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NUCKS Is a Positive Transcriptional Regulator of Insulin Signaling

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SUMMARY

Although much is known about the molecular players in insulin signaling, there is scant information about transcriptional regulation of its key components. We now find that NUCKS is a transcriptional regulator of the insulin signaling components, including the insulin receptor (IR). Knockdown of NUCKS leads to impaired insulin signaling in endocrine cells. NUCKS knockout mice exhibit decreased insulin signaling and increased body weight/fat mass along with impaired glucose tolerance and reduced insulin sensitivity, all of which are further exacerbated by a high-fat diet (HFD). Genome-wide ChIP-seq identifies metabolism and insulin signaling as NUCKS targets. Importantly, NUCKS is downregulated in individuals with a high body mass index and in HFD-fed mice, and conversely, its levels increase upon starvation. Altogether, NUCKS is a physiological regulator of energy homeostasis and glucose metabolism that works by regulating chromatin accessibility and RNA polymerase II recruitment to the promoters of IR and other insulin pathway modulators.

INTRODUCTION

Type 2 diabetes (T2D) and one of its major risk factors, obesity, are pandemic problems (Friedman, 2009). Inherent genetic predispositions in combination with an inappropriate diet and a sedentary lifestyle contribute to the pathogenesis of these disorders (Ramachandrapappa and Farooqi, 2011). T2D is characterized by high blood glucose levels due to relative insulin deficiency, the result of reduced insulin secretion and/or impaired insulin sensitivity (Gustavsson et al., 2008). Defective response

to insulin often leads to serious metabolic disorders, including hyperglycemia, dyslipidemia, and hypertension (Leavens and Birnbaum, 2011). A better understanding of insulin signaling is critical for the development of therapeutic strategies toward T2D and obesity. Insulin activates a diverse array of biological responses by binding to its receptor (IR), which then phosphorylates and recruits different adaptors such as the insulin receptor substrate (IRS) (Vollenweider et al., 2002). Phosphorylated IRS proteins coordinate the activation of numerous well-defined downstream signaling pathways, including the phosphatidylinositol 3-kinase (PI3K) cascade (Bozulic and Hemmings, 2009). PI3K signaling culminates in the activation of a multitude of kinases including Akt/PKB and PKC ζ , which elicit the biological action of insulin such as glycogen synthesis through GSK-3 or protein synthesis via mTOR (Manning and Cantley, 2007). Insulin signaling can be regulated at multiple levels (Taniguchi et al., 2006), with transcriptional control, microRNA-mediated post-transcriptional control (Trajkovski et al., 2011), posttranslational modifications (Zick, 2005), changes in subcellular localization (Inoue et al., 1998), protein degradation (Rui et al., 2002), and modification of phosphoinositol phospholipid all known to play a part (Sleeman et al., 2005). While many studies focus on negative regulators associated with impaired insulin signaling, the positive mechanisms and their dysregulation during insulin resistance are less explored.

As part of a directed screen to identify molecules that respond to dietary cues in adipose tissue during development of obesity and insulin resistance, we report the identification of NUCKS (nuclear ubiquitous casein and cyclin-dependent kinase substrate) as a regulator of insulin signaling. NUCKS was identified as a highly phosphorylated protein ubiquitously expressed in vertebrates (Ostfold et al., 1990). NUCKS can be phosphorylated by casein kinase 2, cyclin-dependent kinases, DNA-activated protein kinase, and second messenger-activated kinases (Meijer et al., 1991). Expression of NUCKS is deregulated in invasive breast cancers (Drosos et al., 2009; Ziłkowski et al., 2009). These studies implicated NUCKS in signal transduction and

regulation of cell-cycle-related processes and DNA repair. However, the function of NUCKS beyond these signaling pathways has not been reported. In this paper, we characterize the function of NUCKS in the regulation of energy homeostasis and glucose metabolism. Expression of NUCKS is inversely correlated with body mass index (BMI) in humans and body fat in mice. Ablation of NUCKS results in weight gain, increased body fat accumulation, glucose intolerance, and insulin resistance. NUCKS plays a critical role in insulin signaling and is required for efficient Akt activation following insulin stimulation. Using chromatin immunoprecipitation sequencing (ChIP-seq), we show that NUCKS is a key chromatin modifier and transcriptional regulator of a number of insulin signaling genes, including IR.

RESULTS

Identification of NUCKS

To identify regulators of energy homeostasis and glucose metabolism, we performed liquid chromatography-mass spectrometry (LC-MS) analysis using white adipose tissue (WAT) lysates from mice fed a low-fat diet (LFD) or a high-fat diet (HFD). We performed reductive dimethyl (DM) labeling experiments (Figure 1A) of mixed peptides from LFD and HFD WAT samples, followed by LC-MS analysis. In addition, the samples were also subjected to label-free analysis. In this screen, we identified more than 5,000 proteins, including several proteins detected in LFD samples with significant iBAQ (intensity based absolute quantification) value but absent in HFD samples (refer to Tables S1 and S2 for complete proteomic analysis). Among these proteins, we decided to focus on NUCKS, which was only detected in LFD WAT samples (Figures 1B and 1C). NUCKS resembles high-mobility group A (HMGA) proteins in amino acid composition and DNA binding domain structure. Despite being the most modified protein in the genome (Wiśniewski et al., 2008), the physiological role of NUCKS is very poorly defined in the literature. The dramatic reduction of NUCKS in WAT from HFD-fed mice prompted us to investigate if NUCKS may have important functions in metabolic regulation.

Reduced NUCKS Levels in WAT of High-BMI Human Subjects

Clinical relevance of NUCKS in obesity was evaluated by measuring NUCKS expression levels in the subcutaneous fat of human subjects with differing BMIs (Figure 1D). Overweight individuals ($\text{BMI} > 25 \text{ kg/m}^2$) displayed more than 40% reduction in NUCKS protein levels when compared to lean individuals ($\text{BMI} < 23 \text{ kg/m}^2$) (Figure 1E). Expression levels of NUCKS showed a strong inverse correlation with BMI (Figure 1F). A significant inverse correlation was also observed between NUCKS expression and HOMA-IR (Figure 1G). Furthermore, like in humans, expression of NUCKS was drastically reduced in the WAT of mice fed an HFD for 16 weeks when compared to age- and sex-matched C57Bl6 mice on an LFD (Figures 1H and 1I). Interestingly, IR levels were also lower in tissues from HFD-fed mice (Figures 1H and 1I), suggesting a positive correlation between NUCKS and IR expression. Besides WAT, NUCKS was also markedly reduced in the liver, hypothalamus, and muscle of HFD-fed mice (Figures S1A–S1F). On the other hand,

NUCKS protein and mRNA levels increased in starved MIHA hepatocytes (Figures S1G and S1H) and several endocrine cells (data not shown), indicating that NUCKS may be a molecular link between physiological cues and signaling that regulates energy homeostasis.

