+), SIK3^{-}, UAS-SIK3 (SIK348, UAS-SIK3/+) and SIK3^{-}, FB> (FB-GAL4, SIK348/ SIK348) flies. (n=97-127 per genotype).

(H) Lipid levels in SIK3 mutant flies with fat body specific expression of wild-type or catalytically inactive (K70M) SIK3, SIK2, and AMPK. Genotypes are: SIK3^{-}, FB> (FB-GAL4, SIK348 / SIK348), SIK3+, FB> (FB-GAL4 /+), SIK3^{-}, FB>SIK3.WT (FB-GAL4, SIK348 / SIK348;UAS-SIK3/+), SIK3+, FB>SIK3.WT (FB-GAL4 /+;UAS-SIK3/+), SIK3^{-}, FB>SIK3.K70M (FB-GAL4, SIK348 / SIK348; UAS-SIK3.K70M/+), SIK3+, FB>SIK3.K70M (FB-GAL4 /+;UAS-SIK3.K70M/+), SIK3^{-}, FB>SIK2 (FB-GAL4, SIK348 / SIK348;UAS-SIK2/+), SIK3+, FB>SIK2 (FB-GAL4/+; UAS-SIK2/+), SIK3^{-}, FB>AMPK (FB-GAL4, SIK348 / SIK348;UAS-AMPKTD/+) and SIK3+, FB>AMPK (FB-GAL4 /+;UAS-AMPKTD/+). (n=12 per genotype). Data are averages ± SEM (**, P<0.01; *, P<0.05).

Figure 2: AKT stimulates SIK3 activity during feeding, also see Figure 9.

(A) Effect of fat-body specific AKT expression on lipid levels in wild-type and SIK3^{-} flies. Genotypes are: FB> (FB-GAL4/+), FB>AKT (FB-GAL4/UAS-AKT), SIK3^{-}, FB> (FB-GAL4, SIK348/ SIK348) and SIK3^{-}, FB>AKT (FB-GAL4,SIK348/UAS-AKT, SIK348). (n=16 per genotype).

(B) SIK3 catalytic activity in 24 hour fasted versus 0.5 hour refed flies, as determined by relative SIK3 autophosphorylation in fat bodies of flies (ppl-GAL4/UAS-SIK3.WT) expressing HA-tagged SIK3. Incorporation of γ32P-labeled ATP by in-vitro kinase assay of anti-HA immunoprecipitates shown. Immunoblot of total SIK3 protein amounts also indicated.

(C) Immunoblot showing relative amounts of phosphorylated SIK3 in fat bodies of wild-type and Chico mutant flies. Phospho-SIK3 levels determined using phospho-AKT substrate antibody (PAS). Total SIK3 levels measured with SIK3 antisera on HA-

immunoprecipitates prepared from CON (ppl-GAL4,UAS-SIK31+) and Chico (chico1; ppl-GAL4,UAS-SIK3/+) flies under 24 hour fasted or 0.5 hour refed conditions.

(D) In vitro kinase assays showing effect of recombinant AKT on phosphorylation of catalytically inactive (K70M) SIK3. Effect of mutating four putative AKT phosphorylation sites (T281/486A.S293/401A; 4A) in SIK3 on 32P incorporation shown. Total protein amounts for wild-type and 4A mutant SIK3 proteins indicated by Coomassie staining.

(E) Immunoblot showing effect of Ala substitutions at putative AKT phosphorylation sites in SIK3 (4A) on SIK3 phosphorylation in Drosophila S2 cells. Immunoblot with PAS antiserum was performed on HA-immunoprecipitates prepared from transfected cells. Exposure to insulin (0.5 hr) indicated. Total amounts of SIK3 shown. Relative amounts of phospho-SIK3, determined by densitometric analysis, indicated below each lane.

(F) Lipid levels in SIK3 mutant flies following fat body specific expression of wild-type or AKT phosphorylation-defective (4A) SIK3. Genotypes are: FB> (FB-GAL4/+), $SIK3^{-}$, FB> (FB-GAL4, SIK348/ SIK348), $SIK3^{-}$, FB>SIK3.WT (FB-GAL4, SIK348 / SIK348;UAS-SIK3/+) and $SIK3^{-}$, FB>SIK3.4A (FB-GAL4, SIK348 / SIK348;UAS-SIK3.4A/+). (n=16 per genotype). Data are averages ± SEM (**, $P<0.01$; *, $P<0.05$).

Figure 3: SIK3 blocks brummer lipase-dependent lipolysis, see also figure 10.

(A) Relative effect of AKH receptor (AKHR) and brummer (bmm) deficiency on lipid levels in wild-type and SIK3 mutant flies. Genotypes are: SIK3+,w^{-} (w), SIK3-,w^{-} (SIK348), SIK3+, $AKHR^{-}$ (AKHR1), $SIK3^{-}$, $AKHR^{-}$ (AKHR1, SIK348), SIK3+,bmm^{-} (bmm1), $SIK3^{-}$,bmm- (SIK348;bmm1). (n=16 per genotype).

(B) Q-PCR analysis of bmm mRNA amounts in wild-type and SIK3 mutant flies under feeding conditions. Effect of expressing wild-type or kinase-dead (K70M) SIK3 in fat body on bmm mRNA amounts indicated. Genotypes are: SIK3+ (FB-GAL4/+), $SIK3^{-}$: (FB-GAL4,SIK348/ SIK348), SIK3+, FB>SIK3.WT (FB-GAL4 /+;UAS-SIK3/+), $SIK3^{-}$, FB>SIK3.WT (FB-GAL4 ,SIK348/ SIK348;UAS-SIK3/+), SIK3+, FB>SIK3.K70M (FB-GAL4/+;UAS-SIK3. K70M/+), $SIK3^{-}$, FB>SIK3.K70M (FB-GAL4 ,SIK348/ SIK348;UAS-SIK3.K70M/+) flies. Data are averages ± SEM (**, $P<0.01$).

(C-E) Time course analysis of lipid depletion (C, n=14-20 per genotype), catabolic gene expression (D), and FOXO as well as AKT and SIK3 dephosphorylation (E) during fasting in adult wild-type flies. For panel E, AKT phosphorylated FOXO migrates slower than the unphosphorylated protein (see Figure 11D). Densitometric analysis ofphospho-SIK3 amounts from two independent experiments shown.

(F) Q-PCR analysis of mRNA amounts for bmm and other fasting-inducible genes (PEPCK, lip3 and 4E-BP) in wild-type (w) and FOXO (FOXO21/FOXO25) mutant flies under fasting (24 hour) or ad libitum feeding conditions.

Figure 4: SIK3 inhibits FOXO activity, also see Figure 11.

(A-B) Immunohistochemical analysis and quantification of FOXO localization in SIK3+ (SIK3+51/ Df(2R)P34) and SIK3^{-} (SIK348/Df(2R)P34) L3 larvae. Arrows point to nuclear-localized FOXO protein. Scale bar; 10 μm. (B) Percentage of cells with nuclear FOXO staining in (A) shown graphically. At least 400 cells were counted for each genotype.

(C,D) Effect of SIK3 depletion (C) or fat body-specific over-expression (D) on FOXO phosphorylation in flies. Genotypes are: w^{-} (w), SIK3^{-} (SIK348), FOXO^{-} (FOXO21/FOXO25), Con (r4-GAL4,UAS-HA-FOXO/+), and SIK3 over-expression (O/E) (r4-GAL4,UAS-HA-FOXO/UAS-SIK3). In panel C, flies were fasted for 24 hours (0) and then refed for 0.5, 1, or 5 hours. In panel D, flies were fasted for 24 hours and refed for 0.5 hours.

(E, F) Effect of FOXO gene disruption on brummer mRNA amounts (E) and lipid levels (F) in SIK3 mutant flies. Genotypes are: SIK3+,w^{-} (w), SIK3^{-},w^{-} (SIK348), SIK3+,FOXO^{-}(FOXO21/FOXO25), and SIK3^{-}, FOXO^{-} (SIK348; FOXO21/FOXO25). (n=24 per genotype). Data are averages ± SEM (**, P<0.01).

Figure 5: SIK3 phosphorylates HDAC4 and promotes its cytoplasmic translocation, also see Figure 12.

(A) Alignment of SIK consensus phosphorylation motif with conserved phosphoacceptor sites in HDAC4 and in mammalian class IIa HDACs (Hdac4/5/7). (SEQ ID NOs:51-55)

(B) Autoradiogram showing relative phosphorylation of wild-type (HDAC4.WT) and phosphorylation defective (HDAC4.3A) HDAC4 polypeptides by SIK3 in in vitro kinase assays using 32P-labeled ATP. Lower panel shows total protein amounts for HDAC4.WT and HDAC4.3A by Coomassie staining.

(C) Immunoblot showing effect of wild-type and PKA phosphorylation defective SIK3 (S563A) on amounts of phospho (Ser239) and total HDAC4 in transfected HepG2 cells. Effect of cAMP agonists (0.5 mM IBMX, 0.5 mM 8-Br-cAMP; 4 hours) on phospho (Ser239) HDAC4 amounts shown. Bottom, diagram of SIK3 showing location of inhibitory PKA phosphorylation site relative to catalytic and ubiquitin binding (UBA) domains indicated. Below, alignment of PKA phosphorylation sites in Drosophila SIK3 and mouse SIK2. (SEQ ID NOs:56-57)

(D) Immunohistochemical analysis of FLAG-HDAC4 localization in fed control (r4-GAL4/+), and ad libitum fed or 10 hour fasted HDAC4-over-expressing (r4-

GAL4/UAS-HDAC4) L3 larvae. HDAC4 protein shown in green, DNA shown in red. Scale bar; 10 μm.

Figure 6: HDAC4 activates FOXO, also see Figure 13.

(A) Immunoblot of FOXO recovered from immunoprecipitates of HDAC4 or control IgG prepared from Drosophila S2 cells. Input amounts of transfected HDAC4 and FOXO shown.

(B) Q-PCR analysis of mRNA amounts for fasting inducible genes (PEPCK, bmm, CPTI) in HDAC4 hypomorphic mutant flies (top) or in flies expressing phosphorylation-defective, constitutively active HDAC4.3A in fat-body (bottom). Genotypes are: FB>(FB-GAL4/+) and FB>HDAC4.3A (FB-GAL4/+;UAS-HDAC4.3A/+) for over-expression assays. For HDAC4 hypomorphic mutant flies, genotypes are HDAC4+ (HDAC4KG09091/+) and HDAC4^{-} (HDAC4KG09091/HDAC4e04575). Effects of HDAC4^{-} (top) examined in 24 hour fasted flies. Effects of HDAC4.3A examined under ad libitum feeding conditions.

(C) Immunoblot showing time course of FOXO phosphorylation and acetylation during refeeding in wild-type (w), SIK3 mutant (SIK348). Ac-FOXO protein detected using anti ac-FOXO1 (K242/245) antibody after immunoprecipitation with anti- acetyl-lysine antibody. Extract from FOXO mutant (FOXO21/FOXO25) flies (FOXO-) included to show specificity of ac-FOXO1 and non-discriminating FOXO antisera.

(D) Top: schematic showing domain organization of FOXO, including DNA-binding domain (DBD), nuclear localization sequence (NLS), nuclear export sequence (NES), and trans-activation domain (TA). Middle: alignment of conserved lysine residues in the DBDs of Drosophila FOXO and human FOXO1. Conserved lysine residues that undergo acetylation (Lys 179,182 in Drosophila; Lys 242, 245 in human) shown. Bottom, Immunoblot showing Ac-FOXO, and unphosphorylated (FOXO) or phospho-FOXO (pFOXO) protein amounts in 24 hour fasted or 1 hour refed wild-type (HDAC4+) and HDAC4-mutant (HDAC4-) flies. Relative amounts of acetylated FOXO, determined by densitometric analysis, indicated above each lane. (SEQ ID NOs:58-59)

Genotypes are: HDAC4+ (HDAC4KG09091/+) and HDAC4^{-} (HDAC4KG09091/HDAC4e04575).

(E) In vitro deacetylation assay showing effect of purified HDAC4 on deacetylation of a GST-FOXO (aa.95-195) polypeptide acetylated in vitro with the histone acetyl transferase P300. Immunoblot shows amounts of acetylated FOXO (Ac-FOXO) using anti-acetyl lysine antibody. Input protein amounts for GST-FOXO (aa. 95-195) and HDAC4 proteins determined by Ponceau S staining.

(F) Effect of oral supplementation with class I/II HDAC inhibitor trichostatin A (TSA) in the food (100μM) on fat body lipid levels in SIK3 mutant flies. (n=10 per genotype). Genotypes are: w^- (w) and $SIK3^-$ (SIK348). Data are averages ± SEM (**, P<0.01; *, P<0.05).

Figure 7: SIK3 inhibits FOXO activity via an HDAC4-dependent mechanism.

(A-C) Effect of HDAC4 gene disruption on lipid levels (A) bmm mRNA amounts (B), and FOXO phosphorylation (C) in SIK3 mutant relative to control flies. Data panels A and B is from ad libitum fed flies. Data in panel C was from 1 hour refed flies. Genotypes are: SIK3+(w), $SIK3^-$ (SIK348), SIK3+, $HDAC4^-$ (HDAC4KG09091/HDAC4e04575), and $SIK3^-$, $HDAC4^-$ (HDAC4KG09091/HDAC4e04575; SIK348), $FOXO^-$(FOXO21/FOXO25). (n=24 per genotype).

(D, E) Effect of fat body-specific depletion of HDAC4 (HDAC4i) on lipid levels (D) and bmm mRNA amounts (E) in SIK3 mutant relative to control flies under feeding conditions. Genotypes are: SIK3+,FB>(FB-GAL4/+), SIK3-,FB> (FB-GAL4,SIK348/ SIK348), SIK3+,FB>HDAC4i(FB-GAL4/+;UAS-HDAC4 RNAi/+), and $SIK3^-$, FB>HDAC4i (FB-GAL4, SIK348/ SIK348; UAS-HDAC4 RNAi/+). (n=14-16 per genotype).

(F) Immunoblot showing amounts of phosphorylated mouse HDAC4 in extracts from primary hepatocytes following RNAi-mediated depletion of SIK2. Effect of exposure to insulin (l00nM) for different times (in minutes) indicated.

(G) Immunoblot showing effect of glucagon (100nM) on HDAC4 phosphorylation at Ser245 and on inhibitory phosphorylation of SIK2 at Ser587 in primary mouse hepatocytes.

(H) Q-PCR analysis of PEPCK, Glucose 6 phosphatase, and NR4A2 mRNA amounts in primary hepatocytes following exposure to glucagon (100nM) for 1 hour. Effect of adenovirus encoded HDAC4/5 RNAis shown.

Figure 8: SIK3 mutant flies phenotypes, related to Figure 1.

(A) Survival rate of control (SIK372/Cyo,ubi-GFP) and SIK3 null (SIK372) during development.

(B) Representative photos of control (upper panel) and SIK372 (lower panel) larvae at 72 hours after egg laying (AEL).

(C) Image of fat-body rescue fly (FB-GAL4,SIK372 /Df(2R)P34;UAS-SIK3/+).

(D,E) Lipid levels of male (D) and female (E) SIK3 heterozygotes and homozygotes relative to wild-type flies. (n= 16 per genotype).

(F,G) Paraquat sensitivities of SIK3+ (SIK3+511 Df(2R)P34) and $SIK3^-$ (SIK348/Df(2R)P34) male (F) and female (G) flies shown. (n=100 per genotype).

Median survival for males (F) was 141h (SIK3+) vs. 117h (SIK3^{-}). Logrank test: P< 0.0001. Median survival for females (G) was 165h (SIK3+) vs. 141 h (SIK3^{-}). Logrank test: P< 0.0001. Data for panels A-E are means + SEM (*, P<0.05).

Figure 9: Insulin signaling activates SIK3 through AKT-mediated phosphorylation, related to Figure 2.

(A) Immunoblot showing amounts of phospho-SIK3 in S2 cells following dsRNA-mediated depletion of AKT. Amounts of phosphorylated SIK3 evaluated with phospho-AKT substrate (PAS) antiserum.

(B) Lipid levels in SIK3 hypomorphic mutant flies that are heterozygous for chico relative to single SIK3 mutant and chico heterozygote. Genotypes are: WT (w), Chico het (chico1/+), SIK3- (SIK348), and Chico het, SIK3- (chico1,SIK348/ SIK348).

(C) In vitro kinase assay of SIK3.K70M, K70M.T486A, K70M.S293/401A, K70M.T486, S293/401A (K70M.3A) and K70M.T281/486A, S293/401A (K70M.4A) polypeptides with recombinant AKT. Immunoblot of total SIK3 amounts also shown.

(D) Sequence alignment of potential AKT phosphorylation sites in Drosophila SIK3 and mouse Sik2. (SEQ ID NOs:1 and 60-65)

(E) Mass spectrometry analysis of phosphorylated residues in SIK3 from refed transgenic flies expressing HA-SIK3 in fat body. Total SIK3 protein coverage was 70%. (SEQ ID NO:66-71)

Figure 10: SIK3 disrupts FOXO dependent transcription in fat body, related to Figure 3.

(A) Effect of 5% soyoil feeding on lipid storage in SIK3^{-} (SIK348) compared to SIK3+(w) flies. (n=16 per genotype).

(B) Q-PCR analysis of mRNAs for lipogenic genes (SREBP, ACC and FAS) in SIK3+(w) and SIK3- (SIK348) flies under fed conditions.

(C) Top, putative FOXO/IRS sites in the brummer promoter shown. Bottom, Effect of phosphorylation-defective, active human FOXO1 and Drosophila FOXO on wild-type and IRS-mutant brummer-luc reporters in HEK293T cells.

(D) Effect of SIK3 over-expression on bmm promoter activity in HEK293T cells. (E, F) brummer mRNA amounts (E) and lipid levels (F) in flies expressing FOXO in fat body (r4>FOXO) relative to controls (r4>). (n=10 per genotype).

Figure 11: SIK3 inhibits FOXO activity, related to Figure 4.

(A,B) Q-PCR analysis of 4E-BP (A) and PEPCK (B) mRNAs in SIK3+ (w) and SIK3^{-}(SIK348) under feeding or 24 hour fasting conditions.

(C) Q-PCR analysis of dilp2 and AKH mRNA amounts in SIK3+ (w) and SIK3^{-} (SIK348) flies under fed conditions.

(D) Effect of insulin on FOXO phosphorylation. Immunoblot showing mobility of wild-type or phosphorylation defective (FOXO.TM) Drosophila FOXO in transfected S2 cells exposed to insulin (100nM) for 15 minutes.

(E) Alignment of SIK consensus motif with sequences flanking Ser66 and Ser531 phosphorylation sites in FOXO. (SEQ ID NO:72-73)

(F,G) Transient reporter assay of HepG2 cells showing 3XIRE-luc reporter activity in cells expressing FOXO.WT and either SIK3 phosphorylation-defective FOXO (S66/531A) (F) or AKT phosphorylation-defective FOXO.TM (G).

Figure 12: SIK3 suppresses HDAC4 activity, related to Figure 5.

(A) Illustration of the adult wing. Position of longtitudinal veins (LV1-6) shown. ACV and PCV refer to anterior and posterior cross-veins, respectively.

(B) Deficiency screen of SIK3 in the wing. Top: RNAi-mediated knockdown of SIK3 in wing (right) causes ectopic veins compared to control (left). Bottom: suppression of SIK3 ectopic vein phenotype by Df(X)BSC713 (left) and HDAC4e04575 (right). Genotypes are: MS>(MS1096-GAL4/+), MS>SIK3i (MS1096-GAL4/+;;UAS-SIK3 RNAi/+), MS>SIK3i,Df(X)BSC713 (MS1096-GAL4/ Df(X)BSC713;;UAS-SIK3 RNAi/+) and MS>SIK3i,HDAC4e04575 (MS1096-GAL4/HDAC4e04575;;UAS-SIK3 RNAi/+).

(C) Genetic interaction of SIK3 and HDAC4 in the wing. Genotypes are: MS>CON (MS1096-GAL4/+), MS>, HDAC4 (MS1096-GAL4/HDAC4d07212), MS>, MS>SIK3,CON (MS1096-GAL4/+;UAS-SIK3/+), MS>SIK3,HDAC4 (MS1096-GAL4/ HDAC4d07212;UAS-SIK3/+), MS>SIK3i,CON (MS1096-GAL4/+;UAS-SIK3 RNAi/+), MS>SIK3i,HDAC4 (MS1096-GAL4/ HDAC4d07212;UAS-SIK3 RNAi/+).

(D) Phenotypes of wild-type (WT) and SIK3-phosphorylation defective (3A) HDAC4 in wing. Genotypes are: MS> (MS 1096-GAL4/+), MS> HDAC4.WT (MS1096-GAL4/+;UAS-HDAC4.WT/+), and MS> HDAC4.3A(MS1096-GAL4/+;UAS-HDAC4.3A/+).

(E) Immunoblot showing effect of PKA defective, active SIK3.S563A on phosphorylation of wild-type HDAC4 and phosphorylation mutant HDAC4.3A in transfected Drosophila S2 cells.

(F) Immunoblot showing effect of FSK on phosphorylation of wild-type and mutant (Ser563Ala) SIK3 at the inhibitory PKA phosphorylation site (Ser563) in HepG2 hepatocytes. Ser563 phosphorylation was detected using phospho-PKA substrate antibody on wild-type and mutant SIK3 immunoprecipitates.

(G) lmmunoblot showing effect of constitutively active SIK3.T196E on subcellular localization of HDAC4 in Hela cells. Nuclear (CREB) and cytoplasmic (ACC) markers shown.

Figure 13. HDAC4 activates FOXO, related to Figure 6.