NUCKS, present in the cytoplasm and nucleus (Grundt et al., 2002, 2007), is composed of two nuclear localization signals (NLS) and one DNA binding domain (DBD) (Figure S1I). We examined whether NUCKS plays a direct role in insulin signaling. First, we generated 3T3-L1 cells with stable knockdown (KD) of NUCKS by using lentivirus-mediated small hairpin RNAs. Insulin-stimulated Akt phosphorylation was reduced in NUCKS KD cells (Figure S1J). To test the generality of our observations regarding the role of NUCKS in insulin signaling and to evaluate whether the KD effects were specific, we transfected another insulin-responsive cell line, AML12 hepatocytes, with three independent small interfering RNAs (siRNAs) against NUCKS and examined insulin-induced Akt phosphorylation in these cells. Lowering NUCKS expression by independent siRNAs resulted in reduced Akt phosphorylation under all conditions (Figure S1K). Indeed, NUCKS was required for insulin-mediated Akt phosphorylation in a dose- (Figure S1L) and time-dependent (Figures S1M and S1N) manner, suggesting that NUCKS is possibly an essential positive regulator of insulin signaling. Taken together, these results indicate a strong inverse relationship between NUCKS expression and obesity in mice and humans and suggest a potential role of NUCKS in regulating insulin signaling.

Increased Body Weight and Reduced Energy Expenditure in NUCKS Knockout Mice

To determine the physiological role of NUCKS in vivo, we generated NUCKS knockout (KO) mice (Figures S2A–S2D). NUCKS KO animals were born at the expected Mendelian ratios and were morphologically indistinguishable from their wild-type (WT) littermates at birth (Figure S2E). NUCKS KO mice progressively gained more weight when compared with WT mice over the following 4 months when fed a normal chow diet (NCD) (Figure 2A). By 3 months of age, as compared to WT mice, NUCKS KO mice show significantly higher body fat (Figure 2B). Weight gain in NUCKS KO mice was mainly from liver and both visceral and subcutaneous fat (Figure 2C). We then performed indirect calorimetry analysis of paired NUCKS KO and WT mice. NUCKS KO mice on the NCD consumed more food (Figure 2D) and exhibited lower energy expenditure (Figure 2E) and lower basal metabolic rates (Figure 2F) as compared to the age-matched WT mice. The respiratory exchange rate was higher in the NUCKS KO mice during the day (Figure 2G). Body temperature was comparable between WT and KO mice fed a normal diet (Figures S2F and S2G). NUCKS KO mice also displayed lower locomotor activities at night and for the whole day as compared to the age-matched WT mice (Figures 2H and 2I). The obese phenotypes in NUCKS KO mice are exacerbated by an HFD. NUCKS KO mice on an HFD gained significantly more body weight (Figure 2J; Figure S2H) and body fat over time (Figure 2K). The perirenal fat pads of the NUCKS KO mice were enlarged (Figure S2I) with larger adipocytes compared to those of the WT animals (Figure S2J). Hematoxylin and eosin staining and oil red O staining showed more lipids in the cytosol of the KO

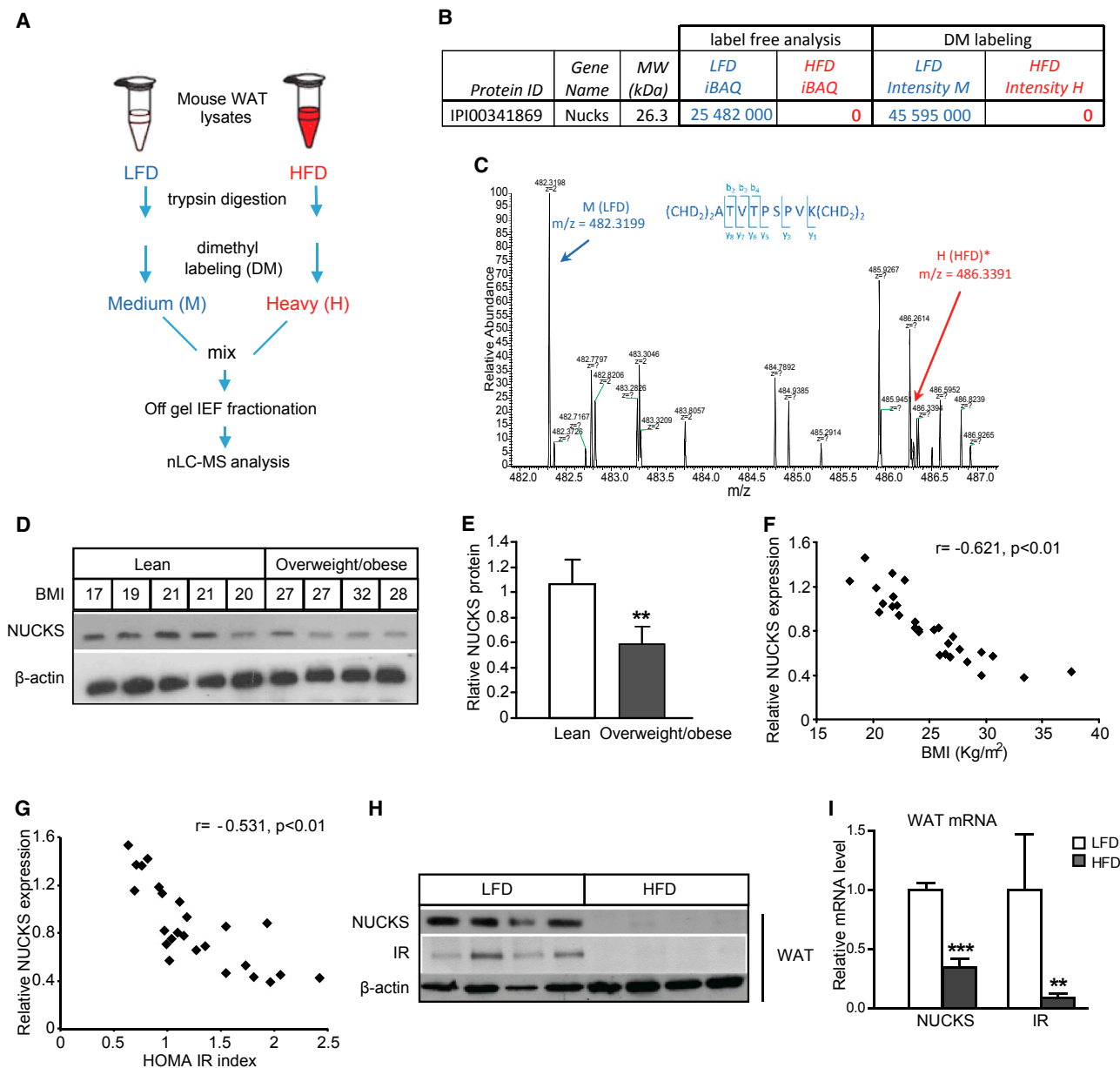


Figure 1. Identification of NUCKS by Mass Spectrometry-Based Whole-Proteomics Analysis

(A) Workflow for mass spectrometry (MS) analysis of adipose tissues from mice on a low-fat diet (LFD) and high-fat diet (HFD). Two approaches (label-free and reductive dimethyl labeling [DM]) were carried out to compare the protein expression levels between LFD and HFD samples.