(A) Immunoblot showing amounts of FOXO recovered from immunoprecipitates of HDAC4 prepared from Hela cells expressing constitutively active SIK3.196E as indicated. Input amounts of each protein shown.

(B) Q-PCR analysis of mRNA amounts for fasting inducible genes (PEPCK, bmm, CPTI) in fat body of flies over-expressing wild-type HDAC4 under fed conditions. Genotypes are: FB>(+/Y;FB-GAL4/+) and FB>HDAC4 (HDAC4d07212/Y;FB-GAL4/+). Data are averages± SEM (**, P<0.01).

(C) Q-PCR analysis of FOXO target gene expression in isolated fat body from control and HDAC4.3A transgenic early L3 larvae. Genotypes are: FB> (FB-GAL4/+) and FB>HDAC4.3A (FB-GAL4/+;UAS-HDAC4.3A/+).

(D) Effect of fat-body expression of phosphorylation-defective HDAC4.3A on fluorescence from a fat-body GAL4-driven UAS-GFP transgene in early L3 larvae.

(E, F) Lipid levels (E) and starvation sensitivity (F) in control and fat body HDAC4.3A over-expressing flies. (n=20 per genotype for lipid measurement and n=100 for starvation assays).

(G) Acetylation of GST-FOXO (aa. 95-198) by P300 in vitro. Immunoblot showing amounts of acetylated GST-FOXO using anti-acetyl lysine antibody. Input protein amounts for GST and GST-FOXO (aa. 95-198) detected by Coomassie blue staining.

(H) Mass spectrometry analysis of HDAC4-associated proteins recovered from transgenic flies expressing Flag epitope-tagged HDAC4 in fat body. Names of associated proteins and relevant mammalian homologs indicated along with numbers of unique peptides recovered for each.

Figure 14. Conservation of the SIK/class II HDAC module in mammals, related to Figure 7.

(A) Left, immunoblot showing amounts of phospho- (Ser245) HDAC4 in primary hepatocytes following adenovirus mediated depletion of SIK2. Exposure to glucagon indicated. Right, Q-PCR analysis of SIK2 mRNA levels in primary hepatocytes expressing adenovirally encoded Sik2 RNAi or non-specific control.

(B) Left, fluorescence images showing relative effect of exposure to FSK or Insulin exposure on subcellular localization of human GFP-HDAC5 in HepG2 hepatoma cells. Scale bar; 10 μm. Right, quantitative analysis of GFP-HDAC5 localization cytoplasmic (C) or nuclear (N) compartments of HepG2 cells. Exposure to Insulin, FSK, or PI3K inhibitor (LY) indicated. 100 cells were scored for each condition. Data are averages± SEM (**, P<0.01; *, P<0.05).

(C) Immunoblot of endogenous Hdac4 recovered from immunoprecipitates of endogenous Foxo1 or control IgG prepared from primary hepatocytes. Input amounts of Hdac4 and Foxo 1 shown.

(D) Q-PCR analysis of G6p, Pck1, Nr4a2, Gapdh, and Foxo1 in primary hepatocytes after 1 hour exposure of glucagon, with or without co-treatment of trichostatin A.

(E) Q-PCR analysis of HDAC4 and HDAC5 mRNA amounts in primary hepatocytes following expression of adenovirally-encoded non-specific (Ad-Uni) or HDAC4/5 RNAis.

(F) Insulin regulates metabolic gene expression via the SIK/class IIa HDAC module. Following their activation by AKT during feeding, SIKs down-regulate FOXO activity by promoting the phosphorylation-dependent cytoplasmic translocation of class IIa HDACs. During fasting, SIKs are inactivated, leading to the dephosphorylation and nuclear translocation of class IIa HDACs and to the subsequent deacetylation and activation of FOXO.

DETAILED DESCRIPTION OF THE INVENTION

Under fasting conditions, metazoans maintain energy balance by shifting from glucose to fat burning. In the fasted state, SIRT1 promotes catabolic gene expression by deacetylating the forkhead factor FOXO in response to stress and nutrient deprivation. The mechanisms by which hormonal signals regulate FOXO deacetylation remain unclear, however. A hormone-dependent module was identified, consisting of the Ser/Thr kinase SIK3 and the class IIa deacetylase HDAC4, which regulates FOXO activity in Drosophila. During feeding, HDAC4 is phosphorylated and sequestered in the cytoplasm by SIK3, whose activity is upregulated in response to insulin. SIK3 is inactivated during fasting, leading to the de-phosphorylation and nuclear translocation of HDAC4, and to FOXO deacetylation. SIK3 mutant flies are starvation-sensitive, reflecting FOXO-dependent increases in lipolysis that deplete triglyceride stores; reducing HDAC4 expression restored lipid accumulation. The results reveal a hormone-regulated pathway that functions in parallel with the nutrient-sensing SIRT1 pathway to maintain energy balance.

During fasting, metazoans maintain energy balance through increases in triglyceride lipolysis by Hormone Sensitive Lipase (HSL) and ATGL in response to hormonal signals. This process is reversed during feeding, when increases in insulin signaling promote fat storage in part through the inhibition of both lipases. Although insulin has been shown to down-regulate HSL activity by upregulating phosphodiesterases that terminate cAMP signaling (Kitamura et al., 1999), the mechanism by which it inhibits ATGL expression is unknown.

Herein is shown that SIK3 is activated by AKT during feeding, when it promotes lipid storage in adult flies by inhibiting FOXO activity and thereby reducing the expression of brummer lipase, the fly homolog of ATGL. Although insulin promotes fat storage in mammals, loss of insulin signaling in chico null flies actually leads to an increase in lipid stores. Loss of chico is thought to promote broad changes in intracellular signaling in multiple tissues (Bohni et al., 1999; Clancy et al., 2001); these changes likely compensate for loss of SIK3 activity in the fat body. Nevertheless, SIK3 activity is used in lipid storage in a wild-type background.

SIK3 did not appear to inhibit FOXO activity directly through phosphorylation, but rather by blocking its deacetylation by HDAC4. It was determined that class IIa HDACs are insulin and cAMP regulated deacetylases that promote FOXO activation and catabolic gene expression during fasting (Figure 14F); they are inhibited through SIK-mediated phosphorylation and cytoplasmic translocation. In certain embodiments, in addition to SIK3, other Ser/Thr kinases including calmodulin-dependent kinases and other members of the AMPK family may also regulate class IIa HDAC shuttling through phosphorylation at the same sites under different conditions.

Fasting hormones disrupt SIK activity through PKA-mediated phosphorylation, leading to the de-phosphorylation and nuclear translocation of class IIa HDACs. The subsequent upregulation of FOXO activity by class IIa HDACs was unexpected because these enzymes are thought to function in muscle and brain as signal-dependent repressors of gene expression (Haberland et al., 2009).

Acetylation has been proposed to modulate FOXO activity by decreasing its DNA binding affinity and thereby enhancing its phosphorylation by AKT (Brent et al., 2008; Matsuzaki et al., 2005). Consistent with this view, it was determined that the acetylation and phosphorylation of FOXO increased contemporaneously during refeeding; both FOXO modifications were decreased in SIK3 mutant animals, reflecting the upregulation of HDAC4 activity. Indeed, disruption of HDAC4 restored FOXO phosphorylation and target gene expression in SIK3 mutant flies. Although fat body-specific rescue experiments demonstrate a SIK3 requirement in the fat body and argue for cell autonomous effects of HDAC4 on FOXO target gene expression, in certain embodiments, there are additional non-cell autonomous effects of SIK3/HDAC4 activity. For example, FOXO target gene induction in response to fat body-specific HDAC4 overexpression, as detected in whole fly RNA, could additionally be occurring in tissues other than the fat body.

Supporting a substantial role for these enzymes in glucose and lipid metabolism, the effects of SIK3 and HDAC4 in Drosophila appear to be conserved in mammals. It was determined that Sik2, the mouse homolog of fly SIK3, mediates the

phosphorylation and inactivation of Hdac4 in mouse hepatocytes in response to insulin. Conversely, exposure to glucagon increased Hdac4 activity through the PKA-mediated inhibition of Sik2. In an accompanying paper, Mihaylova and colleagues (Mihaylova et al., 2011) also show that mammalian class IIa HDACs are fasting regulated deacetylases, which increase FOXO target gene expression in mice.

Consistent with the results in Drosophila, mutations in the HDAC4 gene that result in haploinsufficiency have been associated with obesity in humans, although the underlying mechanism remains unclear (Williams et al.).

Without being bound to theory, in certain embodiments, the class IIa HDACs and SIRT1 represent parallel pathways, which regulate fasting programs following their activation by hormonal and nutrient signals, respectively. In this regard, without being bound to theory, mammalian class IIa HDACs may trigger catabolic gene expression during early fasting, when circulating concentrations of glucagon and catecholamines are typically high, while SIRT1 may function primarily at later "protein-sparing" stages of fasting, when cellular energy levels are low.

In certain embodiments, small molecules or other compounds that specifically modulate, including inhibit, one or more Class IIa HDACs are useful as diabetes therapeutics, such as type 1 diabetes, type 2 diabetes, gestational diabetes, and MODY (maturity onset diabetes of the young) diabetes therapeutics. In certain embodiments, small molecules or other compounds that specifically modulate one or more Class IIa HDACs are useful in the treatment of other metabolic disorders, such as insulin resistance, obesity, metabolic syndrome, hyperglycemia, and impaired glucose tolerance. The terms "metabolic disorder" and "metabolic disease" are used interchangeably herein and typically refer to a disorder characterized by one or more problems with an organism's metabolism. Examples of metabolic disorders include, without limitation, diabetes, insulin resistance, obesity, metabolic syndrome, hyperglycemia, and impaired glucose tolerance.

In yet other embodiments, inhibitors specific to Class IIa HDACs are useful in the treatment of medical conditions characterized by one or more hyperactivated FOXO transcription factors. Examples of such medical conditions include muscle-wasting diseases such as cachexia, muscle wasting, age-related sarcopenia, and muscular dystrophy.

Almost all enzymes known to regulate FOXO transcription factors do so by reducing FOXO transcription factor activity. Therefore, inhibiting these enzymes leads to increased FOXO activity, potentially leading to the development of medical conditions such as muscle wasting and diabetes. By contrast, the instant invention

provides methods of inhibiting FOXO transcription factor activity, by administering to a subject in need thereof an inhibitor specific to a Class IIa HDAC.

An example of a Class IIa-specific HDAC inhibitor is MC1568, which is manufactured by Sigma-Aldrich (catalogue# M1824). In certain embodiments, an inhibitor of one or more Class IIa HDACs is a pharmaceutically acceptable salt of MC1568.

As used herein, the terms "drug," "agent," and "compound" encompass any composition of matter or mixture which provides some pharmacologic effect that can be demonstrated in-vivo or in vitro. This includes small molecules, nucleic acids, antibodies, microbiologicals, vaccines, vitamins, and other beneficial agents. As used herein, the terms further include any physiologically or pharmacologically active substance that produces a localized or systemic effect in a patient.

The term "nucleic acid" encompasses DNA, RNA (e.g., mRNA, tRNA), heteroduplexes, and synthetic molecules capable of encoding a polypeptide and includes all analogs and backbone substitutes such as PNA that one of ordinary skill in the art would recognize as capable of substituting for naturally occurring nucleotides and backbones thereof. Nucleic acids may be single stranded or double stranded, and may be chemical modifications. The terms "nucleic acid" and "polynucleotide" are used interchangeably. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present compositions and methods encompass nucleotide sequences which encode a particular amino acid sequence.

Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

As used herein, the term "amino acid sequence" is synonymous with the terms "polypeptide," "protein," and "peptide," and are used interchangeably. The conventional one-letter or three-letter code for amino acid residues are used herein.

As used herein, a "synthetic" molecule is produced by *in vitro* chemical or enzymatic synthesis rather than by an organism.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

A "gene" refers to the DNA segment encoding a polypeptide or RNA.

By "homolog" is meant an entity having a certain degree of identity with the subject amino acid sequences and the subject nucleotide sequences. As used herein, the term "homolog" covers identity with respect to structure and/or function, for example, the expression product of the resultant nucleotide sequence has the enzymatic activity of

a subject amino acid sequence. With respect to sequence identity, preferably there is at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or even 99% sequence identity. These terms also encompass allelic variations of the sequences. The term, homolog, may apply to the relationship between genes separated by the event of speciation or to the relationship between genes separated by the event of genetic duplication.

Thus, in certain embodiments, the present invention encompasses the use of variants, homologues and derivatives of a Class IIa HDAC nucleic acid and/or amino acid sequence. Examples of Class IIa HDAC nucleic acid sequences include the human Class IIa HDAC nucleic acid sequences available through the National Center for Biotechnology Information (NCBI) website, such as GenBank Nos. NM_006037 (HDAC4); NM_001015053 (HDAC5, transcript variant 3), NM_005474 (HDAC5, transcript variant 1); NM_001098416 (HDAC7, transcript variant 4), NM_015401 (HDAC7, transcript variant 1); and NM_058176 (HDAC9, transcript variant 1) and their corresponding amino acid sequences. In one embodiment, the sequences, such as variants, homologs and derivatives of a human Class IIa HDAC amino acid sequence, may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance.

Relative sequence identity can be determined by commercially available computer programs that can calculate % identity between two or more sequences using any suitable algorithm for determining identity, using, for example, default parameters. A typical example of such a computer program is CLUSTAL. Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail on the NCBI website.

The homologs of the peptides as provided herein typically have structural similarity with such peptides. A homolog of a polypeptide includes one or more conservative amino acid substitutions, which may be selected from the same or different members of the class to which the amino acid belongs.

In one embodiment, the sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar

hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

One embodiment encompasses conservative substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue with an alternative residue) that may occur e.g., like-for-like substitution such as basic for basic, acidic for acidic, polar for polar, etc. Non-conservative substitution may also occur e.g., from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine. Conservative substitutions that may be made are, for example, within the groups of basic amino acids (Arginine, Lysine and Histidine), acidic amino acids (glutamic acid and aspartic acid), aliphatic amino acids (Alanine, Valine, Leucine, Isoleucine), polar amino acids (Glutamine, Asparagine, Serine, Threonine), aromatic amino acids (Phenylalanine, Tryptophan and Tyrosine), hydroxyl amino acids (Serine, Threonine), large amino acids (Phenylalanine and Tryptophan) and small amino acids (Glycine, Alanine).

Another emboidment employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual,* Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology,* ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques,* John Wiley & Sons; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach,* Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press. Each of these general texts is herein incorporated by reference.

Many methods of sequencing genomic DNA are known in the art, and any such method can be used, see for example Sambrook et al., Molecular Cloning; A Laboratory Manual 2d ed. (1989). For example, a DNA fragment of interest can be amplified using the polymerase chain reaction or some other cyclic polymerase mediated amplification reaction. The amplified region of DNA can then be sequenced using any method known in the art. Advantageously, the nucleic acid sequencing is by automated methods (reviewed by Meldrum, Genome Res. September 2000;10(9):1288-303, the disclosure

of which is incorporated by reference in its entirety), for example using a Beckman CEQ 8000 Genetic Analysis System (Beckman Coulter Instruments, Inc.). Methods for sequencing nucleic acids include, but are not limited to, automated fluorescent DNA sequencing (see, e.g., Watts & MacBeath, Methods Mol Biol. 2001;167:153-70 and MacBeath et al., Methods Mol Biol. 2001;167:119-52), capillary electrophoresis (see, e.g., Bosserhoff et al., Comb Chern High Throughput Screen. December 2000;3(6):455-66), DNA sequencing chips (see, e.g., Jain, Pharmacogenomics. August 2000;1(3):289-307), mass spectrometry (see, e.g., Yates, Trends Genet. January 2000;16(1):5-8), pyrosequencing (see, e.g., Ronaghi, Genome Res. January 2001;11(1):3-11), and ultrathin-layer gel electrophoresis (see, e.g., Guttman & Ronai, Electrophoresis. December 2000; 21 (18):3952-64), the disclosures of which are hereby incorporated by reference in their entireties. The sequencing can also be done by any commercial company. Examples of such companies include, but are not limited to, the University of Georgia Molecular Genetics Instrumentation Facility (Athens, Ga.) or Seq Wright DNA Technologies Services (Houston, Tex.).

Any one of the methods known in the art for amplification of DNA may be used, such as for example, the polymerase chain reaction (PCR), the ligase chain reaction (LCR) (Barany, F., Proc. Natl. Acad. Sci. (U.S.A.) 88:189-193 (1991)), the strand displacement assay (SDA), or the oligonucleotide ligation assay ("OLA") (Landegren, U. et al., Science 241:1077-1080 (1988)). Nickerson, D. A. et al. have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D. A. et al., Proc. Natl. Acad. Sci. (U.S.A.) 87:8923-8927 (1990)). Other known nucleic acid amplification procedures, such as transcription-based amplification systems (Malek, L. T. et al., U.S. Pat. No. 5,130,238; Davey, C. et al., European Patent Application 329,822; Schuster et al., U.S. Pat. No. 5,169,766; Miller, H. I. et al., PCT Application W089/06700; Kwoh, D. et al., Proc. Natl. Acad. Sci. (U.S.A.) 86:1173 (1989); Gingeras, T. R. et al., PCT Application W088/10315)), or isothermal amplification methods (Walker, G. T. et al., Proc. Natl. Acad. Sci. (U.S.A.) 89:392-396 (1992)) may also be used.

To perform a cyclic polymerase mediated amplification reaction according to the present invention, the primers are hybridized or annealed to opposite strands of the target DNA, the temperature is then raised to permit the thermostable DNA polymerase to extend the primers and thus replicate the specific segment of DNA spanning the region between the two primers. Then the reaction is thermocycled so that at each cycle the amount of DNA representing the sequences between the two primers is doubled, and specific amplification of gene DNA sequences, if present, results.

Any of a variety of polymerases can be used in the present invention. For thermocyclic reactions, the polymerases are thermostable polymerases such as Taq, KlenTaq, Stoffel Fragment, Deep Vent, Tth, Pfu, Vent, and UITma, each of which are readily available from commercial sources. For non-thermocyclic reactions, and in certain thermocyclic reactions, the polymerase will often be one of many polymerases commonly used in the field, and commercially available, such as DNA pol 1, Klenow fragment, T7 DNA polymerase, and T4 DNA polymerase. Guidance for the use of such polymerases can readily be found in product literature and in general molecular biology guides.

Typically, the annealing of the primers to the target DNA sequence is carried out for about 2 minutes at about 37-55° C, extension of the primer sequence by the polymerase enzyme (such as Taq polymerase) in the presence of nucleoside triphosphates is carried out for about 3 minutes at about 70-75° C, and the denaturing step to release the extended primer is carried out for about 1 minute at about 90-95° C. However, these parameters can be varied, and one of skill in the art would readily know how to adjust the temperature and time parameters of the reaction to achieve the desired results. For example, cycles may be as short as 10, 8, 6, 5, 4.5, 4, 2, 1, 0.5 minutes or less.

Also, "two temperature" techniques can be used where the annealing and extension steps may both be carried out at the same temperature, typically between about 60-65° C, thus reducing the length of each amplification cycle and resulting in a shorter assay time.

Typically, the reactions described herein are repeated until a detectable amount of product is generated. Often, such detectable amounts of product are between about 10 ng and about 100 ng, although larger quantities, e.g. 200 ng, 500 ng, 1 mg or more can also, of course, be detected. In terms of concentration, the amount of detectable product can be from about 0.01 pmol, 0.1 pmol, 1 pmol, 10 pmol, or more. Thus, the number of cycles of the reaction that are performed can be varied, the more cycles are performed, the more amplified product is produced. In certain embodiments, the reaction comprises 2, 5, 10, 15, 20, 30, 40, 50, or more cycles.

For example, the PCR reaction may be carried out using about 25-50 μl samples containing about 0.01 to 1.0 ng of template amplification sequence, about 10 to 100 pmol of each generic primer, about 1.5 units of Taq DNA polymerase (Promega Corp.), about 0.2 mM dDATP, about 0.2 mM dCTP, about 0.2 mM dGTP, about 0.2 mM dTTP, about 15 mM MgCl.sub.2, about 10 mM Tris-HCl (pH 9.0), about 50 mM KCl, about 1 μg/ml gelatin, and about 10 μl/ml Triton X-100 (Saiki, 1988).

Those of ordinary skill in the art are aware of the variety of nucleotides available for use in the cyclic polymerase mediated reactions. Typically, the nucleotides will consist at least in part of deoxynucleotide triphosphates (dNTPs), which are readily commercially available. Parameters for optimal use of dNTPs are also known to those of skill, and are described in the literature. In addition, a large number of nucleotide derivatives are known to those of skill and can be used in the present reaction. Such derivatives include fluorescently labeled nucleotides, allowing the detection of the product including such labeled nucleotides, as described below. Also included in this group are nucleotides that allow the sequencing of nucleic acids including such nucleotides, such as chain-terminating nucleotides, dideoxynucleotides and boronated nuclease-resistant nucleotides. Commercial kits containing the reagents most typically used for these methods of DNA sequencing are available and widely used. Other nucleotide analogs include nucleotides with bromo-, iodo-, or other modifying groups, which affect numerous properties of resulting nucleic acids including their antigenicity, their replicatability, their melting temperatures, their binding properties, etc. In addition, certain nucleotides include reactive side groups, such as sulfhydryl groups, amino groups, N-hydroxysuccinimidyl groups, that allow the further modification of nucleic acids comprising them.

In certain embodiments, oligonucleotides that can be used as primers to amplify specific nucleic acid sequences of a gene in cyclic polymerase-mediated amplification reactions, such as PCR reactions, consist of oligonucleotide fragments. Such fragments should be of sufficient length to enable specific annealing or hybridization to the nucleic acid sample. The sequences typically will be about 8 to about 44 nucleotides in length, but may be longer. Longer sequences, e.g., from about 14 to about 50, are advantageous for certain embodiments.