(B) The table shows iBAQ (intensity-based absolute quantification) values in the label-free approach and peptide intensity in the DM approach. NUCKS was detected in LFD, but not in HFD, samples for both approaches.

(C) MS spectrum of a detected NUCKS peptide further confirms its presence in an LFD sample, but not in an HFD sample. The asterisk (*) in red corresponding to the heavy form of the NUCKS peptide was either below the noise level (very low expression) or not expressed at all.

(D) Immunoblot analysis of NUCKS expression in human subcutaneous fat. β-Actin was used as a loading control. The body mass index (BMI) of individuals is shown on the top.

(E) Quantification of normalized NUCKS protein expression in human subcutaneous fat (n = 29).

(F) Correlation between normalized NUCKS expression and BMI in humans (n = 29).

(G) Correlation between normalized NUCKS expression and the HOMA-IR index in humans.

(H) Immunoblot analysis of NUCKS and IR expression in WAT 6-month-old mice fed a low- or high-fat diet (LFD or HFD, respectively) (n = 4).

(I) Real-time analysis of *NUCKS* and *IR* mRNA expression in WAT 6-month-old mice fed with a low- or high-fat diet (LFD or HFD, respectively) (n = 4).

*p < 0.05, **p < 0.01. See also Figure S1.

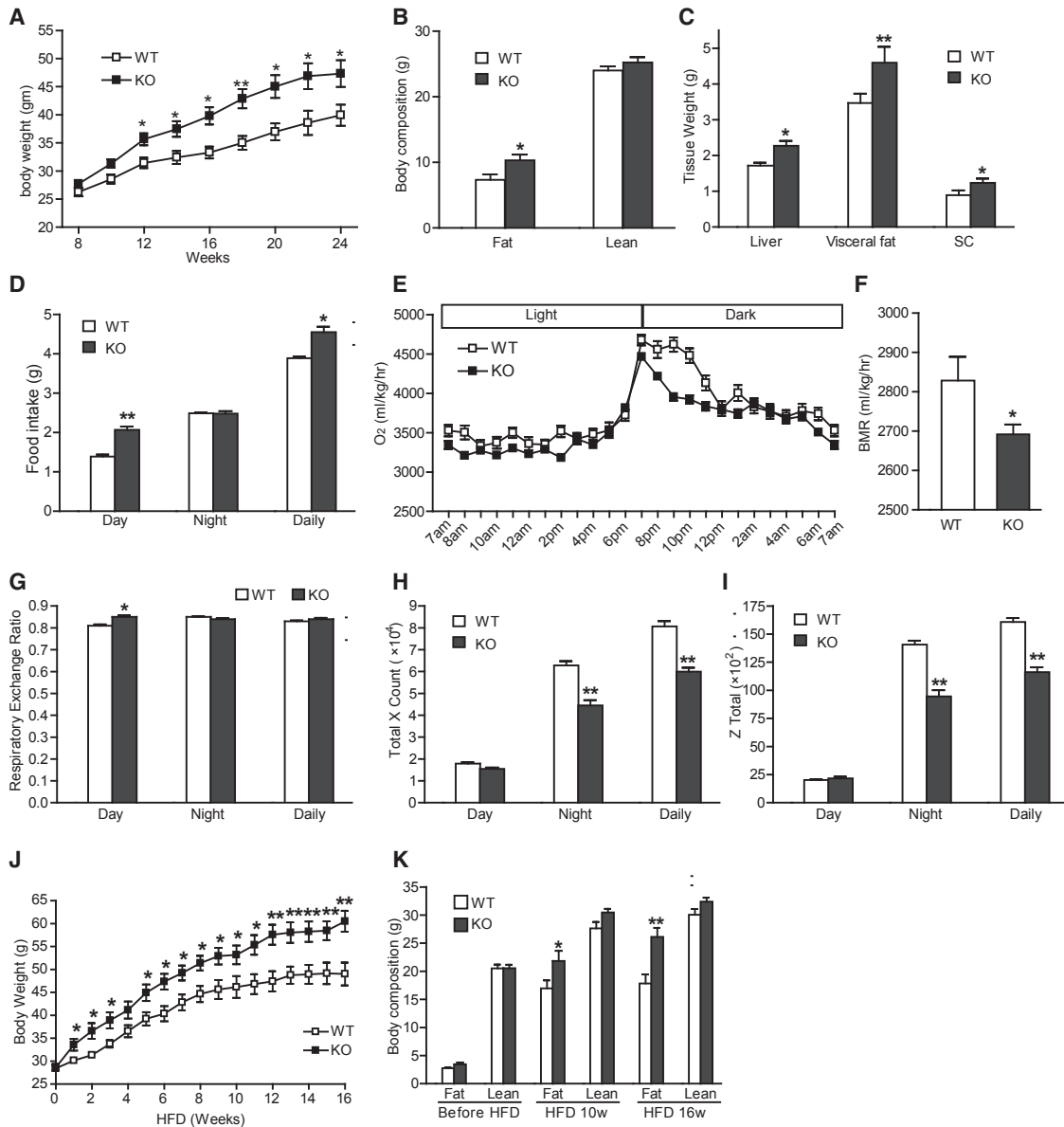


Figure 2. Increased Body Weight and Fat Mass in NUCKS KO Mice Fed an NCD and HFD

- (A) Body weight of WT and KO mice was monitored from 8 to 24 weeks of age (n = 9). Data are representative of three independent cohorts of mice.
- (B) MRI of 3-month-old WT and KO mice. The fat and lean mass were expressed as absolute weight (n = 22).
- (C) The weight of liver and visceral adipose tissues from the perirenal fat pad and subcutaneous fat (SC) from 6-month-old WT and KO mice in absolute weight. Eight pairs of mice from three independent cohorts of mice fed a normal diet were analyzed.
- (D) Food intake by 3-month-old WT and KO mice monitored over 5 days using the metabolic chamber (n = 6). Data are representative of two independent experiments.
- (E) Oxygen consumption as a measure of energy expenditure by 3-month-old WT and KO mice; shown are the average readings from six mice monitored over 24 hr.
- (F) Basal metabolic rate taken as an average of the oxygen consumption rates measured from 7 a.m. to 5 p.m. (n = 6).
- (G) Respiratory exchange rate (RER) of 3-month-old WT and KO mice monitored over 5 days (n = 6).
- (H) Total horizontal (X) activity of 3-month-old WT and KO mice monitored over 5 days (n = 6).
- (I) Total vertical (Z) activity of 3-month-old WT and KO mice monitored over 5 days (n = 6).
- (J) Body weight of WT and KO mice were monitored weekly beginning at 2 months of age for 4 months. Data are representative of three independent cohorts of HFD-fed mice (n = 10).
- (K) MRI of WT and KO mice before (2 months old) and 10 or 16 weeks after HFD (n = 10).