In embodiments where it is desired to amplify a fragment of DNA, primers having contiguous stretches of about 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides from a gene sequence are contemplated.

As used herein, "hybridization" refers to the process by which one strand of nucleic acid base pairs with a complementary strand, as occurs during blot hybridization techniques and PCR techniques.

Whichever probe sequences and hybridization methods are used, one ordinarily skilled in the art can readily determine suitable hybridization conditions, such as temperature and chemical conditions. Such hybridization methods are well known in the art. For example, for applications requiring high selectivity, one will typically desire to employ relatively stringent conditions for the hybridization reactions, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about

0.02 M to about 0.10 M NaCl at temperatures of about 50 °C to about 70 °C. Such high stringency conditions tolerate little, if any, mismatch between the probe and the template or target strand. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide. Other variations in hybridization reaction conditions are well known in the art (see for example, Sambrook et al., Molecular Cloning; A Laboratory Manual 2d ed. (1989)).

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught, e.g., in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5 °C (5 °C below the Tm of the probe); high stringency at about 5 °C to 10 °C below Tm; intermediate stringency at about 10 °C to 20 °C below Tm; and low stringency at about 20 °C to 25 °C below Tm. As will be understood by those of ordinary skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

In one aspect, nucleotide sequences that can hybridize to another nucleotide sequence under stringent conditions (e.g., 65 °C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na3 Citrate pH 7.0) are employed. Where the nucleotide sequence is double-stranded, both strands of the duplex, either individually or in combination, may be employed by the present invention. Where the nucleotide sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included within the scope of the present invention.

Stringency of hybridization refers to conditions under which polynucleic acid hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of ordinary skill in the art, the stability of hybrids is reflected in the melting temperature (Tm) of the hybrid which decreases approximately 1 to 1.5 °C with every 1 % decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency includes conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 1 M Na+ at 65-68 °C. High stringency conditions can be provided, for example, by hybridization in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 Na+ pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non-

specific competitor. Following hybridization, high stringency washing may be done in several steps, with a final wash (about 30 minutes) at the hybridization temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g., formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of ordinary skill in the art as are other suitable hybridization buffers (see, e.g., Sambrook, et al., eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York or Ausubel, et al., eds. (1990) Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). Optimal hybridization conditions are typically determined empirically, as the length and the GC content of the hybridizing pair also play a role.

Nucleic acid molecules that differ from the sequences of the primers and probes disclosed herein, are intended to be within the scope of the invention. Nucleic acid sequences that are complementary to these sequences, or that are hybridizable to the sequences described herein under conditions of standard or stringent hybridization, and also analogs and derivatives are also intended to be within the scope of the invention. Advantageously, such variations will differ from the sequences described herein by only a small number of nucleotides, for example by 1, 2, or 3 nucleotides.

Nucleic acid molecules corresponding to natural allelic variants, homologues (i.e., nucleic acids derived from other species), or other related sequences (e.g., paralogs) of the sequences described herein can be isolated based on their homology to the nucleic acids disclosed herein, for example by performing standard or stringent hybridization reactions using all or a portion of the known sequences as probes. Such methods for nucleic acid hybridization and cloning are well known in the art.

Similarly, a nucleic acid molecule detected in the methods of the invention may include only a fragment of the specific sequences described. Fragments provided herein are defined as sequences of at least 6 (contiguous) nucleic acids, a length sufficient to allow for specific hybridization of nucleic acid primers or probes, and are at most some portion less than a full-length sequence. Fragments may be derived from any contiguous portion of a nucleic acid sequence of choice. Derivatives and analogs may be full length or other than full length, if the derivative or analog contains a modified nucleic acid or amino acid, as described below.

Derivatives, analogs, homologues, and variants of the nucleic acids of the invention include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids of the invention, in various embodiments, by at least about 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or even 99% identity

over a nucleic acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art.

For the purposes of the present invention, sequence identity or homology is determined by comparing the sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. In particular, sequence identity may be determined using any of a number of mathematical algorithms. A nonlimiting example of a mathematical algorithm used for comparison of two sequences is the algorithm of Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1990;87: 2264-2268, modified as in Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1993;90: 5873-5877.

Another example of a mathematical algorithm used for comparison of sequences is the algorithm of Myers & Miller, CABIOS 1988;4: 11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson & Lipman, Proc. Natl. Acad. Sci. USA 1988;85: 2444-2448.

Advantageous for use according to the present invention is the WU-BLAST (Washington University BLAST) version 2.0 software. WU-BLAST version 2.0 executable programs for several UNIX platforms can be downloaded from ftp://blast.wustl.edu/blast/executables. This program is based on WU-BLAST version 1.4, which in turn is based on the public domain NCBI-BLAST version 1.4 (Altschul & Gish, 1996, Local alignment statistics, Doolittle ed., Methods in Enzymology 266: 460-480; Altschul et al., Journal of Molecular Biology 1990;215: 403-410; Gish & States, 1993;Nature Genetics 3: 266-272; Karlin & Altschul, 1993;Proc. Natl. Acad. Sci. USA 90: 5873-5877; all of which are incorporated by reference herein).

In all search programs in the suite the gapped alignment routines are integral to the database search itself. Gapping can be turned off if desired. The default penalty (Q) for a gap of length one is Q=9 for proteins and BLASTP, and Q=10 for BLASTN, but may be changed to any integer. The default per-residue penalty for extending a gap (R) is R=2 for proteins and BLASTP, and R=10 for BLASTN, but may be changed to any integer. Any combination of values for Q and R can be used in order to align sequences so as to maximize overlap and identity while minimizing sequence gaps. The default amino acid comparison matrix is BLOSUM62, but other amino acid comparison matrices such as PAM can be utilized.

Alternatively or additionally, the term "homology" or "identity", for instance, with respect to a nucleotide or amino acid sequence, can indicate a quantitative measure

of homology between two sequences. The percent sequence homology can be calculated as $(N_{ref}-N_{dif})*100/-N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (N N_{ref}=8; N N_{dif} =2). "Homology" or "identity" can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur & Lipman, Proc Natl Acad Sci USA 1983;80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics.TM. Suite, Intelligenetics Inc. CA). When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus, RNA sequences are within the scope of the invention and can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences. Without undue experimentation, the ordinarily skilled artisan can consult with many other programs or references for determining percent homology."Antisense" nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American 262 40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. This interferes with the translation of the mRNA since the cell will not translate an mRNA that is double-stranded. Antisense oligomers of at least about 15, about 20, about 25, about 30, about 35, about 40, or of at least about 50 nucleotides are preferred, since they are easily synthesized and are less likely to cause non-specific interference with translation than larger molecules. The use of antisense methods to inhibit the in vitro translation of genes is well known in the art (Marcus-Sakura Anal. Biochem. 172: 289, 1998).

One embodiment provides for nucleic acids complementary to (e.g., antisense sequences to) cellular modulators of Class IIa HDAC activity. Antisense sequences are capable of inhibiting the transport, splicing or transcription of protein-encoding genes. The inhibition can be effected through the targeting of genomic DNA or messenger RNA. The transcription or function of targeted nucleic acid can be inhibited, for example, by hybridization and/or cleavage. One particularly useful set of inhibitors provided by the present invention includes oligonucleotides which are able to either

bind gene or message, in either case preventing or inhibiting the production or function of the protein. The association can be through sequence specific hybridization. Another useful class of inhibitors includes oligonucleotides that cause inactivation or cleavage of protein message. The oligonucleotide can have enzyme activity which causes such cleavage, such as ribozymes. The oligonucleotide can be chemically modified or conjugated to an enzyme or composition capable of cleaving the complementary nucleic acid. One can screen a pool of many different such oligonucleotides for those with the desired activity.

Short double-stranded RNAs (dsRNAs; typically <30 nucleotides) can be used to silence the expression of target genes in animals and animal cells. Upon introduction, the long dsRNAs enter the RNA interference (RNAi) pathway which involves the production of shorter (20-25 nucleotide) small interfering RNAs (siRNAs) and assembly of the siRNAs into RNA-induced silencing complexes (RISCs). The siRNA strands are then unwound to form activated RISCs, which cleave the target RNA. Double stranded RNA has been shown to be extremely effective in silencing a target RNA. Introduction of double stranded RNA corresponding to, e.g., a Class IIa HDAC gene, would be expected to modify the Class IIa HDAC-related functions discussed herein.

General methods of using antisense, ribozyme technology and RNAi technology, to control gene expression, or of gene therapy methods for expression of an exogenous gene in this manner are well known in the art. Each of these methods utilizes a system, such as a vector, encoding either an antisense or ribozyme transcript. The term "RNAi" stands for RNA interference. This term is understood in the art to encompass technology using RNA molecules that can silence genes. See, for example, McManus, et al. Nature Reviews Genetics 3: 737, 2002. In this application, the term "RNAi" encompasses molecules such as short interfering RNA (siRNA), small hairpin or short hairpin RNA (shRNA), microRNAs, and small temporal RNA (stRNA). Generally speaking, RNA interference results from the interaction of double-stranded RNA with genes.

"Small interfering RNA" (siRNA) refers to double-stranded RNA molecules from about 10 to about 30 nucleotides long that are named for their ability to specifically interfere with protein expression through RNA interference (RNAi). Preferably, siRNA molecules are 12-28 nucleotides long, more preferably 15-25 nucleotides long, still more preferably 19-23 nucleotides long and most preferably 21-23 nucleotides long. Therefore, preferred siRNA molecules are 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 28 or 29 nucleotides in length.

RNAi is a two-step mechanism (Elbashir et al., Genes Dev., 15: 188-200, 2001). First, long dsRNAs are cleaved by an enzyme known as Dicer in 21-23 ribonucleotide (nt) fragments, called small interfering RNAs (siRNAs). Then, siRNAs associate with a ribonuclease complex (termed RISC for RNA Induced Silencing Complex) which target this complex to complementary mRNAs. RISC then cleaves the targeted mRNAs opposite the complementary siRNA, which makes the mRNA susceptible to other RNA degradation pathways.

In one embodiment, siRNAs are designed to interact with a target ribonucleotide sequence, meaning they complement a target sequence sufficiently to bind to the target sequence. The present invention also includes siRNA molecules that have been chemically modified to confer increased stability against nuclease degradation, but retain the ability to bind to target nucleic acids that may be present.

One embodiment provides antisense oligonucleotides capable of binding messenger RNA, e.g., mRNA encoding Class IIa HDAC4, that can inhibit polypeptide activity by targeting mRNA. Strategies for designing antisense oligonucleotides are well described in the scientific and patent literature, and the ordinarily skilled artisan can design such oligonucleotides using the novel reagents of the invention. For example, gene walking/RNA mapping protocols to screen for effective antisense oligonucleotides are well known in the art, see, e.g., Ho, Methods Enzymol. 314: 168-183, 2000, describing an RNA mapping assay, which is based on standard molecular techniques to provide an easy and reliable method for potent antisense sequence selection. See also Smith, Eur. J. Pharm. Sci. 11: 191-198, 2000.

Naturally occurring nucleic acids are typically used as antisense oligonucleotides. The antisense oligonucleotides can be of any length; for example, in alternative aspects, the antisense oligonucleotides are between about 5 to 100, about 10 to 80, about 15 to 60, about 18 to 40. The optimal length can be determined by routine screening. The antisense oligonucleotides can be present at any concentration. The optimal concentration can be determined by routine screening. A wide variety of synthetic, non-naturally occurring nucleotide and nucleic acid analogues are known which can also be used. For example, peptide nucleic acids (PNAs) containing non-ionic backbones, such as N-(2-aminoethyl) glycine units can be used. Antisense oligonucleotides having phosphorothioate linkages can also be used, as described in Mata, Toxicol Appl Pharmacol. 144: 189-197, 1997; Antisense Therapeutics, ed. Agrawal, Humana Press, Totowa, N.J., 1996. Antisense oligonucleotides having synthetic DNA backbone analogues can also include phosphoro-dithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal,

methylene(methylimino), 3'-N-carbamate, and morpholino carbamate nucleic acids, as described above.

Combinatorial chemistry methodology can be used to create vast numbers of oligonucleotides that can be rapidly screened for specific oligonucleotides that have appropriate binding affinities and specificities toward any target, such as the sense and antisense polypeptides sequences of the invention (see, e.g., Gold, J. of Biol. Chem. 270: 13581-13584, 1995).

Certain embodiments relate to animals that have at least one modulated Class IIa HDAC function. Such modulated functions include, among others, altered lipid homeostasis. The ordinarily skilled artisan will also recognize that alterations in an animal's ability to regulate lipolysis may be assessed by various assays, including by way of example, by assessing changes in expression or activity of molecules involved in increasing triglyceride mobilization (and therefore decreasing lipid stores) for example, by measuring expression of FOXO target genes and/or protein expression and/or activity levels of specific fasting response proteins (e.g., proteins induced in response to catecholamine stimulation).

Animals having a modified Class IIa HDAC-related function include transgenic animals showing an altered lipid homeostasis due to transformation with constructs using antisense or siRNA technology that affect transcription or expression from a Class IIa HDAC gene. Such animals exhibit an altered lipid homeostasis, such as, for example, a reduction in lipid levels.

Accordingly, another series of embodiments provide methods of screening or identifying proteins, small molecules or other compounds which are capable of modulating the activity or expression of Class IIa HDAC genes and proteins. The assays may be performed, by way of example, in vitro using transformed or non-transformed cells, immortalized cell lines, or in vivo using transformed animal models enabled herein.

To aid in the detection of a protein or nucleic acid, labels are typically used-such as any readily detectable reporter, for example, a fluorescent, bioluminescent, phosphorescent, radioactive, etc. reporter. For example, labels suitable for use in the methods and compositions of the instant invention include green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, and red fluorescent protein. Examples of such reporters (e.g., green fluorescent protein, red fluorescent protein), their detection, coupling to targets/probes, etc. are disclosed herein, for example, in the non-limiting examples.

Further embodiments contemplate direct and indirect labeling techniques. For example, direct labeling includes incorporating fluorescent dyes directly into a

nucleotide sequence (e.g., dyes are incorporated into nucleotide sequence by enzymatic synthesis in the presence of labeled nucleotides or PCR primers). Direct labeling schemes include using families of fluorescent dyes with similar chemical structures and characteristics. In certain embodiments comprising direct labeling of nucleic acids, cyanine or alexa analogs are utilized. In other embodiments, indirect labeling schemes can be utilized, for example, involving one or more staining procedures and reagents that are used to label a protein in a protein complex (e.g., a fluorescent molecule that binds to an epitope on a protein in the complex, thereby providing a fluorescent signal by virtue of the conjugation of dye molecule to the epitope of the protein).

In another series of embodiments there is provided methods for identifying proteins and other compounds which bind to, or otherwise directly interact with a Class IIa HDAC protein. Thus, in one series of embodiments, High Throughput Screening-derived proteins, DNA chip arrays, cell-lysates or tissue homogenates may be screened for proteins or other compounds which bind to one of the normal or mutant Class IIa HDAC genes. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (e.g., libraries of small molecules or peptides), may be screened for Class IIa HDAC function modulating capacity.

Embodiments also include methods of identifying proteins, small molecules and other compounds capable of modulating the activity of a Class IIa HDAC gene or protein. Using normal cells or animals, the transformed cells and animal models of the present invention, or cells obtained from subjects bearing normal or mutant Class IIa HDAC genes, the present invention provides methods of identifying such compounds on the basis of their ability to affect the expression of a Class IIa HDAC, the activity of a Class IIa HDAC, the activity of proteins that interact with normal or mutant Class IIa HDAC proteins, or other biochemical, histological, or physiological markers that distinguish cells bearing normal and modulated Class IIa HDAC activity in animals.

In accordance with another embodiment, the proteins of the invention can be used as starting points for rational chemical design to provide ligands or other types of small chemical molecules. Alternatively, small molecules or other compounds identified by the above-described screening assays may serve as "lead compounds" in design of modulators of Class IIa HDAC-related traits in animals.

DNA sequences encoding a Class IIa HDAC protein can be expressed in vitro by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny or graft material, for example, of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used.

Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

The terms "recombinant expression vector" or "expression vector" refer to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a genetic sequence. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted sequence. The expression vector typically contains an origin of replication, a promoter, as well as specific genes that allow phenotypic selection of the transformed cells.

Methods that are well known to those ordinarily skilled in the art can be used to construct expression vectors containing a Class IIa HDAC coding sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo recombination/genetic techniques.

A variety of host-expression vector systems may be utilized to express a coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing a coding sequence; yeast transformed with recombinant yeast expression vectors containing a coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing a coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing a coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., retroviruses, adenovirus, vaccinia virus) containing a coding sequence, or transformed animal cell systems engineered for stable expression.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see e.g., Bitter et al. Methods in Enzymology 153, 516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage 7, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used.

The term "operably linked" refers to functional linkage between a promoter sequence and a nucleic acid sequence regulated by the promoter. The operably linked promoter controls the expression of the nucleic acid sequence.

The expression of structural genes may be driven by a number of promoters. Although the endogenous, or native promoter of a structural gene of interest may be utilized for transcriptional regulation of the gene, preferably, the promoter is a foreign regulatory sequence. For mammalian expression vectors, promoters capable of directing expression of the nucleic acid preferentially in a particular cell type may be used (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. Genes Dev. 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. Adv. Immunol. 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. EMBO J. 8: 729-733) and immunoglobulins (Banerji, et al., 1983. Cell 33: 729-740; Queen and Baltimore, 1983. Cell 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. Proc. Natl. Acad. Sci. USA 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. Science 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. Science 249: 374-379) and the α-fetoprotein promoter (Campes and Tilghman, 1989. Genes Dev. 3: 537-546).

Promoters useful in the invention include both natural constitutive and inducible promoters as well as engineered promoters. Examples of inducible promoters useful in animals include those induced by chemical means, such as the yeast metallothionein promoter, which is activated by copper ions (Mett, et al. Proc. Natl. Acad. Sci., U.S.A. 90, 4567, 1993); and the GRE regulatory sequences which are induced by glucocorticoids (Schena, et al. Proc. Natl. Acad. Sci., U.S.A. 88, 10421, 1991). Other promoters, both constitutive and inducible will be known to those of ordinary skill in the art.

Animals included in the invention are any animals amenable to transformation techniques, including vertebrate and non-vertebrate animals and mammals. Examples of mammals include, but are not limited to, pigs, cows, sheep, horses, cats, dogs, chickens, or turkeys.

Compounds tested as modulators of Class IIa HDAC activity can be any small organic molecule, or a biological entity, such as a protein, e.g., an antibody or peptide, a sugar, a nucleic acid, e.g., an antisense oligonucleotide, RNAi, or a ribozyme, or a lipid.

Alternatively, modulators can be genetically altered versions of a cellular modulator of Class IIa HDAC activity. Test compounds may be, without limitation, small organic molecules, nucleic acids, peptides, lipids, and/or lipid analogs.

Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic solutions. In certain embodiments, the assays of the invention are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, Mo.), Aldrich (St. Louis, Mo.), Sigma-Aldrich (St. Louis, Mo.), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

In one embodiment, high throughput screening methods involve providing a combinatorial small organic molecule or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of ordinary skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Pat. No. 5,010,175, Furka, Int. J. Pept. Prot. Res. 37: 487-493, 1991 and Houghton et al., Nature 354: 84-88, 1991). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides

(Hobbs et al., Proc. Nat. Acad. Sci. USA 90: 6909-6913, 1993), vinylogous polypeptides (Hagihara et al., J. Amer. Chem. Soc. 114: 6568, 1992), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann et al., J. Amer. Chem. Soc. 114: 9217-9218, 1992), analogous organic syntheses of small compound libraries (Chen et al., J. Amer. Chem. Soc. 116: 2661, 1994), oligocarbamates (Cho et al., Science 261: 1303, 1993), and/or peptidyl phosphonates (Campbell et al., J. Org. Chem. 59: 658, 1994), nucleic acid libraries (see Ausubel, Berger and Sambrook, all supra), peptide nucleic acid libraries (see, e.g., U.S. Pat. No. 5,539,083), antibody libraries (see, e.g., Vaughn et al, Nature Biotechnology, 14: 309-314, 1996 and PCT/US96/10287), carbohydrate libraries (see, e.g., Liang et al., Science 274: 1520-1522, 1996 and U.S. Pat. No. 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, January 18, page 33 (1993); isoprenoids, U.S. Pat. No. 5,569,588; thiazolidinones and metathiazanones, U.S. Pat. No. 5,549,974; pyrrolidines, U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506,337; benzodiazepines, U.S. Pat. No. 5,288,514, and the like.

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville Ky., Symphony, Rainin, Woburn, Mass., 433A Applied Biosystems, Foster City, Calif., 9050 Plus, Millipore, Bedford, Mass.). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, Mo., ChemStar, Ltd, Moscow, R U, 3D Pharmaceuticals, Exton, Pa., Martek Biosciences, Columbia, Md., etc.).

Candidate compounds are useful as part of a strategy to identify drugs for reducing lipid stores wherein the compounds enhance activity of a Class IIa HDAC, for example, wherein the compound enhances the binding of HDAC4 and/or HDAC5 or a homolog thereof to one or more interacting proteins, such as HDAC3. Screening assays for identifying candidate or test compounds that bind to one or more cellular modulators of Class IIa HDAC activity, or polypeptides or biologically active portions thereof, are also included in the invention. The test compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including, but not limited to, biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach can be used for, e.g., peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, Anticancer Drug Des. 12: 145, 1997). Examples of methods for the synthesis of molecular libraries can be found

in the art, for example in: DeWitt et al., Proc. Natl. Acad. Sci. U.S.A. 90: 6909, 1993; Erb et al., Proc. Natl. Acad. Sci. USA 91: 11422, 1994; Zuckermann et al., J. Med. Chem. 37: 2678, 1994; Cho et al., Science 261: 1303, 1993; Carrell et al., Angew. Chem. Int. Ed. Engl. 33: 2059, 1994; Carell et al., Angew. Chem. Int. Ed. Engl. 33: 2061, 1994; and Gallop et al., J. Med. Chern. 37: 1233, 1994.