*p < 0.05, **p < 0.01. See also [Figures S2](#) and [S3](#).

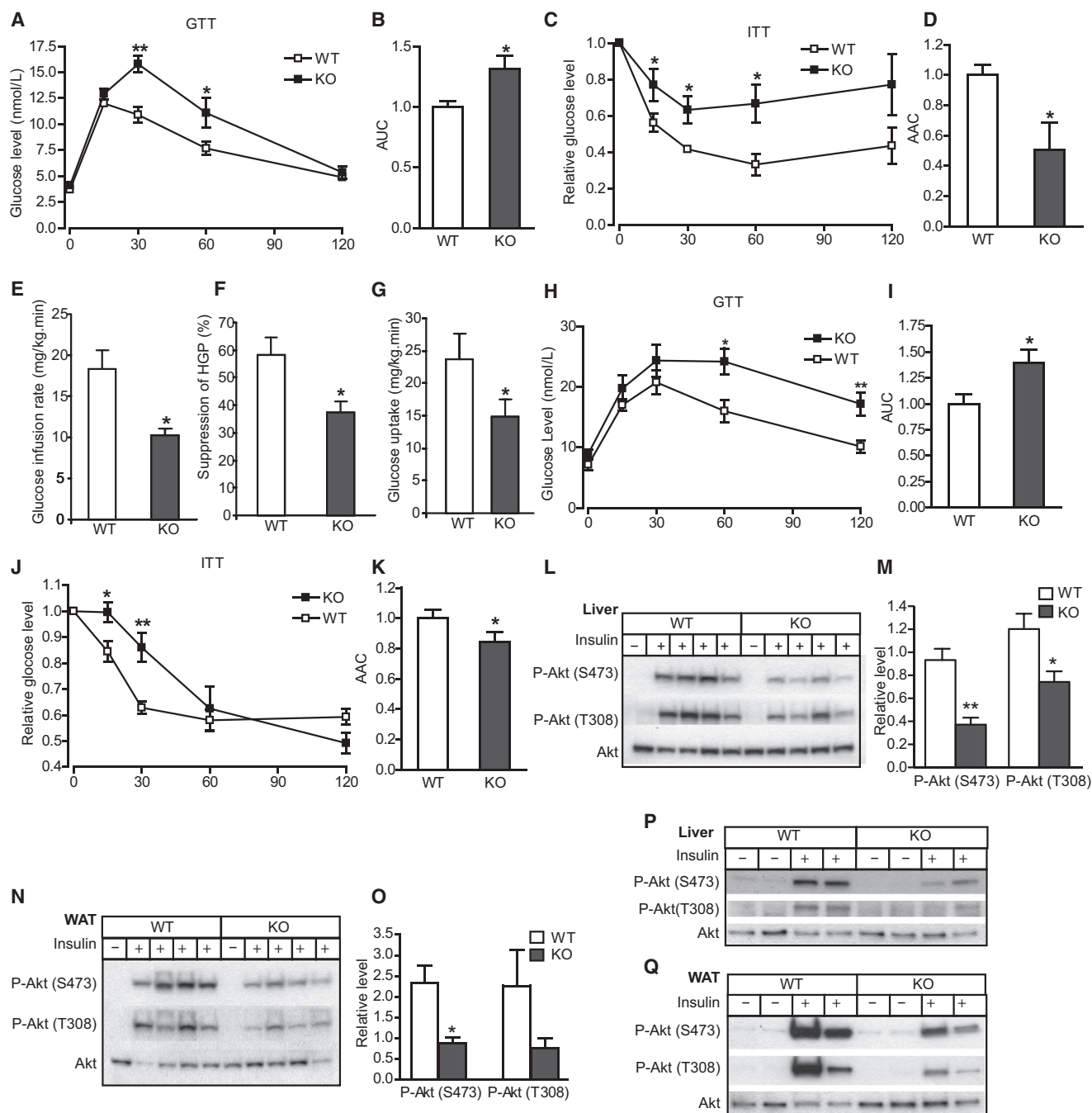


Figure 3. Glucose Intolerance, Impaired Insulin Sensitivity, and Insulin Signaling in NUCKS KO Mice

(A) Glucose tolerance tests performed on 3-month-old WT and KO mice fed a normal diet (n = 7).
 (B) Area under curve (AUC) was calculated based on the GTT data and is shown on the right panel.
 (C) Insulin tolerance tests performed on 3-month-old WT and KO mice fed a normal diet (n = 7). Glucose levels in ITT tests were expressed as fold change relative to time 0 before insulin injection.
 (D) Area above the curve (AAC) was calculated based on the ITT data.
 (E–G) Hyperinsulinemic euglycemic clamping of 10-week-old NUCKS WT mice (n = 5) and NUCKS KO mice (n = 4). Glucose infusion rate (GIR) was expressed as mg/kg.min (E). Percent suppression of hepatic glucose production by insulin was expressed as percentage (F). Glucose disappearance rate was expressed as mg/kg.min (G).
 (H–K) GTT (H–I) and (J and K) ITT of WT and KO mice fed a HFD for 4 months (n = 7). AUC and AAC are shown on the right of these panels, respectively.
 (L) Phosphorylation of Akt in liver were checked before and 15 min after insulin stimulation (1 U/kg) in 3-month-old NUCKS WT and KO mice. Each lane represents extracts obtained from individual mouse, and data are representative of three independent cohorts of mice (n = 4 for each experiment).

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livers (Figures S2K and S2L). No significant differences were observed in liver triglyceride (TG), high-density lipoprotein cholesterol, or low-density lipoprotein/very low-density lipoprotein cholesterol levels between WT and KO mice on an HFD (Figures S3A and S3B) and in serum triglyceride (TG) or free fatty acid (FFA) levels between the normal NCD-fed KO and WT mice (Figures S3C and S3D). Serum leptin and adiponectin levels were comparable between WT and KO mice fed with the NCD (Figures S3E and S3F). Consistent with the obese phenotype, leptin levels were elevated while those of adiponectin were decreased in HFD-fed NUCKS KO mice (Figure S3G and S3H). Together, these results, especially the finding that nutrient overload exacerbates the metabolic phenotypes in the absence of NUCKS, suggest that NUCKS plays a role in metabolic homeostasis in vivo.

Impaired Glucose Tolerance and Reduced Insulin Sensitivity in NUCKS KO Mice

To test whether NUCKS is directly involved in the regulation of glucose metabolism and insulin signaling at the organism level, we first measured resting and fasting glucose and insulin levels of NCD-fed NUCKS KO and WT mice. There was no difference in resting or fasting glucose levels between the two groups, but resting insulin levels were higher in NUCKS KO mice compared with WT mice (Figures S4A and S4B; data not shown). Glucose tolerance tests (GTT) showed that NCD-fed NUCKS KO mice had higher blood glucose levels at the 30 and 60 min time points and significantly worse maintenance of glucose levels during the 120 min testing period (Figures 3A and 3B). Consistent with reduced insulin sensitivity, the KO mice exhibited slower reduction of glucose levels after insulin injection in insulin tolerance tests (ITT) (Figures 3C and 3D). To confirm the insulin-resistance phenotype and the role of NUCKS in regulating glucose homeostasis, we performed hyperinsulinemic euglycemic clamp experiments. NUCKS mice exhibited a reduced glucose infusion rate (Figure 3E), enhanced hepatic glucose production (Figure 3F), and impaired glucose uptake (Figure 3G). Resting blood glucose levels in HFD-fed NUCKS KO mice were significantly elevated compared with HFD-fed WT mice (Figure S4C). Resting plasma insulin levels were significantly elevated in both HFD-fed NUCKS KO and WT mice, with NUCKS KO mice showing more than twice the levels of plasma insulin than the WT mice (Figure S4D). Similar to the body-weight phenotype, glucose intolerance and insulin resistance were also markedly exacerbated in HFD-fed NUCKS KO mice when compared with HFD-fed WT mice (Figures 3H–3K). To investigate the molecular basis underlying the observed metabolic phenotype, including reduced insulin sensitivity in vivo, we next examined insulin signaling in tissues of WT and KO mice. Consistent with decreased insulin signaling observed in cell lines (Figures S1J–S1N), insulin-stimulated phosphorylation of Akt was significantly attenuated in livers and WAT of NUCKS KO mice when compared with WT mice (Fig-

ures 3L–3O). Similarly, insulin-dependent Akt phosphorylation was also suppressed in the WAT and livers of NUCKS KO mice on an HFD (Figures 3P and 3Q). Consistent with lower p-Akt activation in NUCKS KO livers, we observed higher levels of mRNAs encoding gluconeogenic enzymes fructose 1, 6 bisphosphatase 1 (*Fbp1*) and glucose-6-phosphatase (*G6P*) in the KO livers (Figure S4E) and higher glucose output in the KO mouse primary hepatocytes (Figure S4F). Taken together, these results support the notion that NUCKS is a positive regulator of glucose metabolism and insulin signaling in vivo.

Genome-wide Approach Identifies NUCKS as a Key Integrator of Metabolic Signaling

It is clear from our assays that NUCKS has an important role in regulating glucose homeostasis and loss of NUCKS leads to obesity. To get an unbiased handle on the plausible mechanism(s) of NUCKS action, we performed a genome-wide ChIP-seq for NUCKS in primary hepatocytes. We successfully mapped 25 mln reads to the mm9 genome and detected NUCKS binding at 10,203 sites, 60% of which were located in the proximity of a transcription start site (TSS) (Figure 4A). The peaks of NUCKS occupancy were often broad, with some around 1 kb (Figure 4B), suggesting that multiple NUCKS molecules could bind cooperatively to the same genomic loci. Gene Ontology and signaling pathway analyses of NUCKS-bound genes revealed that 16 of the top 20 pathways were metabolic and biosynthetic processes and that the insulin signaling pathway was represented with high significance in the top categories, with cytokine secretion/signaling being the other most prominent module (Figures 4C and 4D). We also performed de novo motif discovery to identify specific sequences bound by NUCKS (Figure 4E). Interestingly, we identified motifs resembling those bound by SP1, which has been previously shown to be involved in the regulation of the IR (Brunetti et al., 2001; Foti et al., 2003, 2005). Since ChIP sequencing revealed that NUCKS bound the TSS of a few members of the insulin signaling pathway (Table S3), we validated the ChIP data and also checked whether NUCKS binding to these genes regulates their expression. Indeed, NUCKS could bind the promoter regions of a few genes in the insulin pathway, including *IR β* , *IRS1*, *IRS2*, and *PDK1* (Figure 4F). These results suggest that NUCKS-mediated regulation of key insulin signaling genes like *IR* and some other targets like *PDK1*, *Rictor*, and *Deptor* contributes to the metabolic phenotype of the NUCKS KO mice.

NUCKS Regulates *IR* Transcription by Opening Chromatin and Enhancing Pol II Recruitment

We next examined the molecular mechanism of NUCKS-mediated transcription, especially that of *IR β* . We generated luciferase reporter constructs driven by the first 2,000 bp of the mouse or human *IR* promoter region. Overexpression of NUCKS led to increased *IR* transcription, whereas NUCKS- Δ C, a C-terminal deletion mutant lacking the DBD (Figure S1I), failed to

(M) Quantification of P-Akt normalized to total Akt is shown on the right (n = 4).

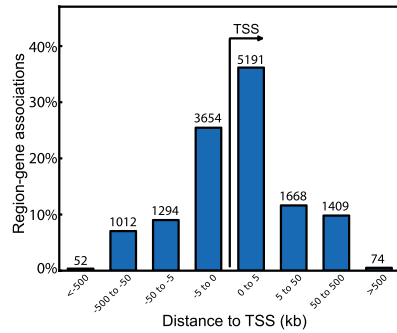
(N) Strength of insulin signaling in WAT as judged by Akt phosphorylation after 15 min of insulin stimulation (1 U/kg). Each lane represents an individual mouse.

(O) Quantification of P-Akt normalized to total Akt is shown on the right (n = 4). Data are representative of two independent cohorts of mice.

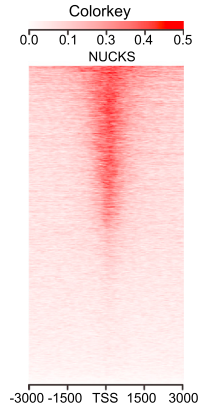
(P and Q) Akt phosphorylation before and 15 min after insulin stimulation in liver, and WAT obtained from mice fed an HFD for 4 months.