Libraries of compounds can be presented in solution (e.g., Houghten, Bio/Techniques 13: 412-421, 1992), or on beads (Lam, Nature 354: 82-84, 1991), chips (Fodor, Nature 364: 555-556, 1993), bacteria (U.S. Pat. No. 5,223,409), spores (U.S. Pat. Nos. 5,571,698, 5,403,484, and 5,223,409), plasmids (Cull et al., Proc. Natl. Acad. Sci. USA 89: 1865-1869, 1992) or on phage (Scott et al., Science 249: 386-390, 1990; Devlin, Science 249: 404-406, 1990; Cwirla et al., Proc. Natl. Acad. Sci. USA 87: 6378-6382, 1990; and Felici, J. Mol. Biol. 222: 301-310, 1991).

Another embodiment pertains to novel agents identified by the herein-described screening assays and uses thereof for treatments as described herein, for example, for the treatment of hyperglycemia in an animal, including humans.

One embodiment provides soluble assays using an agonist of a Class IIa HDAC activity, or a cell or tissue expressing a cellular agonist of a Class IIa HDAC activity, either naturally occurring or recombinant. In another embodiment, the invention provides solid phase based in vitro assays in a high throughput format, where a cellular agonist of a Class IIa HDAC activity is attached to a solid phase substrate via covalent or non-covalent interactions.

"Inhibitors," "activators," and "modulators" of a Class IIa HDAC activity in cells encompass inhibitory, activating, or modulating molecules, respectively, identified using in vitro and in vivo assays for Class IIa HDAC activity, e.g., ligands, agonists, antagonists, and their homologs and mimetics.

"Activity" with respect to a protein includes any activity of the protein, including binding and/or enzymatic activity of the protein.

"Modulator" includes inhibitors and activators. Inhibitors are agents that, e.g., bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate Class IIa HDAC activity, e.g., antagonists. Activators are agents that, e.g., bind to, stimulate, increase, open, activate, facilitate, enhance activation, sensitize or up-regulate a Class IIa HDAC activity, e.g., agonists. Modulators include genetically modified versions of biological molecules with a Class IIa HDAC activity, e.g., with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like.

"Cell-based assays" for inhibitors and activators include, e.g., applying putative modulator compounds to a biological sample having a Class IIa HDAC activity and then

determining the functional effects on the Class IIa HDAC activity, as described herein. "Cell based assays" include, but are not limited to, in vivo tissue or cell samples from a mammalian subject or in vitro cell-based assays comprising a biological sample having Class IIa HDAC activity that are treated with a potential activator, inhibitor, or modulator and are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition.

"Compound" or "test compound" refers to any compound tested as a modulator of Class IIa HDAC activity. The test compound can be any small organic molecule, or a biological entity, such as a protein, e.g., an antibody or peptide, a sugar, a nucleic acid, e.g., an antisense oligonucleotide, RNAi, or a ribozyme, or a lipid. Alternatively, a test compound can be modulators of biological activities that affect a Class IIa HDAC activity. Test compounds may be, without limitation, small organic molecules, nucleic acids, peptides, lipids, and/or lipid analogs.

Methods of delivery of a compound for treatment of a metabolic disease according to the invention include but are not limited to, oral, intra-arterial, intramuscular, intravenous, intranasal, and inhalation routes. In certain embodiments, the delivery route is oral. Suitable modes of delivery will be apparent based upon the particular combination of drugs employed and their known administration forms. A compound for treatment of a metabolic disorder may be administered by any suitable route, including without limitation, oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, penile, parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary.

Therapeutic amounts can be empirically determined and may vary with the particular metabolic condition being treated, the subject, the particular formulation components, dosage form, and the like. The actual dose to be administered may vary depending upon the age, weight, and general condition of the subject as well as the severity of the metabolic condition being treated, along with the judgment of the health care professional. Therapeutically effective amounts can be determined by those ordinarily skilled in the art, and will be adjusted to the requirements of each particular case.

The invention will now be described by way of the following non-limiting examples.

EXAMPLE 1

SIK3 Promotes Lipid Storage

Within the AMPK family of Ser/Thr kinases, the Salt-inducible kinases (SIKs) have been shown to regulate hepatic glucose metabolism in mammals following their phosphorylation and activation by LKB1, a master kinase for the AMPK family (Dentin

et al., 2007; Koo et al., 2005). The SIK kinases are highly conserved through evolution; there are two SIKs encoded by the Drosophila genome: CG4290 (fly SIK2) and CG15072 (fly SIK3) (Okamoto et al., 2004). The physiological roles of SIK3 are herein investigating and described by mutating this gene using P element-mediated imprecise excision (see Experimental Procedures).

A series of deletions were generated affecting the SIK3 gene (Figure 1A). Because SIK3 null mutants (SIK372) die during early larval stages, a viable SIK3 hypomorphic allele (SIK348) was used (Figures 1A-C and 8A-C). SIK348 homozygous mutants showed markedly decreased lipid stores, and they were more sensitive to starvation than control (SIK3+51) flies (Figure 1D-E). By contrast with its effects on fasting, loss of SIK3 only modestly increased sensitivity (15-17%) to oxidative stress (Figure 8F-G). SIK348/SIK372 and SIK348/Df(2R)P34 trans-heterozygotes showed a similar lipid phenotype to SIK348 homozygotes, suggesting that SIK348 is a strong hypomorphic allele (Figure 8D-E). Further supporting this idea, SIK3 mRNA and protein levels are dramatically reduced in SIK348 flies (Figure 1B-C).

SIK3 shows high levels of expression in the fat body, the fly equivalent of the mammalian liver and adipose tissue. Targeted transgenic expression of wild-type SIK3 in fat body restored lipid stores and starvation resistance and an associated developmental delay in SIK3 mutants (Figures 1F-G and data not shown). Transgenic expression of SIK3 in fat body also rescued the lethality of SIK372 null mutant flies (Figure 8C). By contrast, a kinase-dead form of SIK3 (SIK3.K70M) had no rescuing effect in this setting, indicating that SIK3 catalytic activity is required to maintain lipid balance in vivo (Figure 1H). Indeed, LKB1 phosphorylation-defective SIK3 (SIK3.T196A) also failed to rescue the metabolic phenotype in fat body (data not shown).

Because they share similar substrate specificities, other AMPK family members might be predicted to substitute for SIK3 in fat body. Supporting this idea, expression of SIK2 in fat body fully rescued lipid accumulation (Figure 1H); but AMPK did not, indicating that SIK kinases have distinct regulatory properties from other members of this family.

EXAMPLE 2

Insulin Regulates SIK3 Activity

Having found that SIK3 mutant flies are sensitive to starvation, it was believed that SIK3 activity may be required for insulin effects on lipid accumulation during feeding. Supporting a genetic link between SIK3 and the insulin signaling pathway, fat body specific over-expression of the insulin-responsive Ser/Thr kinase AKT increased

lipid levels in control flies (Verdu et al., 1999), but it had no effect in SIK3 mutants (Figure 2A).

Because SIK3 appears to be required for lipid accumulation, it was tested whether insulin regulates SIK3 activity. Supporting this notion, SIK3 catalytic activity in fat body was elevated during refeeding and decreased in response to fasting by in vitro kinase assay of HA-tagged SIK3 immunoprecipitates prepared from HA-SIK3 transgenic flies (Figure 2B). Moreover, exposure of Drosophila S2 cells to insulin increased the phosphorylation of SIK3 at potential AKT phosphorylation sites, as evaluated using a phospho-AKT substrate antibody (PAS); these effects were diminished when cells were depleted of AKT with a double-stranded AKT oligonucleotide (Figure 9A). Consistent with the proposed role of insulin signaling on SIK3 activity, disruption of chico, the fly homolog of insulin receptor substrate (Bohni et al., 1999), also blocked SIK3 phosphorylation (Figure 2C). Although it had no effect on fat stores by itself, disruption of one chico allele reduced lipid levels in SIK3 hypomorphic mutant flies, demonstrating a genetic interaction between these effectors (Figure 9B).

To identify residues in SIK3 that undergo phosphorylation during feeding, mass spectrometry studies were performed on HA-SIK3 immunoprecipitates from transgenic flies expressing HA-tagged SIK3 in the fat body. This analysis revealed a number of phosphorylation sites, including the LKB1 site at Thr196, and two residues (Ser401, Thr 486) that, along with Thr 281 and Ser293, correspond to consensus (RXRXXS/T; SEQ ID NO:1) or near-consensus AKT phosphorylation sites in SIK3 and in its mammalian homolog Sik2 (Figure 9D,E). In keeping with these results, recombinant active AKT appeared to phosphorylate each of the four residues in SIK3 in vitro, by mutational analysis of a kinase-dead (K70M) form of SIK3 (Figure 9C). Furthermore, mutant SIK3.4A containing alanine substitutions at all 4 sites, was not phosphorylated by AKT in vitro or in cells exposed to insulin (Figure 2D-E). Next, the rescuing activities of wild-type and phosphorylation-defective SIK3 in fat body were compared. Although expression of wild-type SIK3 rescued lipid accumulation in SIK3 mutant flies, AKT phosphorylation defective SIK3.4A did not (Figure 2F), indicating that insulin promotes lipid accumulation during feeding in part through the AKT-mediated phosphorylation of SIK3. These studies support the notion that AKT is the physiological upstream kinase of SIK3 in fat body.

EXAMPLE 3

SIK3 Inhibits Expression of Brummer Lipase

In principle, the loss of SIK3 expression could reduce lipid accumulation by blocking lipogenesis or by increasing lipolysis. Arguing against an effect on lipid

absorption or on adipogenesis, high-fat feeding with soy oil rescued the fat storage defects and accompanying developmental delay in SIK3 mutants (Figure 10A and data not shown). Moreover, lipogenic gene expression (SREBP, ACC and FAS) in SIK3 mutants was comparable to wild-type, suggesting that triglyceride synthesis is not altered in SIK3 mutants (Figure 10B).

Under fasting conditions, increases in circulating AKH stimulate the mobilization of triglycerides by triggering the AKH receptor (AKHR) and stimulating cAMP signaling in fat body (Gronke et al., 2007). In parallel, decreases in insulin signaling lead to upregulation of Brummer (bmm) lipase, the fly homolog of adipose triglyceride lipase (ATGL) in mammals (Gronke et al., 2005). To evaluate the regulatory contributions of each pathway, SIK3 mutant flies were crossed to AKHR or bmm mutants.

Deletion of AKHR only partially rescued lipid accumulation in SIK3 mutant flies (Figure 3A), arguing against a direct effect of SIK3 on the AKH pathway per se. By contrast, SIK3/bmm double mutant flies were as obese as bmm single mutants, indicating that bmm likely acts downstream of SIK3. Supporting this notion, levels of bmm mRNA were upregulated in SIK3 mutant flies compared to wild-type (Figure 3B); elevated bmm mRNA levels were reduced following fat body specific expression of wild-type but not kinase-dead (K70M) SIK3. Collectively, these results indicate that SIK3 promotes lipid accumulation during feeding by down-regulating the bmm gene.

EXAMPLE 4

SIK3 Modulates FOXO Activity

Under fasting conditions, decreases in insulin signaling have been found to stimulate catabolic gene expression through the dephosphorylation and nuclear entry of the forkhead activator FOXO (Junger et al., 2003). Based on the presence of consensus FOXO binding sites at -56 and -46 in the bmm promoter (Figure 10C), FOXO may regulate bmm gene expression.

The kinetics of FOXO activation and lipid utilization in fasted flies (Figure 3C-E) were examined. Amounts of phosphorylated AKT fell over a 48 hour period after food withdrawal, leading to corresponding decreases in amounts of phosphorylated SIK3. Amounts of dephosphorylated, active FOXO increased in parallel with SIK3 inactivation in response to starvation; these were accompanied by increases in bmm mRNA levels and in lipid disposal. Consistent with a regulatory role for FOXO, fasting-associated increases in bmm gene expression were blocked in FOXO mutant flies (Figure 3F). Conversely, FOXO over-expression enhanced bmm promoter activity in reporter assays; these effects were blocked by mutation of the -46 FOXO binding site on the bmm promoter or by over-expression of SIK3 (Figure 10C-D). FOXO over-

expression in fat body was also sufficient to enhance bmm gene expression and to promote lipid depletion (Figure 10E-F).

Having seen that bmm gene expression is elevated in SIK3 mutant flies, does SIK3 negatively regulate FOXO activity.? Supporting this idea, amounts of nuclear-localized active FOXO were increased 5-fold in ad libitum fed SIK3 mutant larvae compared to controls (Figure 4A-B). As a result, mRNA amounts for other FOXO target genes (4E-BP, PEPCK) were upregulated in SIK3 mutants relative to control flies (Figure 11A-B).

In the fed state, increases in insulin signaling reduce catabolic gene expression through AKT-mediated phosphorylation and cytoplasmic translocation of FOXO (Teleman et al., 2008). In principle, the increase in FOXO activity in SIK3 mutant flies could reflect decreases in either insulin expression or in insulin signaling. Arguing these possibilities, mRNA amounts for insulin like peptide 2 (Ilp2) were similar in wild-type and SIK3 mutant flies (Figure 11C); and refeeding upregulated AKT phosphorylation comparably in both groups (Figure 4C).

By contrast with its effects in wild-type flies, refeeding increased FOXO phosphorylation only modestly in SIK3 mutants (Figures 4C and 11D). Conversely, refeeding stimulated FOXO phosphorylation more robustly in transgenic flies over-expressing SIK3 in fat body relative to controls (Figure 4D). Pointing to a genetic link between SIK3 and FOXO, disruption of the FOXO gene restored both bmm expression and lipid accumulation in SIK3 mutant flies (Figure 4E-F). Taken together, these results suggest that SIK3 inhibits lipolysis during feeding by reducing FOXO activity and thereby lowering bmm gene expression in fat body.

EXAMPLE 5

SIK3 Regulates a Class IIa HDAC

SIK3 could modulate FOXO activity directly through phosphorylation. Although purified SIK3 was capable of phosphorylating recombinant FOXO in vitro, it did so with low stoichiometry and at non-consensus sites (Ser66, Ser531), which, when mutated to alanine, did not appear to enhance FOXO activity (Figure 11E-G). These results suggest that SIK3 likely regulates FOXO activity through an intermediary factor.

To identify this putative regulator, a genetic screen using a set of 270 chromosomal deletions covering 95% of the Drosophila genome was performed. By capitalizing on an ectopic wing vein phenotype caused by wing-specific knock-down of SIK3 function as an easily scorable output for SIK3 activity, we identified one deletion, Df(X)BSC713, which strongly suppressed the loss of SIK3 function in wing (Figure 12A-B). One of the genes deleted in Df(X)BSC713 is HDAC4, which has previously been identified as a substrate for SIK phosphorylation in both C. elegans and mammals

(Berdeaux et al., 2007; van der Linden et al., 2006). Supporting a functional link in Drosophila, removal of one copy of HDAC4 (HDAC4e04575) suppressed effects of SIK3 loss of function (LOF) in the wing. Conversely, gain of HDAC4 function (GOF) by over-expression in wing enhanced SIK3 LOF, and it suppressed SIK3 GOF (Figure 12C). Taken together, these results suggest that HDAC4 acts downstream of SIK3 in Drosophila.

Phosphorylation has been shown to sequester class IIa HDACs in the cytoplasm by stimulating their interaction with 14-3-3 proteins (Bassel-Duby and Olson, 2006). Following their de-phosphorylation, class IIa HDACs migrate to the nucleus where they modulate cellular gene expression by associating with relevant transcription factors. Consistent with the presence of three conserved consensus SIK phosphorylation sites known to be phosphorylated by SIKs in C. elegans and mammals (Berdeaux et al., 2007; van der Linden et al., 2006), wild-type fly HDAC4 was efficiently phosphorylated in vitro and in HepG2 and Drosophila S2 cells by SIK3 (Figure 5A-C and 12E). In contrast, SIK3 did not phosphorylate a mutant HDAC4 polypeptide containing alanine substitutions at all three putative Ser phospho-acceptor sites (Figure 5B-C). Indeed, phosphorylation-defective HDAC4 was more active than wild-type in promoting the ectopic wing vein phenotype (Figure 12D). In keeping with the ability of the cAMP dependent kinase PKA to disrupt the activity of the SIKs through phosphorylation at a conserved Ser residue corresponding to Ser563 in SIK3 (Screaton et al., 2004), exposure to cAMP agonist reduced amounts of phosphorylated HDAC4 in cells expressing wild-type SIK3 (Figures 5C and 12F). By contrast, exposure to cAMP agonist actually increased HDAC4 phosphorylation in cells expressing PKA phosphorylation-defective S563A mutant SIK3.

Realizing that HDAC4 phosphorylation promotes its nucleocytoplasmic shuttling, it was tested whether HDAC4 subcellular localization is regulated by dietary status. Under feeding conditions, HDAC4 was largely confined to the cytoplasm of larval fat body cells; fasting triggered nuclear shuttling of HDAC4 (Figure 5D). To determine whether SIK3 is sufficient to promote HDAC4 shuttling, Hela cells, which are defective in the AMPK family master kinase LKB1 (Hawley et al., 2003) were employed. Consistent with the absence of endogenous SIK-related activities, HDAC4 was primarily nuclear-localized in Hela cells. Over-expression of constitutively active SIK3T196E increased the cytoplasmic localization of HDAC4 (Figure 12G). Taken together, these results indicate that HDAC4 activity in fat body is regulated by phosphorylation or dephosphorylation in response to feeding and fasting signals.

EXAMPLE 6

HDAC4 Activates FOXO

Given the finding that SIK3 promotes the translocation of HDAC4 to the cytoplasm, did nuclear HDAC4 modulates FOXO activity? Supporting this possibility, HDAC4 was found to associate with FOXO in co-immunoprecipitation studies of Drosophila S2 and Hela cells expressing epitope-tagged HDAC4 and FOXO (Figure 6A). Consistent with its proposed role in regulating HDAC4 localization, over-expression of constitutively active SIK3T.196E disrupted the FOXO:HDAC interaction (Figure 13A). Furthermore, FOXO target gene expression was down-regulated in HDAC4 hypomorphic mutant flies (Figure 6B, top). Conversely, mRNA amounts for PEPCK, bmm, and CPTI were increased in transgenic flies over-expressing wild-type HDAC4 in fat body (Figure 13B) whereas non-FOXO target genes (eg. HSL) were relatively unchanged.

To test whether HDAC4 regulates FOXO target gene expression in the fat body itself, bmm mRNA levels were measured in fat bodies isolated from early L3 larvae expressing a constitutively active HDAC4.3A transgene in fat body. bmm mRNA amounts were elevated in fat bodies from transgenic compared to control larvae, although the extent of bmm upregulation in larval fat bodies was more modest than in adult flies (Figure 13C).

Arguing against a general effect of this deacetylase on cellular gene transcription, GAL4 driven expression of a GFP transgene was unaffected by HDAC4 over-expression (Figure 13D). Pointing to the importance of nuclear HDAC4, phosphorylation-defective HDAC4 was more active than wild-type HDAC4 in stimulating FOXO target gene expression in fat body (Figure 6B, bottom). Consistent with the substantial upregulation of bmm in adult flies, HDAC4.3A transgenic flies also had lower lipid stores, and they were more sensitive to starvation compared to controls (Figure 13E-F). Taken together, these results indicate that HDAC4 is both necessary and sufficient to activate FOXO.

Under feeding conditions, P300 and CBP have been shown to inhibit catabolic gene expression by acetylating FOXO1 at conserved lysine residues within the forkhead domain (Daitoku et al., 2004). Acetylation appears to reduce FOXO activity by reducing its DNA binding affinity and thereby enhancing its phosphorylation by AKT (Frescas et al., 2005; Matsuzaki et al., 2005). Conversely, stress and nutrient deprivation have been found to increase FOXO activity during starvation through deacetylation by the NAD+ dependent deacetylase SirTl and by trichostatin A-sensitive class I/II histone deacetylases (Brunet et al., 2004; Matsuzaki et al., 2005).

In parallel with its stimulatory effects on FOXO phosphorylation, refeeding also enhanced FOXO acetylation in wild-type flies, but not in SIK3 mutants (Figure 6C). By contrast, amounts of acetylated FOXO were substantially increased in HDAC4

hypomorphic mutants after 24 hours of fasting, and they remained elevated during refeeding (Figure 6D).

In principle, HDAC4 could reduce amounts of acetylated FOXO by blocking its association with P300/CBP or by catalyzing its deacetylation. Arguing for the latter scenario, purified HDAC4 was capable of deacetylating FOXO, which had been acetylated in vitro by the HAT coactivator P300 (Figure 13G and 6E). Superimposed on its direct effects, HDAC4 may also regulate FOXO activity through an association with HDAC complexes. In mass spectrometry studies to identify HDAC4-associated proteins in fat body, we recovered SMRTER, EBI, and HDAC3, all components of a deacetylase complex that have been shown to interact with class IIa HDACs in mammalian cells (Fischle et al., 2002) (Figure 13H). Consistent with the ability of HDAC4 to promote FOXO deacetylation, either directly or through its association with the SMRTER complex, addition of the Class I/II HDAC inhibitor trichostatin A (TSA) to the food increased lipid levels in SIK3 mutant flies (Figure 6F).