*p < 0.05, **p < 0.01. See also Figure S4.

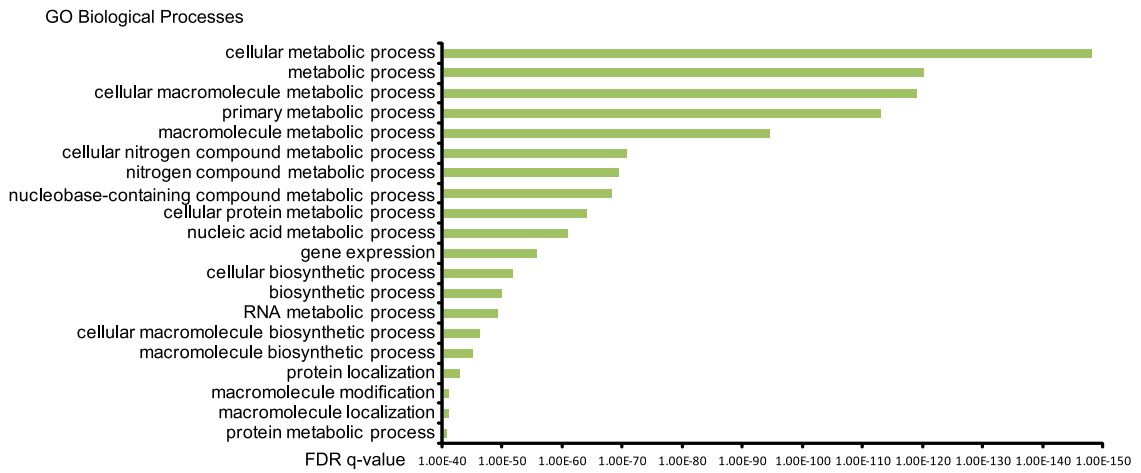
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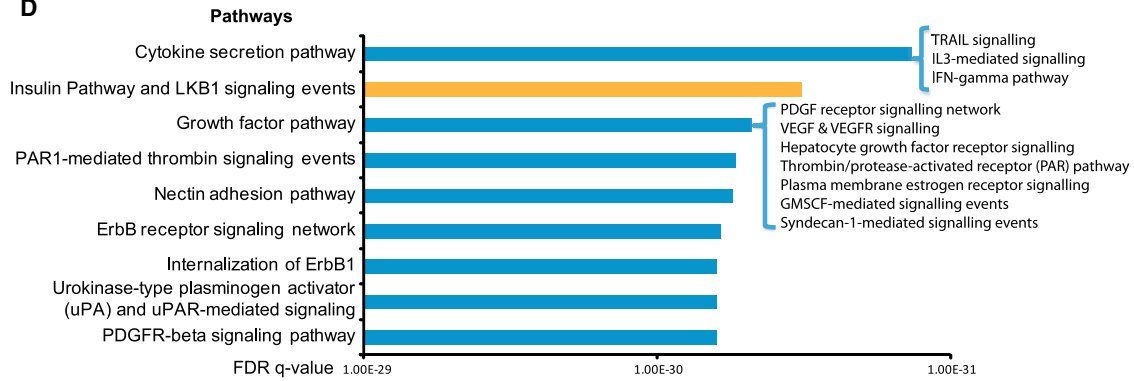
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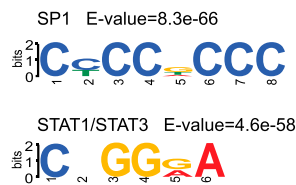
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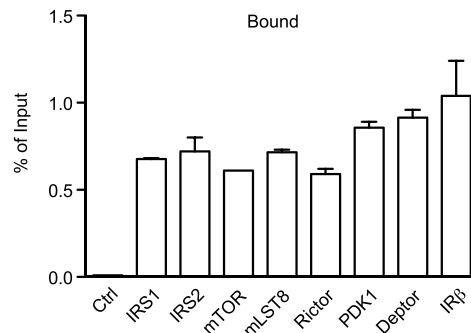
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E



F



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induce the *IR* promoter (Figure 5A), suggesting direct binding of NUCKS to the *IR* promoter/enhancer is essential for *IR* transcription. According to a previous report, HMGA1, one of the high-mobility group family members, binds to a specific probe from the *IR* promoter and regulates *IR* transcription (Foti et al., 2003). An electrophoretic mobility shift assay found that NUCKS could directly bind to the human and mouse *IR* probes (Figure 5B). The specificity of the interaction was confirmed using cold competition (Figure 5B, lane 3). To gain further mechanistic insights, we checked if NUCKS regulates openness of chromatin and Polymerase II (Pol II) recruitment to the *IR* promoter. Using the formaldehyde-assisted isolation of regulatory elements (FAIRE) method (Simon et al., 2012), which assesses the level of open chromatin, we found that depletion of NUCKS in primary hepatocytes decreased the degree of open chromatin in the *IR* promoter at the TSS (Figure 5C). Concomitantly, loss of NUCKS led to reduce Pol II recruitment at the TSS (Figure 5D). We checked *IR* mRNA levels in primary hepatocytes from WT and KO mice fed an NCD and HFD and loss of NUCKS leads to a reduction of *IR* mRNA (Figure 5E). We made use of in vitro mouse embryonic fibroblast (MEF)-differentiated adipocyte culture derived from WT and KO mice. Akt phosphorylation was significantly attenuated in NUCKS KO MEF-differentiated adipocytes (Figures 5F and 5G). Moreover, we found that IR β protein expression was dramatically reduced in the KO MEF adipocytes when compared with WT cells (Figures 5F and 5G), along with a corresponding reduction in IR β transcript levels (Figure 5H). Consistent with the findings in primary cells (Figures 5E–5H), we also observed dramatic changes of *IR* in adipose and liver tissue from HFD-fed mice (Figures 5I–5K). As the ChIP-sequencing results showed that NUCKS also binds to members of the insulin signaling pathway, we checked the mRNA and protein levels of those members between WT and KO mouse hepatocytes and found that NUCKS also regulates the expression of Deptor and PDK1 (Figures S5A and S5B), suggesting that NUCKS might target a few key players in the insulin signaling pathway (Figure 5L). Further study will delineate how NUCKS regulates these genes in a context- and cell-type-specific manner. Taken together, these results define the molecular mechanism of NUCKS action and explain how it transcriptionally regulates *IR* and other members of the insulin signaling pathway and hence impacts insulin signaling.

DISCUSSION

Numerous studies have focused on the transcriptional, translational, and posttranslational modifications of insulin, insulin

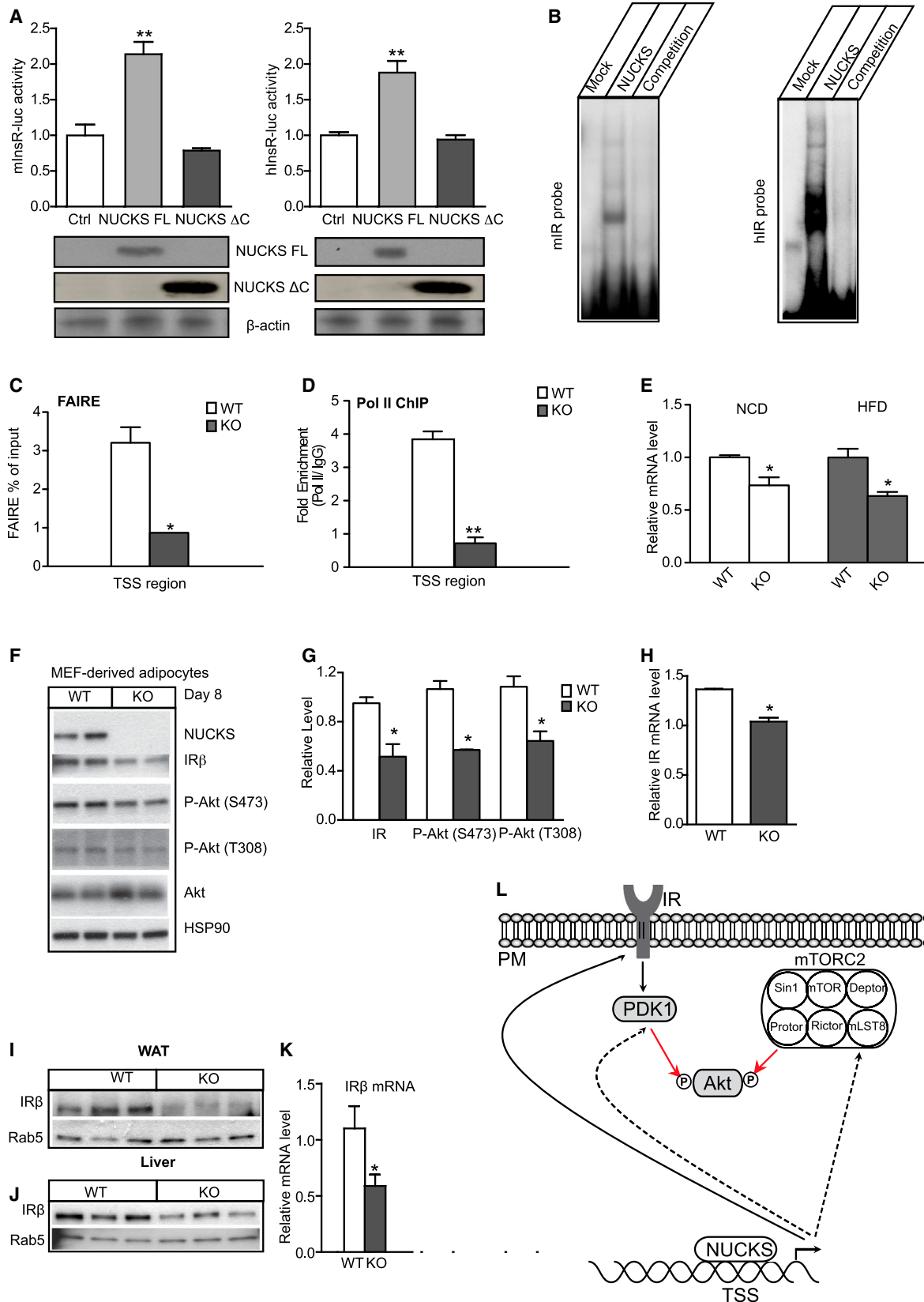
secretion, and insulin-driven signal transduction (Emanuelli et al., 2000; González-Rodríguez et al., 2010; Taniguchi et al., 2006). However, mechanisms that respond to dietary cues and/or contribute to direct transcriptional regulation of insulin signaling components are poorly elucidated. In this study, by using unbiased proteomic and genomic approaches, we report the identification and characterization of NUCKS as a transcriptional regulator of metabolic processes and insulin signaling. This study demonstrates that NUCKS regulates energy and glucose homeostasis. NUCKS expression inversely correlates with obesity in both humans and mice, and it is sensitive to starvation or HFD. Mice deficient in NUCKS in all the tissues are obese, with increased body fat content, and display decreased locomotor activities, hyperphagia, and reduced energy expenditure. Whole-body deletion of NUCKS also leads to glucose intolerance and impaired insulin sensitivity, accompanied by elevated serum insulin levels. We further show that NUCKS regulates transcription of members of the insulin signaling pathway, including IR (by binding to IR promoter/enhancer sequences), and regulates its chromatin context to allow Pol II recruitment at the TSS. Much like NUCKS, it is well documented that IR expression levels correlate with starvation and nutritional status both in tissue culture cells and in animals (Hatada et al., 1989; Puig and Tjian, 2005). The genome-wide ChIP-seq approach also showed with very high confidence (false discovery rate q -value = 6.27616×10^{-31}) that insulin signaling is among the top signaling pathways regulated by NUCKS. This unbiased approach not only ratified our observations that the insulin promoter is bound and regulated by NUCKS but also identified a few key players in insulin signaling that could be both bound and regulated by NUCKS. These findings could explain how NUCKS positively regulates AKT downstream of insulin in an IR-dependent and IR-independent manner and may suggest a more physiological manner in which these processes are regulated in nature.

Although complete loss of IR leads to early lethality in mice, loss of IR in different tissues or within regions of a given tissue has very distinct phenotypes (Kitamura et al., 2003). While it is tempting to speculate that NUCKS-mediated effects are largely due to loss of IR, it is also important to discern that NUCKS loss is not synonymous with IR loss. NUCKS deletion leads only to partial loss of IR, and other HMGA proteins may compensate for loss of NUCKS in some tissues or cells. In addition, NUCKS has other targets that regulate insulin signaling (Figure 5L), and it is not uncommon for transcription factors like nuclear factor κ B or Myc to regulate multiple members of a signaling cascade to regulate the physiological effect (Ang and Tergaonkar, 2007; Cildir et al.,

Figure 4. Genome-wide ChIP Sequencing of NUCKS

- (A) Genome-wide distribution of NUCKS-bound sites. Approximately 60% of peaks are within 5 kb of a known transcription start site (TSS).
 (B) Density of NUCKS binding across all RefSeq annotated TSSs.
 (C) NUCKS-bound promoter regions of genes enriched very significantly for metabolic processes.
 (D) Gene Ontology analysis of pathways from NUCKS ChIP-seq results. These peaks are also highly enriched for genes involved in several pathways (with similar significance), including the insulin pathway (false discovery rate q -value = 6.27616×10^{-31}).
 (E) NUCKS-bound sites were enriched for motifs associated with SP1 (e -value = 8.3×10^{-66}) and STAT1/3 (e -value = 4.6×10^{-58}).
 (F) Quantification of DNA enrichment after immunoprecipitation with NUCKS-specific antibody from mouse primary hepatocytes. ChIP DNA was quantified by quantitative PCR and normalized to DNA input. Chromatin enrichment of each motif has been documented as fold change over enrichment of a control region elsewhere in the chromosome.