EXAMPLE 7

SIK3 Inhibits HDAC4-Mediated Deacetylation of FOXO

Having observed that HDAC4 regulates FOXO activity, it was tested whether SIK3 modulates FOXO target gene expression via an HDAC4-dependent mechanism. Disrupting HDAC4 expression, either through P-element mediated insertion (Figure 7A-C) or through RNAi-mediated depletion in fat body (Figure 7D-E), restored lipid levels in SIK3 mutant flies. Consistent with the increase in fat stores, bmm gene expression was also reduced to wild-type levels in HDAC4/SIK3 double-mutants. In keeping with the proposed role of FOXO in this process, amounts of phosphorylated, inactive FOXO were increased in HDAC4/SIK3 double mutants under refed conditions (Figure 7C). Taken together, these results suggest that SIK3 inhibits lipolysis during feeding by phosphorylating HDAC4 and blocking the deacetylation of FOXO.

Based on the proposed role of SIK3 and HDAC4 in fasting metabolism, it was tested whether this regulatory module in also conserved in mammals. Exposure to insulin triggered the phosphorylation of mammalian HDAC4 at Ser245 in wild-type mouse hepatocytes; these effects were disrupted following RNAi-mediated depletion of SIK2, the mammalian homolog of fly SIK3, which is expressed at highest levels in adipose and liver (Dentin et al., 2007; Horike et al., 2003) (Figure 7F and 14A). Conversely, exposure of hepatocytes to glucagon promoted the dephosphorylation of HDAC4 at Ser245, reflecting the inhibitory PKA-mediated phosphorylation of SIK2 at Ser587 (Figures 7G and 14A).

Having seen effects of insulin and glucagon on phosphorylation of a mammalian class IIa HDAC, we tested whether fasting or feeding signals also promote

shuttling of these deacetylases. Exposure of HepG2 hepatocytes to insulin promoted cytoplasmic translocation of a mammalian HDAC5-GFP fusion protein (Figure 14B). Conversely, exposure to inhibitors of SIK activity, such as PI3K inhibitor (LY294002) or FSK, stimulated the nuclear translocation of HDAC5-GFP (Figure 14B).

It was examined whether mammalian class IIa HDACs also regulate FOXO activity. Similar to Drosophila, endogenous mouse HDAC4 was found to associate with endogenous Foxo1 in primary hepatocytes by co-immunoprecipitation assay (Figure 14C). Inhibiting class IIa HDACs, either by treatment with trichostatin A or by RNAi-mediated depletion of HDAC4 and HDAC5, disrupted the induction of gluconeogenic FOXO target genes (Pckl, G6p) in response to glucagon (Figures 7H and 14D-E). Collectively, these studies demonstrate that the SIK/class IIa HDAC module is conserved in mammals, where it promotes catabolic gene expression through the upregulation ofFOXO activity (Figure 14F).

EXAMPLE 8

EXPERIMENTAL PROCEDURES

Fly strains: P element insertion EY06260 (Bloomington # 15962) was used to generate SIK3 deletions by imprecise excision. Two SIK3 deletion alleles were characterized: SIK348 is a hypomorphic allele that removes the first exon, resulting in a significant reduction of SIK3 mRNA; SIK372 is a null allele that deletes the first two exons, removing part of the kinase domain. Both SIK372 homozygotes and SIK372/Df(2R)P34 are lethal in larval stages. The deletion endpoints for each SIK3 mutant were determined by PCR and DNA sequencing. Flies were maintained on standard fly food at 25oC with light-dark cycle (Percival Incubator, Model: 136VL). 3-5 day old flies were used in each experiment. For refeeding studies, flies were fasted for 24 hours and then shifted to standard fly food for times indicated. For high-fat or trichostatin A feeding studies, 5% soy oil (Sigma #S7381) or 10 uM trichostatin A (Sigma# T1952) was mixed with the standard fly food.

Lipid measurement: TAG measurement was performed as described (Palanker et al., 2009). Samples were assayed using a Modulus microplate spectrophotometer at 560nm. Lipid levels were normalized to protein amounts in each homogenate using a Bradford assay (Bio-Rad). Measurements were performed in ad libitum fed adult flies unless otherwise indicated.

Starvation assay: 3- to 5-day-old flies were transferred to vials of 1% agar/PBS with filter papers soaked with H2O, and dead flies were scored every 4-8 hours. For protein, RNA, and lipid analysis, flies are fasted for about 24 hours unless otherwise indicated.

Cell culture and transfection: Drosophila S2, HEK293T, and HepG2 cells were maintained as previously described (Wang et al., 2008). Hela cells were cultured in DMEM (Mediatech, #10-017-CV) containing 10% FBS at 37oC with 5% CO2. Transfections were performed as reported (Wang et al., 2008). Human GFP-HDAC5 was employed for cell localization studies as described (Berdeaux et al., 2007). Primary hepatocytes were cultured as described (Dentin et al., 2007). Ad-Hdac4/5i viruses (Mihaylova et al., 2011) and Ad-Sik2i virus (Dentin et al., 2007) were generated as reported. 10 p.f.u./cell of adenovirally encoded Ad-Uni, Ad-Sik2i, Ad-Hdac4i and Ad-Hdac5i were used for RNAi-mediated knockdown experiments. After 2-3 days infection, cells were collected for protein or RNA analysis.

Western blot: Cells or flies were lysed on ice in lysis-buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 6 mM EGTA, 20 mM NaF, 1% TritonX-100, and protease inhibitors) for 15-20 min. After centrifugation at 13000 rpm for 15 min, supernatants were reserved for protein determinations and SDS-PAGE analysis. The following antibodies were used: anti-phospho-AKT substrate, anti-AKT, anti-Foxo1, anti-pS239-HDAC4, anti-Ac-lysine, anti-ACC, and anti-phosphor-PKA substrate antibodies are from Cell Signaling Technology. Anti-Hsp90, anti-HA and anti-HDAC4 antibodies are from Santa Cruz Biotechnology, anti-FLAG antibody is from Sigma, and anti-SIK2 antibody is from Abgent. Drosophila FOXO antibodies are from O. Puig, and anti-Ac-FOXO1K242/245 antibody is from A. Fukamizu. Anti-SIK3 (Drosophila) rabbit polyclonal antiserum was prepared and affinity purified as described (Wagner et al., 2000) using recombinant GST-SIK3 (aa. 381-695) as immunogen. Densitometric analysis is carried out using Scion Image software.

Statistical analyses: Statistical significance was determined using an unpaired two-tailed Student's t test with unequal variance. Data are presented as the average ± SEM.

EXAMPLE 9

Supplemental Experimental Procedures:

Fly strains: The SIK3 coding sequence was amplified using following primers: 5'GAATTCATGGCCACCACACCAACG-3' (SEQ ID NO:2) and 5'-GCGGCCGCTTATAATATTTTAGTTAGCCTC-3' (SEQ ID NO:3). The resulting fragment was inserted into HA-pcDNA3 vector using EcoRI and Not1, to generate HA-SIK3 construct. Various point mutants (K70M, T196A and 4A) were generated by PCR-based mutagenesis and confirmed by DNA sequencing. The UAS-SIK3 wild-type and mutants were made by PCR amplifying the HA-SIK3 using following primers: 5'-GCGGCCGCATGTACCCATACGATGTTCCA-3' (SEQ ID NO:4) and 5'-GCGGCCGCTTATAATATTTTAGTTAGCCTC-3' (SEQ ID NO:5). Then fragments

were inserted into pUAST vector. The HDAC4 coding sequence was amplified and inserted into FLAG-pCMV vector to generate FLAG-HDAC4 construct. Phosphorylation defective mutant 3A (S239/573/748A) were generated by PCR-based mutagenesis and confirmed by DNA sequencing. The UAS-HDAC4 wild-type and 3A mutant were generated by PCR amplifying the HA-SIK3 fragments and inserted into pUAST vector. Transgenic flies were established through standard methods (BestGene). Other fly stocks are: Df(2R)P34 (Bloomington #757), Df(X)BSC713 (Bloomington #26565), MS1096-GAL4 (Bloomington #8696), UASInR. A1325D (Bloomington #8263), UAS-AKT (Bloomington #8191), UAS-FOXO (Bloomington #9575), chico I (Bloomington #10738, backcrossed in w background for 5 generations), UAS-SIK3 RNAi (VDRC #39864 and #39866), UAS-HDAC4 RNAi (VDRC #20522 and Bloomington #28549), HDAC4KG09091 (Bloomington# 15159), HDAC4e04575 and HDAC4d07212 (Exelixis), Sir2[2A-7-11] (Bloomington# 8838), Sir2EP2384 (Bloomington # 24848), Sir2EP2300 (Bloomington# 24849), FOX021 and FOX025 (Ernst Hafen), UASAMPKTD (Jongkyeong Chung), ppl-GAL4, FB-GFP (FB-GAL4,UAS-GFP), AKHR1 and bmm1 (Ronald P. Külnlein), ptc-GAL4 (Matthew P. Scott) and r4-GAL4 (Jae H. Park).

Oxidative stress: Three- to 5-day-old flies were starved in 1% agar/PBS for 4 hr and then transferred to vials of 20mM Paraquat/10% sucrose/1% agar/PBS, and dead flies were scored every 4-8 hr. Kaplan-Meier curve analysis was employed in MedCalc.

qPCR Analysis: RNA from adult flies, fat body tissues of early L3 larvae or primary hepatocytes was extracted using RNease Mini Kit (QIAGEN). Total RNA (1ug) was reverse-transcribed by Superscript II transcriptase (Invitrogen) and the generated cDNA used for real time PT-PCR (Roche LightCycler 480 Real-Time PCR system, SYBRGreen), using 2 ng of cDNA template and a primer concentration of 400 nM. Values were normalized to rp49. Data are presented as fold changes as relative to controls.

Primers for Drosophila genes are:

SIK3-F	GCAACTGGACTCTGTGGTAA (SEQ ID NO:6)
SIK3-R	CCTTTGCTGCAGCTTGTCAT (SEQ ID NO:7)
SREBP-F	CGCAGTTTGTCGCCTGATG (SEQ ID NO:8)
SREBP-R	CAGACTCCTGTCCAAGAGCTGTT (SEQ ID NO:9)
ACC-F	GTGCAACTGTTGGCAGATCAGTA (SEQ ID NO:10)
ACC-R	TTTCTGATGACGACGCTGGAT (SEQ ID NO:11)
FAS-F	CCCCAGGAGGTGAACTCTATCA (SEQ ID NO:12)
FAS-R	GACTTGACCGATCCGATCAAC (SEQ ID NO:13)
FOXO-F	GGTCTCTCGCCGAACTCAGTAA (SEQ ID NO:14)

FOXO-R ACCACGTTGCTGTACGTGTT (SEQ ID NO:15)
PEPCK-F GTCCGCGCCCTACACTCT (SEQ ID NO:16)
PEPCK-R GCCGTAGCCCGATCCGTAA (SEQ ID NO:17)
4EBP-F CAATCAAATTGTCGCAGCCATA (SEQ ID NO:18)
4EBP-R TTAGCTGATTGATTGGATTGGTTTC (SEQ ID NO:19)
Lip3-F TCATGCACGGCATGCTGAG (SEQ ID NO:20)
Lip3-R GCCTTGGAGTAGGTGTT (SEQ ID NO:21)
CPTI-F ACACGATGACCAGGTACTT (SEQ ID NO:22)
CPTI-R TTGGTGGACCACCAGCTCTTGA (SEQ ID NO:23)
bmm-F GTCCCTTCAGTCCCTCCTTC (SEQ ID NO:24)
bmm-R TATGAAGCACGCACACAACA (SEQ ID NO:25)
HSL-F CCTACTGCCCAACTCTCGTC (SEQ ID NO:26)
HSL-R CGACTGGAGTTCAAGCTTCC (SEQ ID NO:27)
HDAC4-F ACAACGCGTCCAGTAACTCC (SEQ ID NO:28)
HDAC4-R CCAGTGTCGGGAATCTGACT (SEQ ID NO:29)
Dilp2-F ACGAGGTGCTGAGTATGGTGTGCG (SEQ ID NO:30)
Dilp2-R CACTTCGCAGCGGTTCCGATATCG (SEQ ID NO:31)
AKH-F TTTCGAGACACAGCAGGGCA (SEQ ID NO:32)
AKH-R GGTGCTTGCAGTCCAGAAA (SEQ ID NO:33)
rp49-F GCTAAGCTGTCGCACAAATG (SEQ ID NO:33)
rp49-R GTTCGATCCGTAACCGATGT (SEQ ID NO:34)

Primers for mouse genes are:

Sik2-F AACCCCTCCCTTGAGAGTGT (SEQ ID NO:35)
Sik2-R TTGGCAGGTCTGAGGCTTAT (SEQ ID NO:36)
Hdac4-F AAGCCCTCCTACTGGAGCA (SEQ ID NO:37)
Hdac4-R GGACTGACATGGGGAAGGT (SEQ ID NO:38)
Hdac5-F TTACAGCCCCTCCAGGTGTA (SEQ ID NO:39)
Hdac5-R GTGCTTCAGCATGAACGTGT (SEQ ID NO:40)
Foxo1-F TTTCTAAGTGGCCTGCGAGT (SEQ ID NO:41)
Foxo1-R GGTGGATACACCAGGGAATG (SEQ ID NO:42)
G6p-F CGACTCGCTATCTCCAAGTGA (SEQ ID NO:43)
G6p-R GTTGAACCAGTCTCCGACCA (SEQ ID NO:44)
Pck1-F CTGCATAACGGTCTGGACTTC (SEQ ID NO:45)
Pck1-R CAGCAACTGCCCGTACTCC (SEQ ID NO:46)
Nr4a2-F GTGTTCAGGCGCAGTATGG (SEQ ID NO:47)
Nr4a2-R TGGCAGTAATTTCAGTTGGT (SEQ ID NO:48)
36B4-F TTTGGGCATCACCACGAAAA (SEQ ID NO:49)

36B4-R GGACACCCTCCAGAAAGCGA (SEQ ID NO:50)

In vitro kinase assay: SIK3 protein, immunoprecipitated from transfected cells or flies, was incubated with 370 kBq [γ-32P] ATP in 25 μl of kinase buffer (25 mM HEPES, pH 7.5, 50 mM Tris, 50 mM MgCl2, 5 mM MnCl2, and 5 mM dithiothreitol) and recombinant AKT protein (Invitrogen; PV3184) at 30oC for 15 min. Reactions were terminated by heating at 100°C for 5 min. The reaction mixture was boiled then resolved by 8% SDS-PAGE. After electrophoresis, the gel was dried and autoradiographed. Autophosphorylation activity of SIK3 was examined in the absence of AKT. For measurement of FOXO phosphorylation, GST and GST-FOXO (including its deletion fragments and mutants) proteins were incubated with purified SIK3 or recombinant mammalian SIK2 protein (Invitrogen; PV4792).

In vitro acetylation and deacetylation: 5 ug of GST-FOXO fragment were incubated with purified recombinant HAT fragment of p300 (Upstate Biotechnology, #14-418) or purified full-length p300 in the presence of acetyl-CoA (50 uM) for one hour at 30°C. The reactions were performed in a buffer containing 50 mM Tris HCl (pH 8), 0.1 mM EDTA, 1 mM DTT, 10% glycerol. An aliquot was resolved on SDS-PAGE and detected by anti-ac-lysine antibody, the left were further purified by GST beads. Ac-FOXO fragment was incubated in deacetylation buffer (25 mM Tris HCl (pH 8), 137 mM NaCl, 2.7 mM KCl, 1mM MgCl2) in the presence of purified HDAC4. The reactions were resolved on SDS-PAGE and analyzed by western-blotting.

Mass Spectrometry: Mass spectrometry analysis of SIK3 phosphorylation sites were performed on immunoprecipitates of HA-SIK3 prepared from refed transgenic flies expressing HA-SIK3 in fat body as previously described on an LTQ XL from Thermo Scientific (Screaton et al., 2004). MS data was searched against the most resent versions of the concatenated Drosophila and human protein databases with the differential modification of +80.0 on serine and threonine as previously described except for this analysis the modified and unmodified peptides were individually searched as being tryptic, half-tryptic and fully-tryptic using DTASelect2 software. In each of these sub-groups the distribution of Xcorr and DeltaCN values for a direct (to the direct database) and decoy (reversed database) were separated by quadratic discriminant analysis (Meier et al., 2009). Spectral matches were retained with Xcorr and deltaCN values that produced a maximum peptide false positive rate of 1% which was derived from the frequency of matches to the decoy reverse database (number of decoy database hits/number of filtered peptides identified x 100) (Cociorva et al., 2007; Tabb et al., 2002). This value is calculated by the DTASelect2 software. Mass spectrometry studies were also performed on HA-SIK3 immunoprecipitates as previously described (Wang et al., 2009). Mass spectrometry studies of HDAC4

associated proteins were also performed on FLAG-HDAC4 immunoprecipitates in fat body similarly.

Bibliograpy:

Baker, K. D., and Thummel, C. S. (2007). Cell Metab 6, 257-266.

Barthel, A., et al. (2005). Trends Endocrinol Metab 16, 183-189.

Bassel-Duby, R., and Olson, E. N. (2006). Annu Rev Biochem 75, 19-37.

Berdeaux, R., et al. (2007). Nat Med 13, 597-603.

Bohni, R., et al. (1999). Cell 97, 865-875.

Brazil, D.P., and Hemmings, B. A. (2001). Trends Biochem Sci 26, 657-664.

Brent, M. M., et al. (2008). Structure 16, 1407-1416.

Brunet, A., et al. (2004). Science 303, 2011-2015.

Clancy, D. J., et al. (2001). Science 292, 104-106.

Cociorva, D., et al. (2007). Curr Protoc Bioinformatics Chapter 13, Unit 13 14.

Daitoku, H., et al. (2004). Proc Natl Acad Sci U S A 101, 10042-10047.

Dentin, R., et al. (2007). Nature 449, 366-369.

Fischle, W., et al. (2002). Mol Cell 9, 45-57.

Frescas, D., et al. (2005). J Biol Chern 280, 20589-20595.

Fukuoka, M., et al.. (2003). Int J Mol Med 12, 503-508.

Gronke, S., et al. (2005). Cell Metab 1, 323-330.

Gronke, S., et al. (2007). PLoS Biol 5, e137.

Haberland, M., et al. (2009). Nat Rev Genet 10, 32-42.

Hawley, S. A., et al.. (2003). J Biol 2, 28.

Horike, N., et al. (2003). J Biol Chem 278, 18440-18447.

Ikeya, T., et al. (2002). Curr Biol 12, 1293-1300.

Junger, M.A., et al. (2003). J Biol 2, 20.

Kim, J. K., et al. (2004). J Clin Invest 114, 823-827.

Kim, S. K., and Rulifson, E. J. (2004). Nature 4 31, 316-320.

Kitamura, T., et al. (1999). Mol Cell Biol 19, 6286-6296.

Koo, S. H., et al. (2005). Nature 437, 1109-1111.

Matsuzaki, H., et al. (2005). Proc Natl Acad Sci U S A 102, 11278-11283.

Meier, J. L., et al. (2009). ACS Chem Biol 4, 948-957.

Mihaylova, M., et al. (2011). Cell In the Press.

Okamoto, M., et al. (2004). Trends Endocrinol Metab 15, 21-26.

Palanker, L., et al. (2009). Cell Metab 9, 228-239.

Reiter, L. T., and Bier, E. (2002). Expert Opin Ther Targets 6, 387-399.

Screaton, R. A., et al. (2004). Cell 119, 61-74.

Tabb, D. L., et al. (2002). J Proteome Res 1, 21-26.

Teleman, A. et al. (2008). Cell Metab 7, 21-32.
van der Linden, et al. (2006). Embo J.
Verdu, J., et al. (1999). Nat Cell Biol 1, 500-506.
Wagner, B., et al. (2000). J Biol Chem 275, 8263-8266.
Wang, B., et al. (2008). Cell Metab 7, 434-444.
Wang, Y., et al. (2009). Nature 460, 534-537.
Williams, S. R., et al. (2010). Am J Hum Genet 87, 219-228.

Having thus described in detail embodiments of the present invention, it is to be understood that the invention defined by the above paragraphs is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

Each patent, patent application, and publication cited or described in the present application is hereby incorporated by reference in its entirety as if each individual patent, patent application or publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method of treating obesity, comprising administering to a subject in need thereof an agonist of a Class IIa histone deacetylase (HDAC).

2. The method of claim 1, wherein the Class IIa HDAC is selected from the group consisting of HDAC 4, 5, 7, and 9.

3. The method of claim 1, wherein the Class IIa HDAC agonist enhances the activity of at least one Class IIa HDAC selected from the group consisting of HDAC 4, 5, 7, and 9.

4. The method of claim 3, wherein the Class IIa HDAC agonist enhances the activity of HDAC4.

5. The method of claim 1, wherein the Class IIa HDAC agonist lowers lipid levels in the subject.

6. The method of claim 1, wherein administration of the Class IIa HDAC agonist results in an increase in lipolysis in the subject.

7. The method of claim 1, wherein administration of the Class IIa HDAC agonist results in a reduction of one or more triglyceride stores in the subject.

8. The method of claim 1, wherein the Class IIa HDAC agonist enhances expression of a Class IIa HDAC.

9. The method of claim 1, wherein the Class IIa HDAC agonist enhances nuclear localization of a Class IIa HDAC.

10. The method of claim 1, wherein the Class IIa HDAC agonist enhances dephosphorylation of a Class IIa HDAC.

11. The method of claim 1, wherein the Class IIa HDAC agonist enhances Class IIa HDAC mediated deacetylation of a transcription factor.