All data shown are representative of three independent experiments.



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2013; Dang, 2012; Tergaonkar, 2006). Since loss of NUCKS is also seen in individuals with a high BMI, and since NUCKS levels could be modulated by diet, this offers the exciting possibility that NUCKS could be a worthwhile molecular target for therapeutic intervention. Given the number of signaling cascades that covalently modify NUCKS (Wiśniewski et al., 2008), it is highly likely that modifications of NUCKS could link insulin signaling and energy homeostasis to changes in myriad cellular processes regulated by these enzymes.

In summary, we have identified NUCKS as a positive regulator of members of the insulin signaling pathway, including *IR* transcription, that could play a role in regulating insulin signaling. Future studies directed at understanding its tissue- and stimulus-specific downstream targets may provide promising therapeutic approaches in the treatment of obesity and insulin resistance. We also hypothesize that unlike studying the function of mice with complete depletion of single-candidate targets like *IR*, which are not reflective of human physiology, molecules like NUCKS, which are central regulators of a few key molecules and levels of which respond clinically in response to diet, provide a better understanding of physiology. Given that signaling components are shared among many pathways, the subtle regulation of multiple inputs rather than “on-off” regulation of a key member ensures easy return to homeostasis. Hence, nature uses rheostats like NUCKS that fine-tune response via signaling through additive inputs.

EXPERIMENTAL PROCEDURES

Animal Welfare

All animals were kept on a 12 hr light-dark cycle. All procedures involving animal experimentation were approved by the Institutional Animal Care and Use Committee of A*STAR.

Chromatin Immunoprecipitation Assays, Formaldehyde-Assisted Isolation of Regulatory Elements Analysis, and ChIP-Seq Library Preparation

Cells were crosslinked with 1% paraformaldehyde for 20 min at room temperature. The reaction was quenched for 10 min at room temperature by adding

0.125 M glycine and the washed cell pellet frozen at -80°C . Cell pellets were lysed and centrifuged at $12,000 \times g$ for 1 min at 4°C . Sheared chromatin generated from cell pellets by sonication was incubated with NUCKS antibody or control rabbit immunoglobulin G overnight at 4°C . The immunocomplexes were precipitated with 20 μl preblocked protein A-agarose beads (1 hr at 4°C) and washed extensively. Reversion of crosslinking was performed overnight by heating samples and input at 65°C , and DNA was purified using the QIAquick spin kit (QIAGEN). Open chromatin was prepared by the FAIRE method (Simon et al., 2012) and followed by quantitative PCR analysis. Libraries were prepared using a standard Illumina pipeline and sequenced using a 50 bp single-end format on an Illumina HiSeq 2000 as previously described with minor variations (Migliori et al., 2012). The Sample Prep oligonucleotide kit (Illumina) was used for barcoding/multiplexing.

Statistics

Statistical analysis between two groups was performed by Student's *t* test using PRISM software. Statistical significance of ITT and GTT curves was determined by comparing differences in area above the curve or under the curve. Data are presented as mean \pm SEM (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). For further details, please refer to [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

The GEO accession number for the genome-wide ChIP sequencing of NUCKS data reported in this paper is GSE58100.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.030>.

AUTHOR CONTRIBUTIONS

B.Q., X.S., and E.T.W. designed and performed experiments, analyzed data, and wrote the paper. J.L., S.C.A., M.L., and J.M.L.T. performed parts of cell-based analysis. M.B., D.L., and Q.Z. contributed to ChIP-seq and bioinformatics data analysis. H.L.F.S. and J.G. contributed to mass spectrometry analysis. K.K.Y.C. and K.S.L.L. performed human sample analysis and the clamp study. A.X., E.G., M.I., and W. Hong gave constructive suggestions, designed the experimental details in relevant sections, and contributed with funding. W. Han and V.T. supervised the project and edited the manuscript.

Figure 5. NUCKS Positively Regulates Insulin Receptor Transcription and Protein Expression

(A) 293T cells were transfected with the indicated plasmids and harvested for a dual-luciferase assay as per the manufacturer's instructions. Cell lysates were used for luciferase activity measurement, and firefly luciferase activity was normalized to Renilla luciferase activity. NUCKS increases transcription of both human insulin receptor (*hInsR-luc*) and mouse insulin receptor (*mInsR-luc*) promoter fusions with luciferase gene as a reporter, while C-terminal deletion of NUCKS eliminates this effect. Levels of NUCKS proteins are shown in the panel below.

(B) Electrophoretic mobility shift assay with NUCKS binding site probes with lysates from cells expressing NUCKS. Unlabeled probes were used as competition (the sequences are shown below the figure).

(C) Open chromatin (% of input) in the mouse *IR* promoter region in WT and KO mouse primary hepatocytes by FAIRE analysis.

(D) Fold enrichment of Pol II binding to the mouse *IR* TSS region in WT and KO mouse primary hepatocytes.

(E) *IR* mRNA levels in WT and KO mouse primary hepatocytes with the NCD and HFD.

(F) WT and KO MEFs were differentiated into adipocytes and harvested on day 8 after initiation of the differentiation process for western analysis. Each sample is an independent clone of an MEF.

(G) Quantification normalized levels of *IR*, p-Akt(S473), and pAkt(T308) in (F).

(H) Transcript level of *IR* in day 8 adipocyte culture quantified by quantitative RT-PCR. Expression is normalized to the *RplpO* gene (*n* = 2). All data shown are representative of two or three independent experiments.

(I and J) Western analysis of liver and WAT extracts obtained from mice on an HFD for 4 months. Data are representative of three independent cohorts of mice (liver, *n* = 16; adipose, *n* = 11).

(K) Quantitative RT-PCR analysis of *IR* transcript in WAT (*n* = 9). Expression level of *IR* is normalized to the *RplpO* transcript level for adipose tissues.

(L) Scheme of NUCKS's mechanism to regulate insulin signaling. Black arrows stand for transcription regulation of specific genes, and red arrows stand for Akt phosphorylation by upstream kinase.

p* < 0.05, *p* < 0.01. See also [Figure S5](#).

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