12. The method of claim 11, wherein the transcription factor is a FOXO transcription factor.

13. The method of claim 1, wherein the Class IIa agonist enhances the interaction of one or more Class IIa HDACs with an interacting protein.

14. The method of claim 13, wherein the interacting protein is selected from the group consisting of:HDAC3, SMRTER, EBI, and/or a homolog thereof.

15. A method of screening for a compound for treating obesity, comprising expressing a Class IIa HDAC in the fat body of a fly, wherein the fly is *Drosophila,* in the absence and presence of a test compound, and evaluating the activity of the Class IIa HDAC in the absence and presence of the test compound.

16. The method of claim 15, wherein the activity of the Class IIa HDAC is selected from the group consisting of expression, localization, phosphorylation state, and FOXO transcription factor deacetylation.

17. The method of claim 15, wherein a compound for treating obesity is identified where the compound increases the activity of the Class IIa HDAC compared to the activity of the Class IIa HDAC in the absence of the compound.

18. The method of claim 17, wherein the compound lowers lipid levels in the subject.

19. The method of claim 17, wherein the compound increases lipolysis.

20. The method of claim 17, wherein the compound decreases one or more triglyceride stores.

21. A method of treating a metabolic disease, comprising administering to a subject in need thereof a modulator of a Class IIa histone deacetylase (HDAC), wherein the modulator modulates the activity of a Class IIa HDAC interacting protein.

22. The method of claim 21, wherein the Class IIa HDAC interacting protein is SIK3 or a homolog thereof.

23. The method of claim 22, wherein the Class IIa HDAC modulator modulates the activity of HDAC 4.

24. The method of claim 21, wherein the Class IIa HDAC modulator is an inhibitor of SIK3 or a homolog thereof.

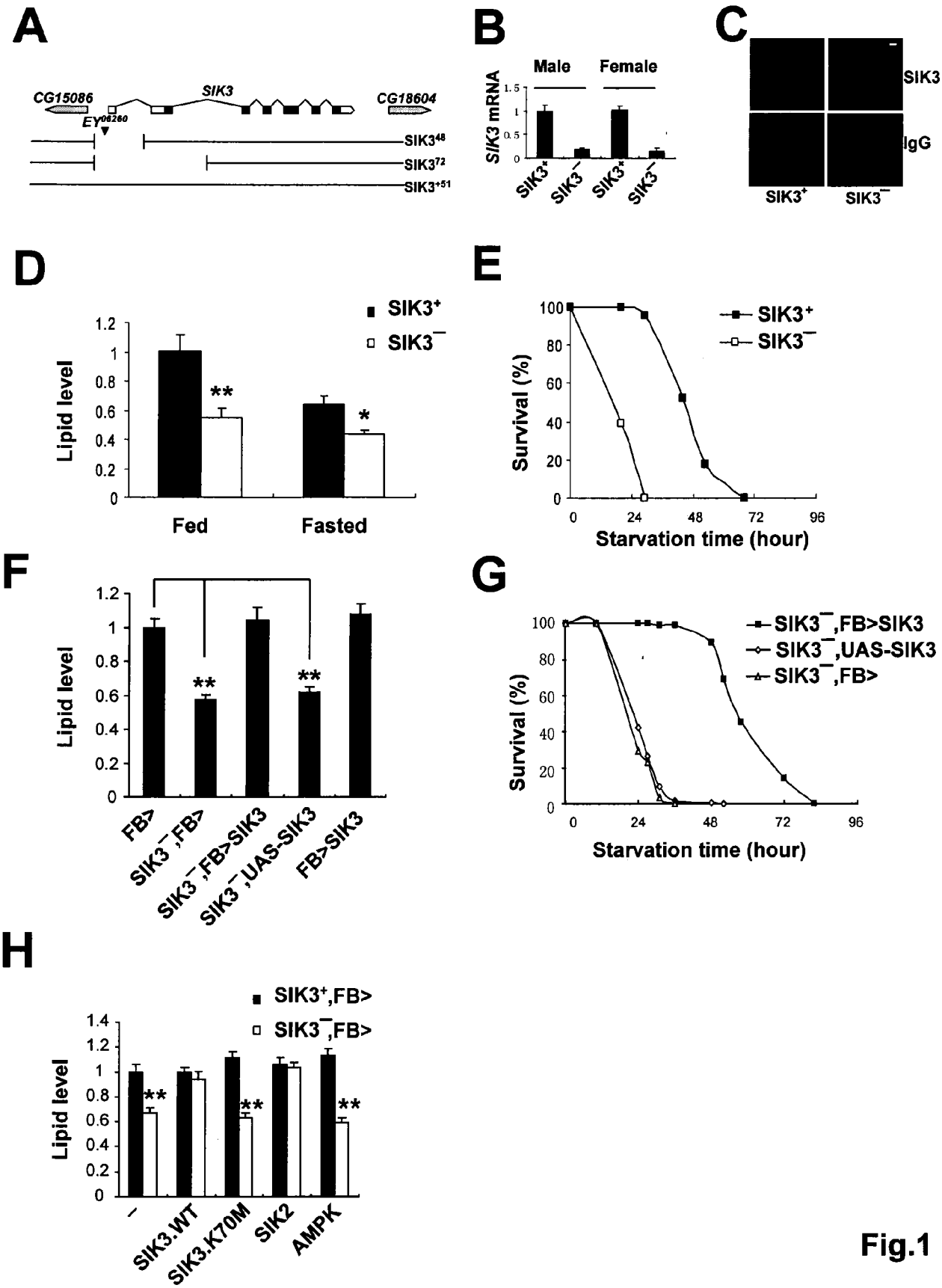

A
CG15086
SIK3
CG18604
EY06260
SIK3^48
SIK3^72
SIK3^+51
B
Male
Female
SIK3 mRNA
SIK3+
SIK3−
C
SIK3
IgG
D
SIK3+
SIK3−
Lipid level
Fed
Fasted
E
Survival (%)
Starvation time (hour)
F
Lipid level
FB>
SIK3−,FB>
SIK3−,FB>SIK3
SIK3−,UAS-SIK3
FB>SIK3
G
SIK3−,FB>SIK3
SIK3−,UAS-SIK3
SIK3−,FB>
Survival (%)
Starvation time (hour)
H
SIK3+,FB>
SIK3−,FB>
Lipid level
–
SIK3.WT
SIK3.K70M
SIK2
AMPK

Fig.1

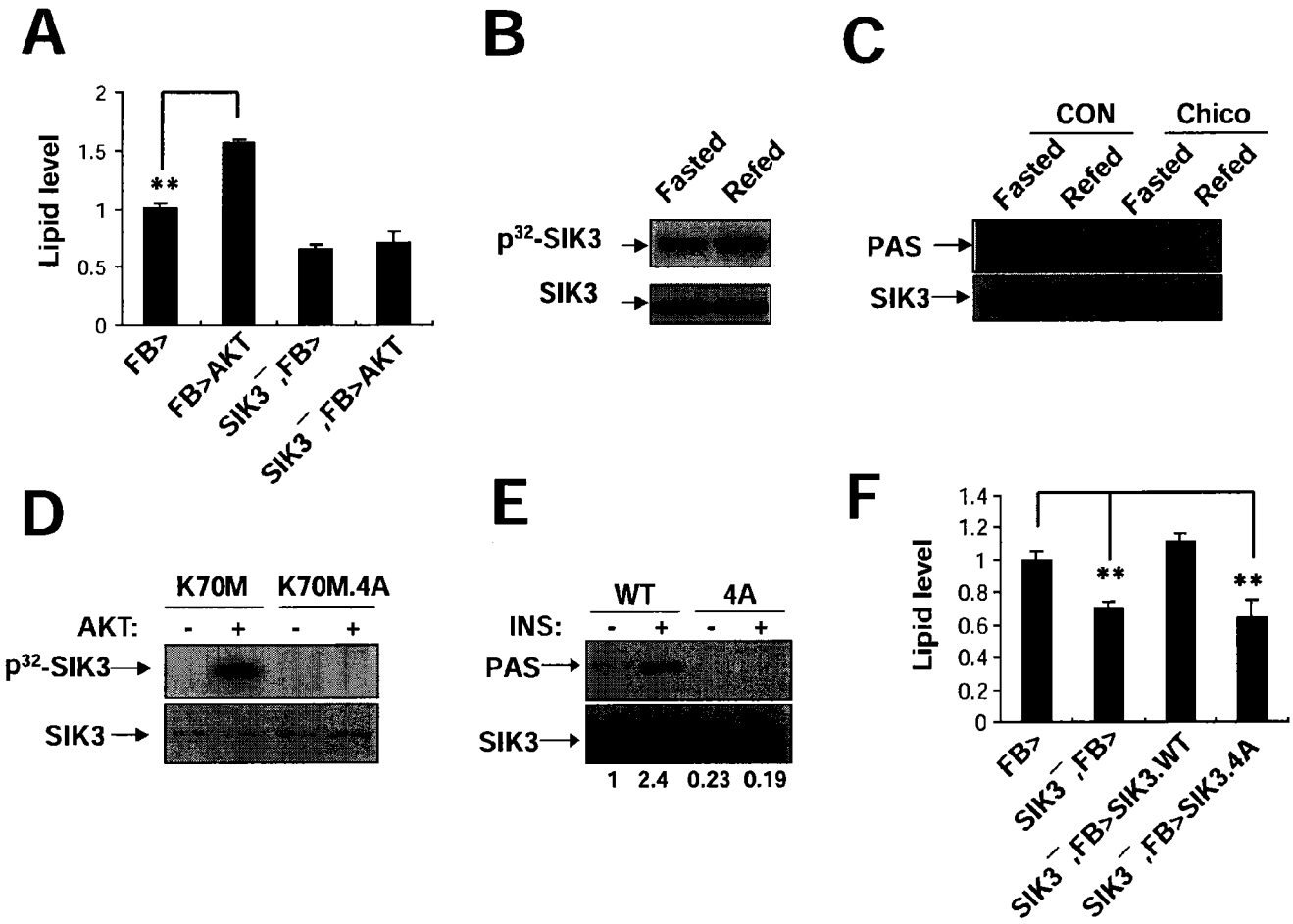

A
Lipid level
2
1.5
1
0.5
0
**
FB>
FB>AKT
SIK3⁻,FB>
SIK3⁻,FB>AKT
B
Fasted
Refed
p32-SIK3
SIK3
C
CON
Chico
Fasted
Refed
Fasted
Refed
PAS
SIK3
D
K70M
K70M.4A
AKT: - + - +
p32-SIK3
SIK3
E
WT
4A
INS: - + - +
PAS
SIK3
1 2.4 0.23 0.19
F
Lipid level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
FB>
SIK3⁻,FB>
SIK3⁻,FB>SIK3.WT
SIK3⁻,FB>SIK3.4A

Fig.2

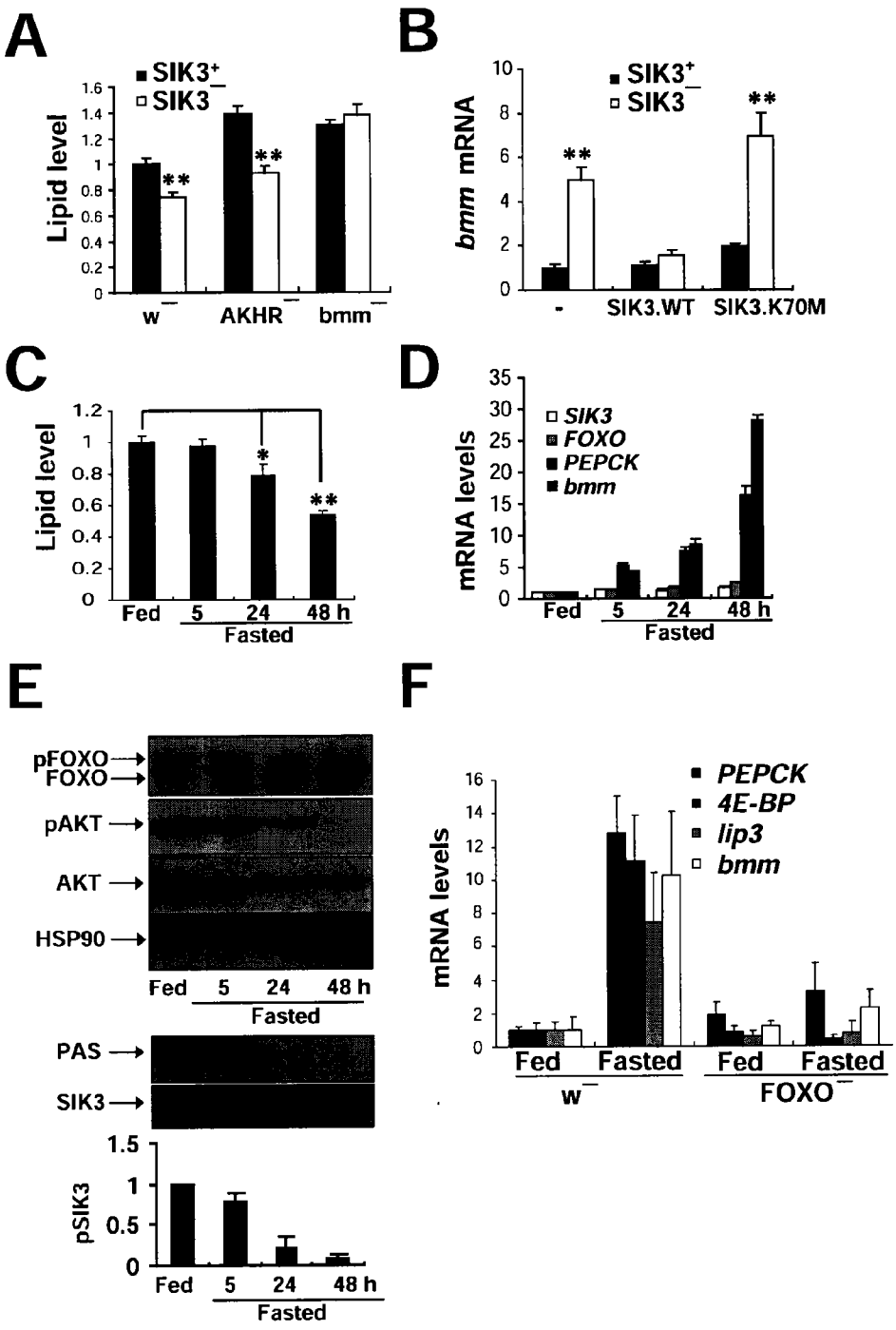

A
SIK3+
SIK3−
Lipid level
w−
AKHR−
bmm−
B
bmm mRNA
-
SIK3.WT
SIK3.K70M
C
Lipid level
Fed
5
24
48 h
Fasted
D
SIK3
FOXO
PEPCK
bmm
mRNA levels
E
pFOXO
FOXO
pAKT
AKT
HSP90
PAS
SIK3
pSIK3
F
PEPCK
4E-BP
lip3
bmm
mRNA levels
Fed
Fasted
w−
FOXO−

Fig.3

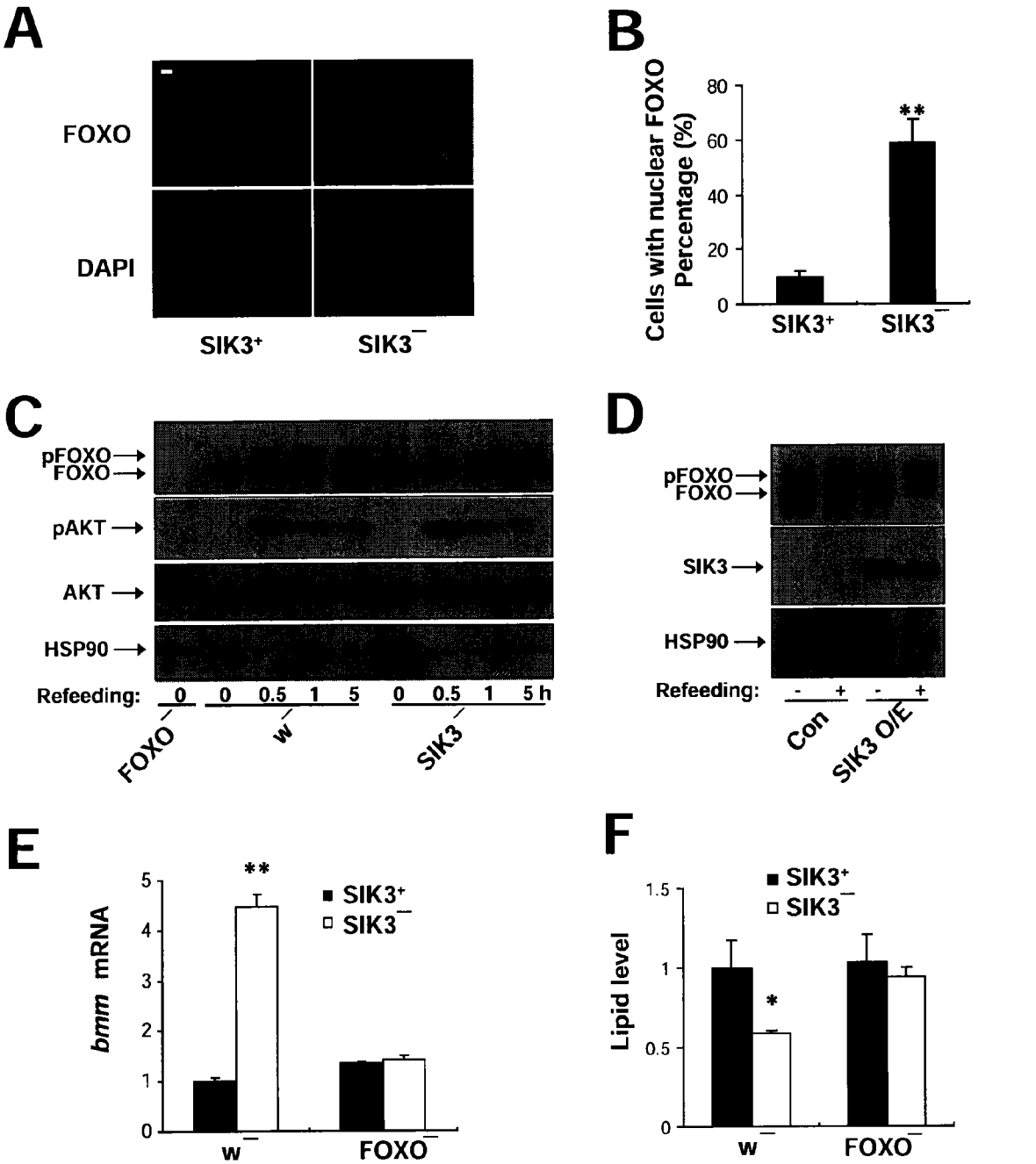

A
FOXO
DAPI
SIK3+
SIK3−
B
Cells with nuclear FOXO Percentage (%)
80
60
40
20
0
**
SIK3+
SIK3−
C
pFOXO
FOXO
pAKT
AKT
HSP90
Refeeding: 0 0 0.5 1 5 0 0.5 1 5 h
FOXO−
w−
SIK3−
D
pFOXO
FOXO
SIK3
HSP90
Refeeding: - + - +
Con
SIK3 O/E
E
bmm mRNA
5
4
3
2
1
0
**
SIK3+
SIK3−
w−
FOXO−
F
Lipid level
1.5
1
0.5
0
*
SIK3+
SIK3−
w−
FOXO−

Fig.4

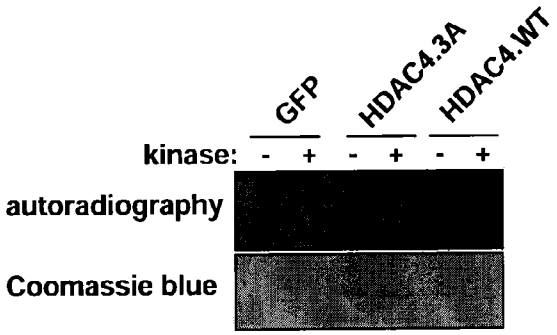
A

SIK consensus site:

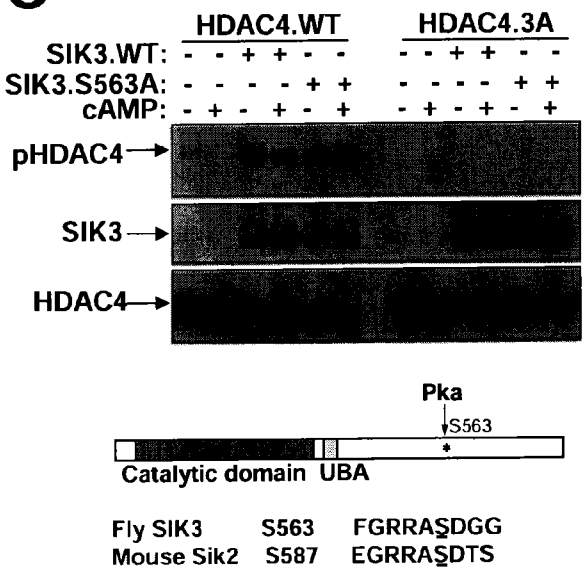
LX[R/K][T/S]XSXXX[L/V/P/M]

Fly HDAC4	S239	LRKTASEPNL
Mouse Hdac4	S245	LRKTASEPNL
Mouse Hdac5	S251	LRKTASEPNL
Mouse Hdac7	S178	LRKTVSEPNL
Fly HDAC4	S573	LGRTQSAPLP
Mouse Hdac4	S465	LGRTQSAPLP
Mouse Hdac5	S490	LSRTQSAPLP
Mouse Hdac7	S178	LSRTQSSPAA
Fly HDAC4	S748	LSRTLSSPLV
Mouse Hdac4	S629	LSRAQSSPAS

B



C



Fly SIK3	S563	FGRRASDGG
Mouse Sik2	S587	EGRRASDTS

D

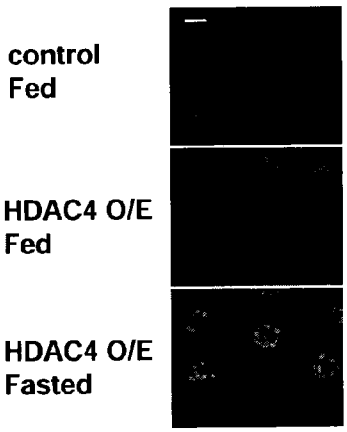


Fig.5

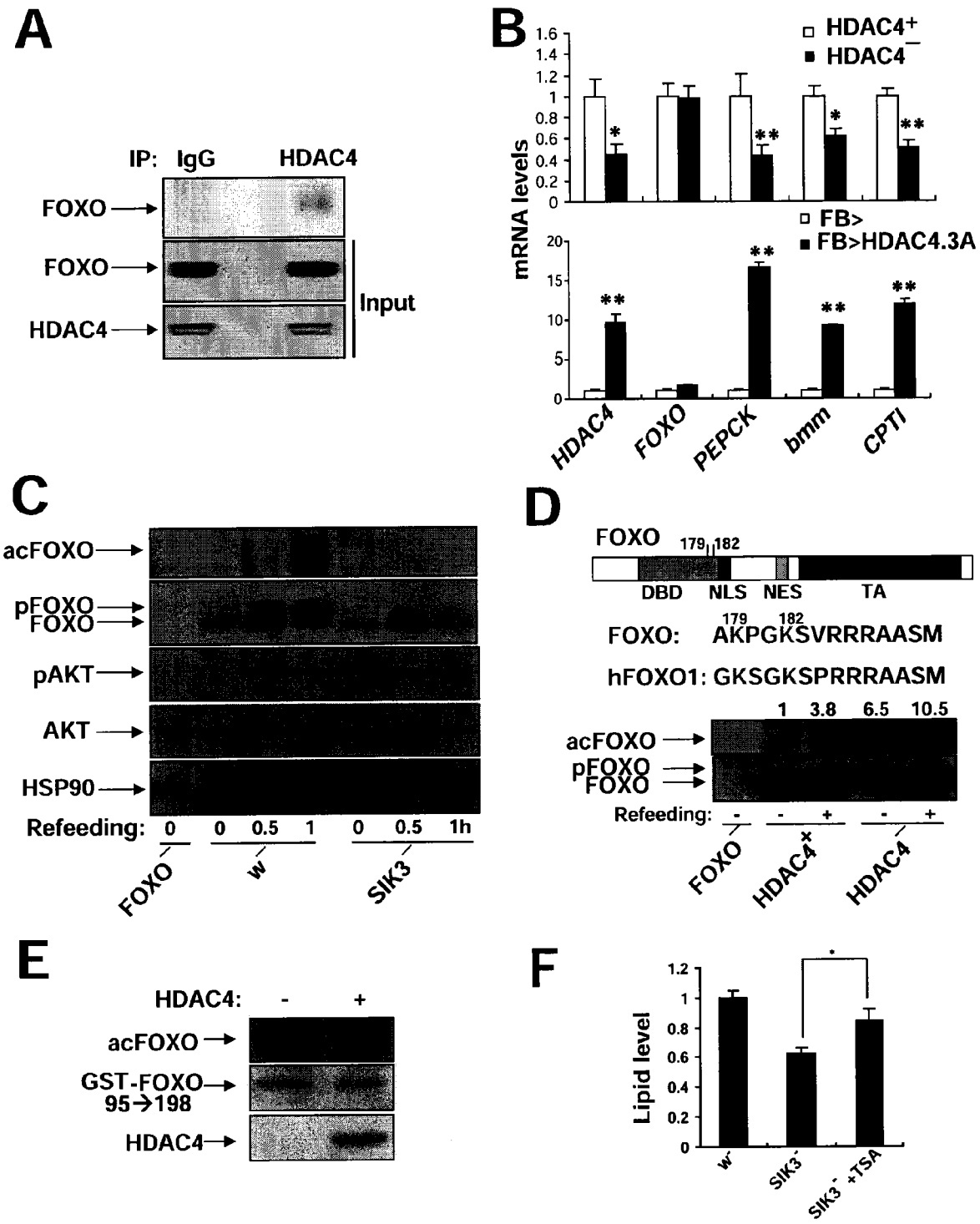

A
IP: IgG HDAC4
FOXO
FOXO
HDAC4
Input
B
HDAC4+
HDAC4-
mRNA levels
FB>
FB>HDAC4.3A
HDAC4
FOXO
PEPCK
bmm
CPTI
C
acFOXO
pFOXO
FOXO
pAKT
AKT
HSP90
Refeeding: 0 0 0.5 1 0 0.5 1h
FOXO
w-
SIK3-
D
FOXO
179 182
DBD NLS NES TA
FOXO: AKPGKSVRRRAASM
hFOXO1: GKSGKSPRRRAASM
1 3.8 6.5 10.5
acFOXO
pFOXO
FOXO
Refeeding: - - + - +
FOXO
HDAC4+
HDAC4-
E
HDAC4: - +
acFOXO
GST-FOXO 95→198
HDAC4
F
Lipid level
w-
SIK3-
SIK3- +TSA

Fig.6

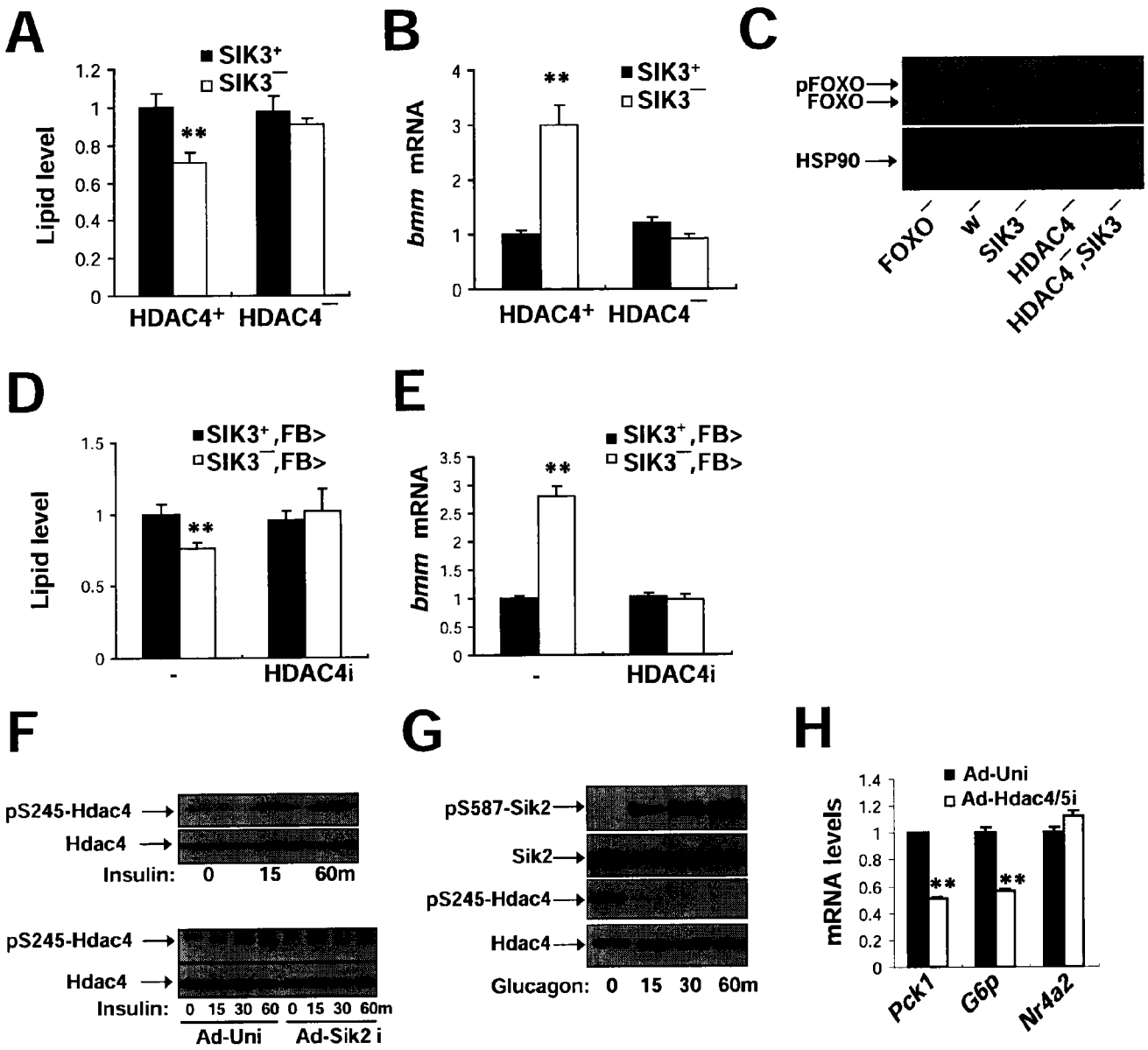

A
SIK3+
SIK3−
Lipid level
1.2
1
0.8
0.6
0.4
0.2
0
**
HDAC4+ HDAC4−
B
SIK3+
SIK3−
bmm mRNA
4
3
2
1
0
**
HDAC4+ HDAC4−
C
pFOXO
FOXO
HSP90
FOXO−/−
w−/−
SIK3−/−
HDAC4−/−
HDAC4−/−,SIK3−/−
D
SIK3+,FB>
SIK3−,FB>
Lipid level
1.5
1
0.5
0
**
-
HDAC4i
E
SIK3+,FB>
SIK3−,FB>
bmm mRNA
3.5
3
2.5
2
1.5
1
0.5
0
**
-
HDAC4i
F
pS245-Hdac4
Hdac4
Insulin: 0 15 60m
pS245-Hdac4
Hdac4
Insulin: 0 15 30 60 0 15 30 60m
Ad-Uni
Ad-Sik2 i
G
pS587-Sik2
Sik2
pS245-Hdac4
Hdac4
Glucagon: 0 15 30 60m
H
Ad-Uni
Ad-Hdac4/5i
mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Pck1
G6p
Nr4a2

Fig.7

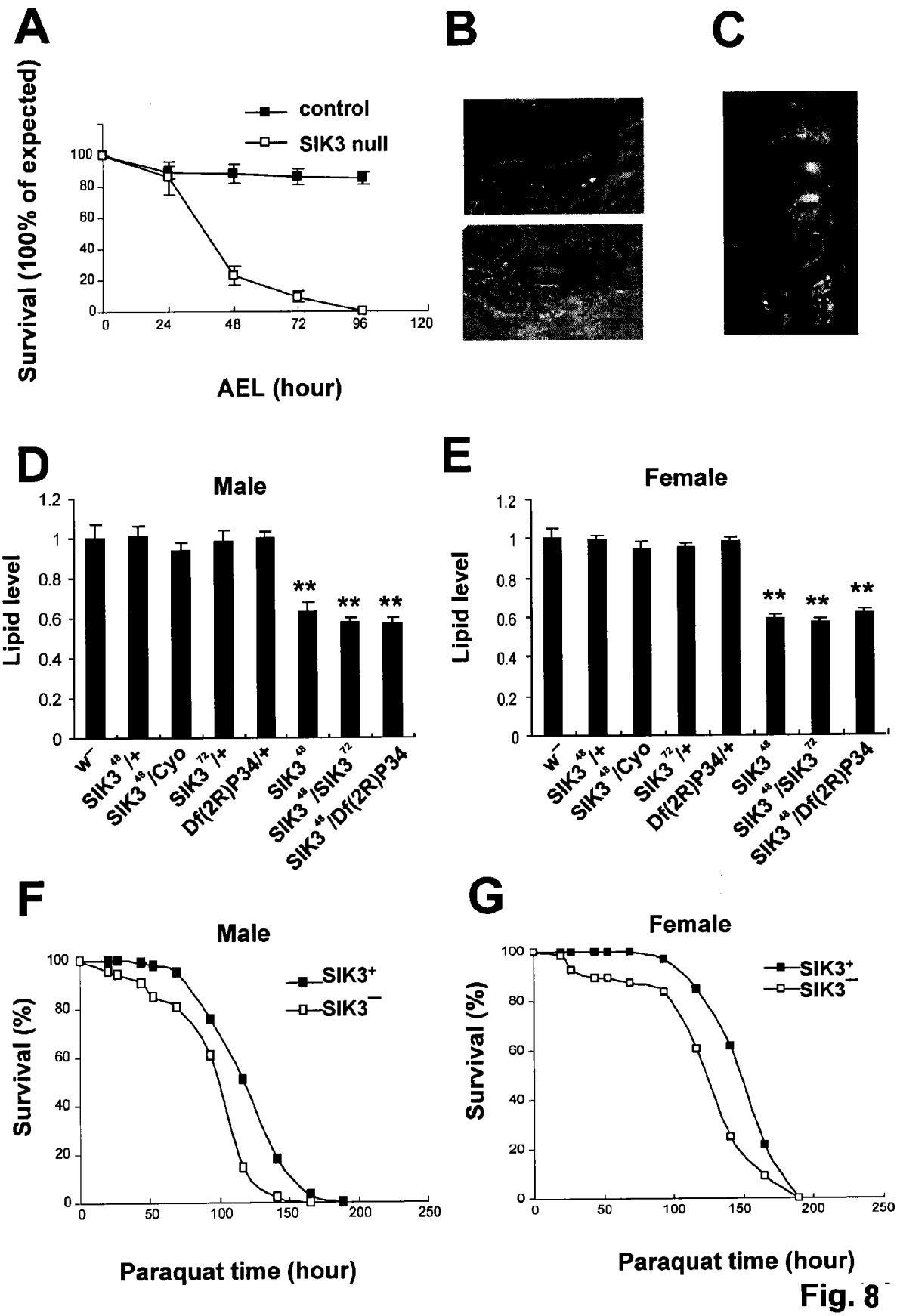
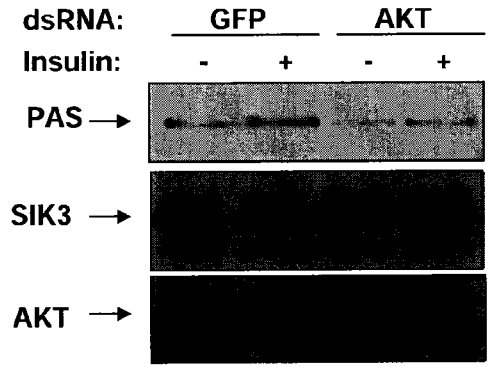
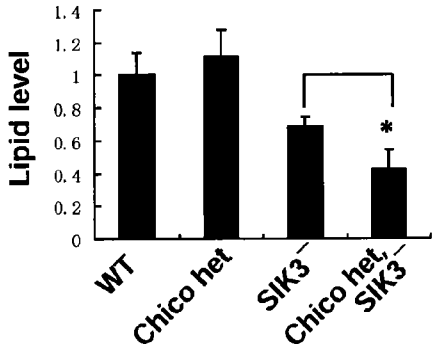

A
Survival (100% of expected)
control
SIK3 null
AEL (hour)
B
C
D
Male
Lipid level
E
Female
F
Male
Survival (%)
SIK3+
SIK3−
Paraquat time (hour)
G
Female
Survival (%)
SIK3+
SIK3−
Paraquat time (hour)

Fig. 8

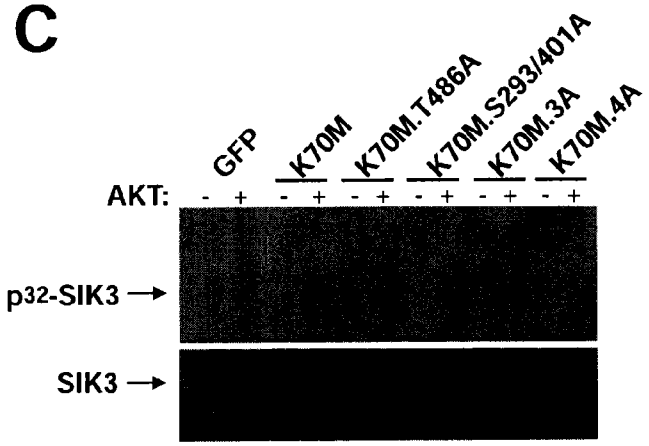
A



B



C



D

AKT consensus motif: RXRXXS/T

Fly SIK3	S293	KHRWLSE
Fly SIK3	S401	RSRKTSI
Mouse Sik2	S358	RWRRPST
Fly SIK3	T281	PDRRYTI
Fly SIK3	T486	NTRRHTV
Mouse Sik2	T484	GQRRHIL

E

Table: Mass spectrometry analysis of SIK3 phosphorylation sties.

S/T	Peptide sequence	# peptides
T196	K.LADFGFSNHYEEGATLKT*WCGSPPYAAPEVFQGLEYDGPK.S	1
S312	R.FGDMSPGS*GTVSK.S	3
S401	R.KTS*ITTGVVDR.S	10
S422	R.SEPVKQESLDRLS*PLSNANASSSALGFGWSDVAVDLEK.Y	4
T486	R.RHT*VGPGDVAHEQALANPNVPPIDFK.C	6
S547	K.DQHLLKPPVVMGASS*FGR.R	1

Fig. 9

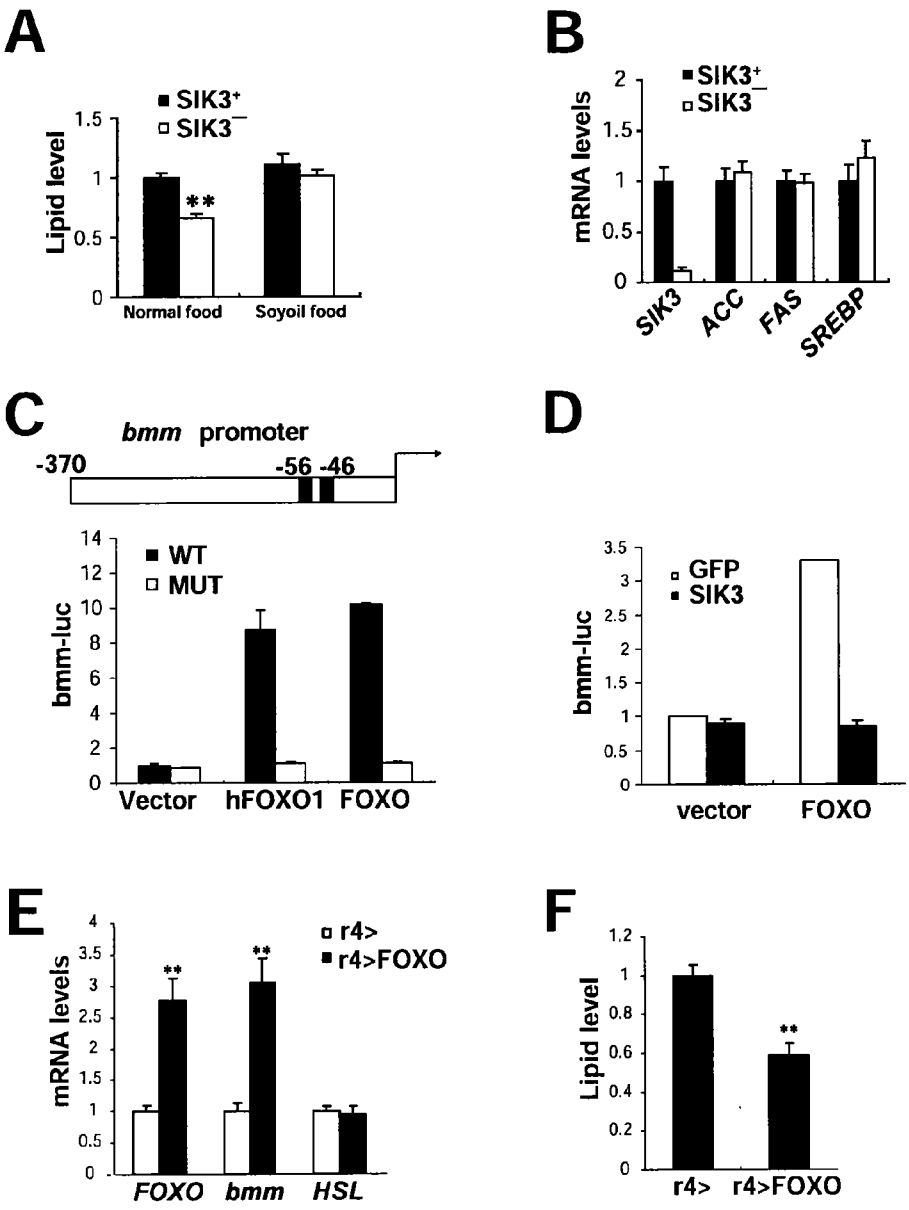

A
SIK3+
SIK3−
Lipid level
1.5
1
0.5
0
**
Normal food
Soyoil food
B
SIK3+
SIK3−
mRNA levels
2
1.5
1
0.5
0
SIK3
ACC
FAS
SREBP
C
bmm promoter
-370
-56 -46
WT
MUT
bmm-luc
14
12
10
8
6
4
2
0
Vector
hFOXO1
FOXO
D
GFP
SIK3
bmm-luc
3.5
3
2.5
2
1.5
1
0.5
0
vector
FOXO
E
r4>
r4>FOXO
mRNA levels
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
FOXO
bmm
HSL
F
Lipid level
1.2
1
0.8
0.6
0.4
0.2
0
**
r4>
r4>FOXO

Fig. 10

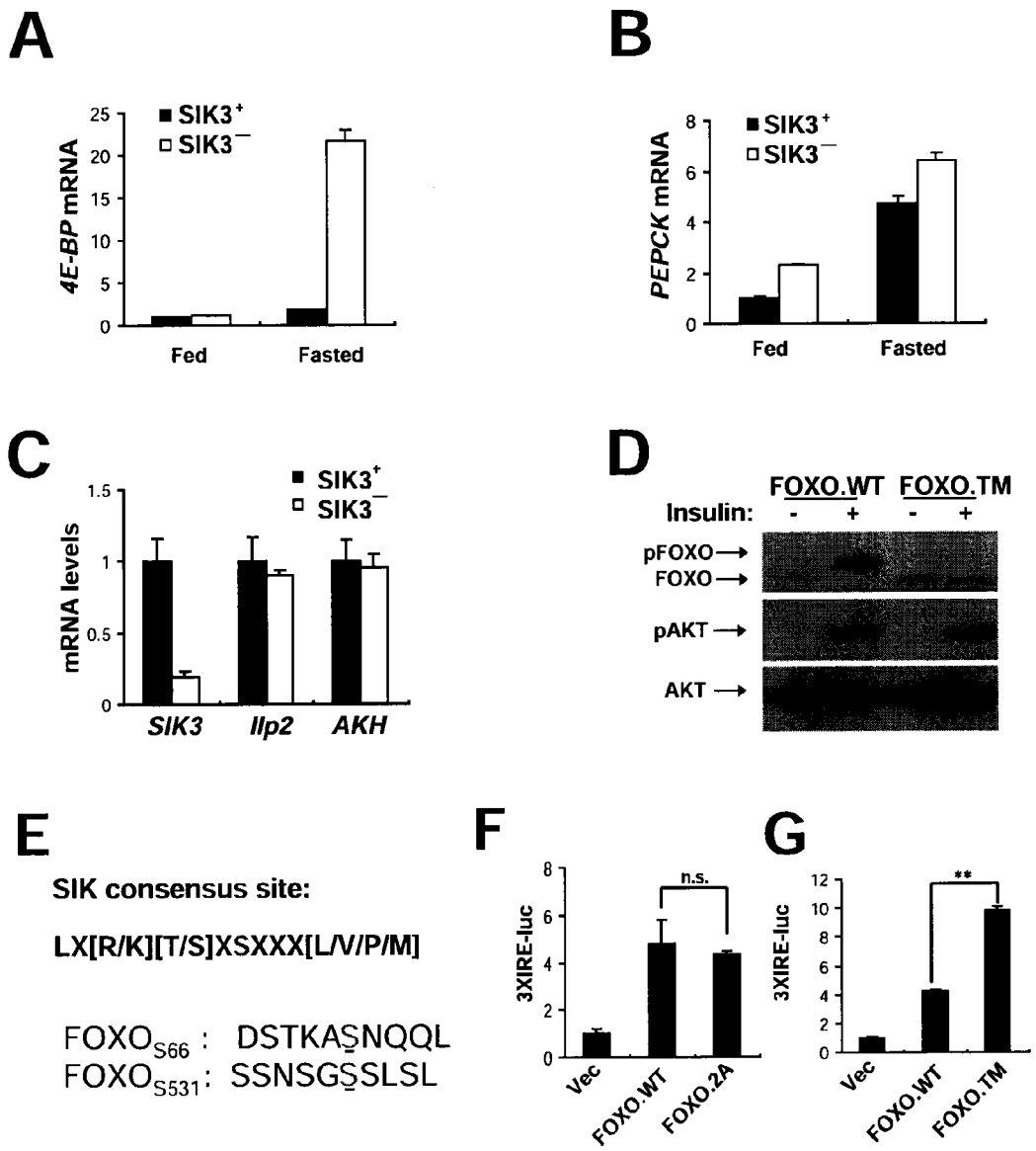

A
4E-BP mRNA
SIK3+
SIK3−
Fed
Fasted
B
PEPCK mRNA
SIK3+
SIK3−
Fed
Fasted
C
mRNA levels
SIK3+
SIK3−
SIK3
Ilp2
AKH
D
FOXO.WT FOXO.TM
Insulin: - + - +
pFOXO
FOXO
pAKT
AKT
E
SIK consensus site:
LX[R/K][T/S]XSXXX[L/V/P/M]
FOXOS66 : DSTKASNQQL
FOXOS531: SSNSGSSLSL
F
3XIRE-luc
n.s.
Vec
FOXO.WT
FOXO.2A
G
3XIRE-luc
**
Vec
FOXO.WT
FOXO.TM

Fig. 11

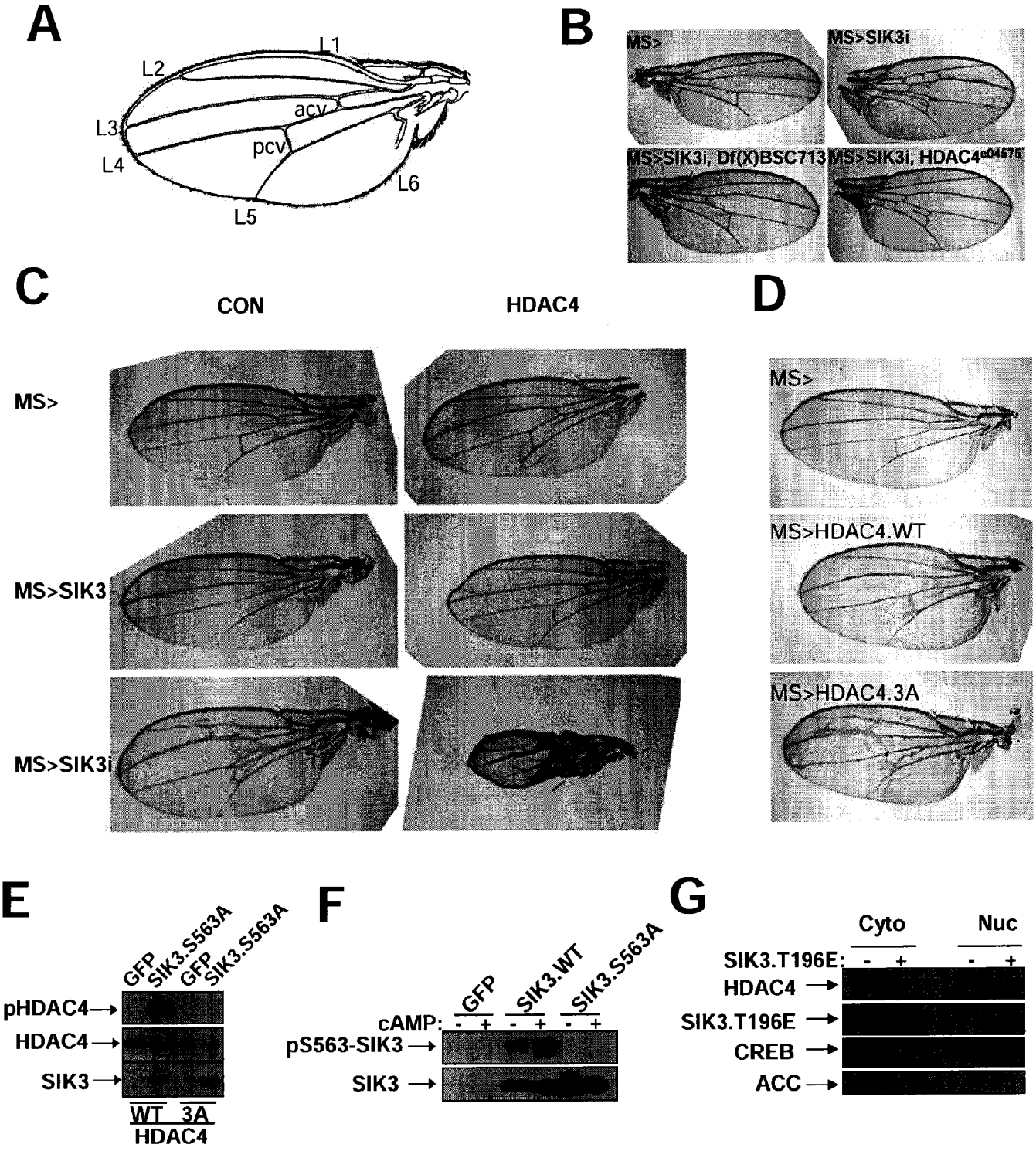
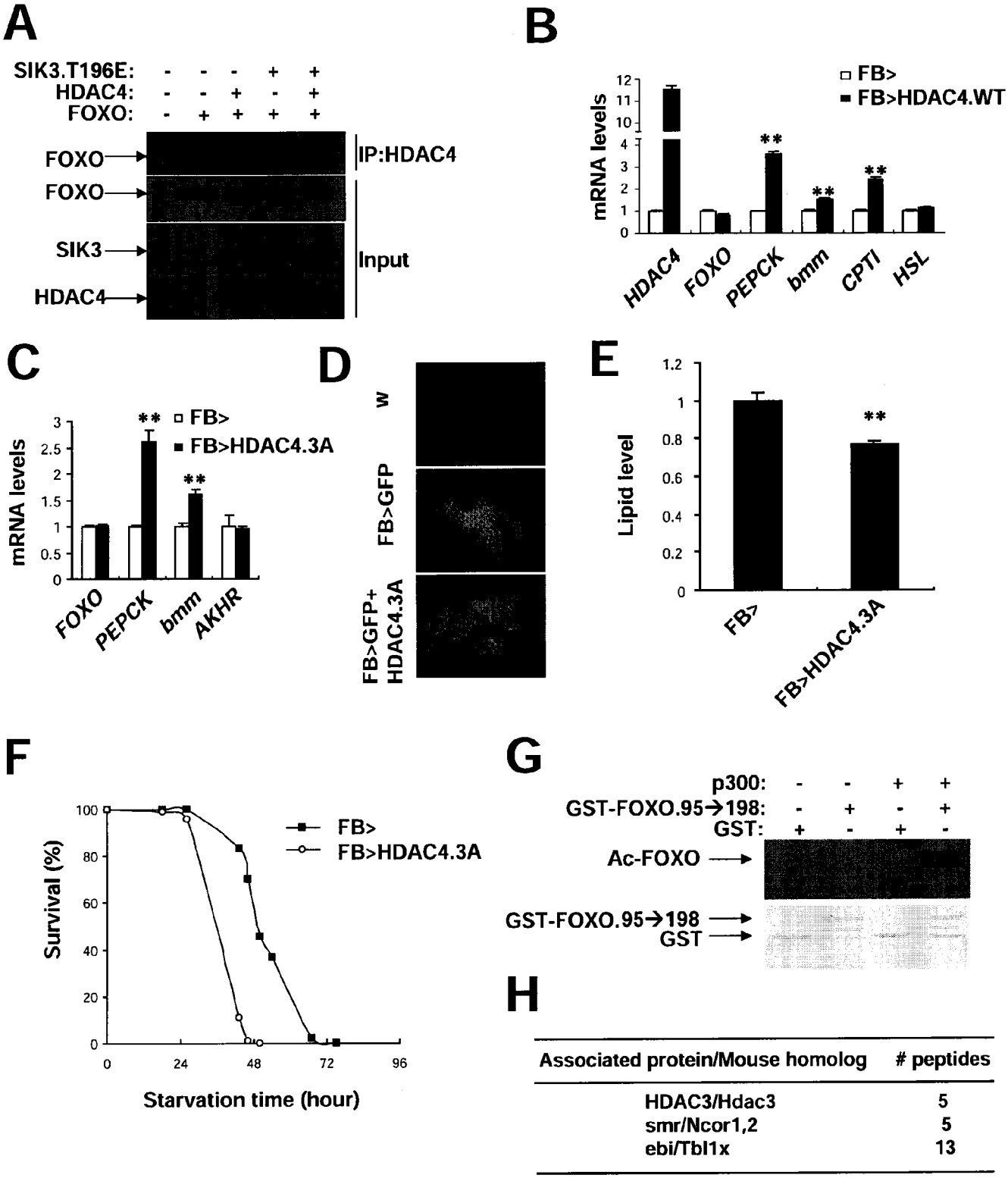

A
L1
L2
acv
L3
pcv
L4
L6
L5
B
MS>
MS>SIK3i
MS>SIK3i, Df(X)BSC713
MS>SIK3i, HDAC4e04575
C
CON
HDAC4
MS>
MS>SIK3
MS>SIK3i
D
MS>
MS>HDAC4.WT
MS>HDAC4.3A
E
GFP
SIK3.S563A
GFP
SIK3.S563A
pHDAC4
HDAC4
SIK3
WT
3A
HDAC4
F
GFP
SIK3.WT
SIK3.S563A
cAMP: - + - + - +
pS563-SIK3
SIK3
G
Cyto
Nuc
SIK3.T196E: - + - +
HDAC4
SIK3.T196E
CREB
ACC

Fig. 12



Associated protein/Mouse homolog	# peptides
HDAC3/Hdac3	5
smr/Ncor1,2	5
ebi/Tbl1x	13

Fig. 13

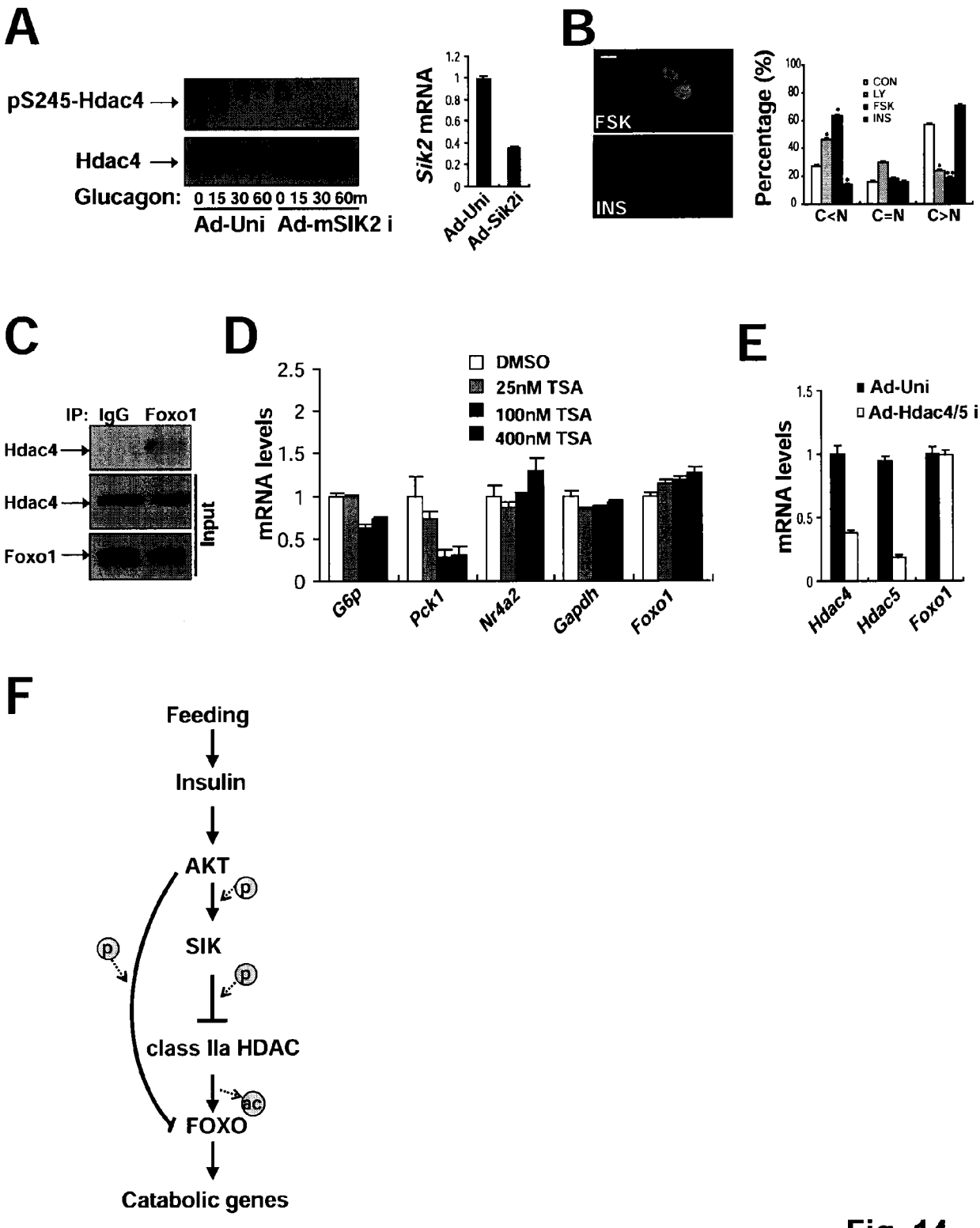

A
pS245-Hdac4
Hdac4
Glucagon: 0 15 30 60 0 15 30 60m
Ad-Uni Ad-mSIK2 i
Sik2 mRNA
Ad-Uni
Ad-Sik2i
B
FSK
INS
Percentage (%)
CON
LY
FSK
INS
C<N
C=N
C>N
C
IP: IgG Foxo1
Hdac4
Hdac4
Foxo1
Input
D
DMSO
25nM TSA
100nM TSA
400nM TSA
mRNA levels
G6p
Pck1
Nr4a2
Gapdh
Foxo1
E
Ad-Uni
Ad-Hdac4/5 i
mRNA levels
Hdac4
Hdac5
Foxo1
F
Feeding
Insulin
AKT
SIK
class IIa HDAC
FOXO
Catabolic genes

Fig. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/37578

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 9/14; C12Q 1/68 (2012.01)
USPC - 435/195; 435/375; 435/6.13
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 9/14; C12Q 1/68 (2012.01)
USPC: 435/195; 435/375; 435/6.13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/195; 435/375; 435/6.13; Journal of Molecular and Cellular Cardiology, February 2011, Vol 50; Molecular and Cellular Biology, 2001, Vol 21 ; Molecular Biol of the Cell. 2006, Vol 17; Journal of Biol Chem. January 2011, Vol 286

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB; USPT, EPAB, JPAB); Google Scholar; Google Web; esp@cenet; NCBI: HDAC, agonist, obesity, lipolysis, HDAC4, triglyceride, obese, class IIa, drosophila, HDAC5, HDAC7, HDAC9, hypertrophy, cardiac hypertrophy, L19, QSK, SIK3, screen, interacting protein, SMRT, FOXO, fat, deacetylation, Salk, Montminy, John Thomas, smrter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0106654 A1 (OLSON et al.) 19 May 2005 (19.05.2005), abstract; para [0023], [0057], [0135], [0300], [0301].	1-14, 21-24
Y	KURDI et al., Three 4-letter words of hypertension-related cardiac hypertrophy: TRPC, mTOR, and HDAC. Journal of Molecular and Cellular Cardiology, June 2011 (published online 19 February 2011), Vol 50, No 6, Pages 964-971. Especially abstract; pg 968, col 1, para 2.	1-14, 21-24
Y	US 2004/0176294 A1 (KELLY) 9 September 2004 (09.09.2004), abstract; para [0004], [0028], [0041], [0043], [0056].	5-7, 18-20
Y	US 2008/0200566 A1 (VERDIN et al.) 21 August 2008 (21.08.2008), abstract; para [0002], [0095], [0233].	10
Y	US 2009/0215681 A1 (KAHN et al.) 27 August 2009 (27.08.2009), abstract; para [0010], [0015], [0099], [0100], [0104], [0110], [0199], [0122].	12
Y	GUENTHER et al., The SMRT and N-CoR Corepressors Are Activating Cofactors for Histone Deacetylase 3. Molecular and Cellular Biology, September 2001, Vol 21, No 18, Pages 6091-6101. Especially abstract; pg 6091, col 2, para 2.	14

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2012 (06.08.2012)	31 AUG 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/37578

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIU et al., Regulation of Histone Deacetylase 4 Expression by the SP Family of Transcription Factors. Molecular Biol of the Cell, February 2006, Vol 17, No 2, Pages 585-597. Especially abstract; pg 586, col 2, para 5.	15-20
Y	US 2004/0097457 A1 (EULENBERG et al.) 20 May 2004 (20.05.2004), abstract; para [0141].	15-20
Y	WEEMS et al., Class II Histone Deacetylases Limit GLUT4 Gene Expression during Adipocyte Differentiation. Journal of Biol Chem. 7 January 2011, Vol 286, No 1, Pages 460-468. Especially abstract; pg 466, col 2, para 1; pg 468, col 1, para 2.	15-20
Y	VAN DER LINDEN et al., The EGL-4 PKG Acts With KIN-29 Salt-Inducible Kinase and Protein Kinase A to Regulate Chemoreceptor Gene Expression and Sensory Behaviors in Caenorhabditis elegans. Genetics, November 2008, Vol 180, No 3, Pages 1475-1491. Especially pg 1476, col 1, para 1; pg 1480, col 2, para 2.	22-24
Y	CHOI et al., Drosophila Salt-inducible Kinase (SIK) Regulates Starvation Resistance through cAMP-response Element-binding Protein (CREB)-regulated Transcription Coactivator (CRTC). Journal of Biol Chem. 28 January 2011, Vol 286, No 4, Pages 2658-2664. Especially abstract; pg 2658, col 2, para 1; pg 2664, col 1, para 2.	22-24
Y	US 2006/0246418 A1 (MONTMINY) 2 November 2006 (02.11.2006), para [0091].	22-24
X,P	WANG et al., A Hormone-Dependent Module Regulating Energy Balance. Cell, 13 May 2011, Vol 145, No 4, Pages 596-606. Especially abstract.	1