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A Role of DNA-PK for the Metabolic Gene Regulation in Response to Insulin

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SUMMARY

Fatty acid synthase (FAS) is a central enzyme in lipogenesis and transcriptionally activated in response to feeding and insulin signaling. The transcription factor USF is required for the activation of FAS transcription, and we show here that USF phosphorylation by DNA-PK, which is dephosphorylated by PP1 in response to feeding, triggers a switch-like mechanism. Under fasting conditions, USF-1 is deacetylated by HDAC9, causing promoter inactivation. In contrast, feeding induces the recruitment of DNA-PK to USF-1 and its phosphorylation, which then allows recruitment of P/CAF, resulting in USF-1 acetylation and FAS promoter activation. DNA break/repair components associated with USF induce transient DNA breaks during FAS activation. In DNA-PK-deficient SCID mice, feeding-induced USF-1 phosphorylation/acetylation, DNA breaks, and FAS activation leading to lipogenesis are impaired, resulting in decreased triglyceride levels. Our study demonstrates that a kinase central to the DNA damage response mediates metabolic gene activation.

INTRODUCTION

To meet the constant energy requirement in the face of highly variable food supply, mammals employ intricate and precise mechanisms for energy storage. During feeding, excess carbohydrates are converted to fatty acids (de novo lipogenesis) for synthesis/storage of triacylglycerol, which can then be utilized during energy shortage, i.e., fasting. Lipogenesis is under tight nutritional and hormonal control (Sul and Wang, 1998). Enzymes involved in fatty acid and triglyceride synthesis, such as fatty acid synthase (FAS) (Paulauskis and Sul, 1988; Paulauskis and Sul, 1989) and mitochondrial glycerol-3-phosphate acyltransferase (mGPAT) (Dircks and Sul, 1997; Jerkins et al., 1995; Shin et al., 1991; Yet et al., 1993, 1995), are coordinately regulated during fasting/feeding. The expression of the lipogenic enzymes is very low in fasting and is drastically upregulated during feeding accompanied by an increase in insulin secretion (Sul et al., 2000; Wang and Sul, 1998). Thus, precise temporal changes in patterns of gene repression and activation are required for lipogenic gene regulation during fasting and feeding/insulin treatment.

By catalyzing seven reactions in fatty acid synthesis, FAS is a central enzyme in lipogenesis. Regulation of FAS is mainly at the transcriptional level. We have been studying the FAS promoter as a model system to dissect the transcriptional activation by feeding/insulin. We mapped the insulin response sequence (IRS) of the FAS promoter in cultured cells at the −65 E box (Moustaid et al., 1993, 1994), where upstream stimulatory factor (USF)-1/2 heterodimer binds (Moustaid and Sul, 1991; Sawadogo and Roeder, 1985; Wang and Sul, 1995, 1997). Functional analysis and chromatin immunoprecipitation (ChIP) in mice transgenic for various 5′ deletions and mutations of the FAS promoter-CAT reporter gene (Latasa et al., 2000; Moon et al., 2000; Soncini et al., 1995), however, showed that both USF binding to the E box and sterol regulatory element-binding protein-1c (SREBP-1c) binding to the nearby sterol response element (SRE) are required for feeding/insulin-mediated FAS promoter activation in vivo. Furthermore, although increased expression of SREBP-1c (Shimomura et al., 1999), mainly through insulin activation of the PI3K pathway (Engelman et al., 2006; Taniguchi et al., 2006), to bind the FAS promoter is critical for feeding/insulin response, SREBP-1c itself cannot bind its SRE without being recruited by USF, which is constitutively bound to the −65 E box (Griffin et al., 2007; Latasa et al., 2003). Many of the lipogenic promoters contain closely spaced E box and SRE at the proximal promoter region, and we documented a similar mechanism for activation of FAS and mGPAT promoters (Griffin et al., 2007). Thus, USF, along with SREBP-1c, play a critical role in mediating the transcriptional activation of lipogenesis in response to feeding/insulin.

The requirement of USF in induction of lipogenic genes, such as FAS, has been demonstrated in USF-deficient mice (Casado et al., 1999). In humans, SNP studies have implicated USF-1 as a prime candidate of familial combined hyperlipidemia (FCHL) (Pajukanta et al., 2004). How does USF regulate lipogenic gene transcription? USF levels do not change during fasting/feeding, and it is constitutively bound to the FAS promoter in both conditions (Wang and Sul, 1995). It is possible that posttranslational modifications of USF underlie its function during fasting/feeding. Insulin regulates metabolism primarily through protein phosphorylation by the well-characterized PI3K cascades (Engelman et al., 2006). Many of the metabolic effects of insulin are also

mediated by protein dephosphorylation catalyzed mainly by protein phosphatase-1 (PP1) (Brady and Saltiel, 2001). In this regard, USF has been previously reported to be phosphorylated by various kinases (Corre and Galibert, 2005). However, the significance of USF phosphorylation in lipogenic gene transcription during feeding/insulin is not known. Moreover, USF may not independently function to regulate transcription but recruit coactivators/corepressors. Such recruited factors may also include signaling molecules that transduce extracellular signals to bring about covalent modifications of USF. Thus, it can be postulated that USF and/or its potentially recruited cofactors need to be regulated by dynamic modifications such as phosphorylation/dephosphorylation in response to feeding/insulin.

Here, we report a novel mechanism for the sensing of nutritional/hormonal status by USF to regulate lipogenic gene transcription. We demonstrate that USF-1 phosphorylation by DNA-dependent protein kinase (DNA-PK), which is first dephosphorylated/activated by PP1, is an immediate response to feeding/insulin treatment. Phosphorylation of USF-1 also allows recruitment and acetylation by p300 associated factor (P/CAF). In contrast, during fasting, USF-1 association with histone deacetylase 9 (HDAC9) leads to USF-1 deacetylation. Thus, upon feeding, DNA-PK-deficient SCID mice show impaired USF-1 phosphorylation/acetylation, DNA break, transcriptional activation of the FAS gene, and lipogenesis. Our present study shows that DNA-PK is critical for the feeding-dependent activation of lipogenic genes, linking DNA-PK to the insulin-signaling pathway.

RESULTS

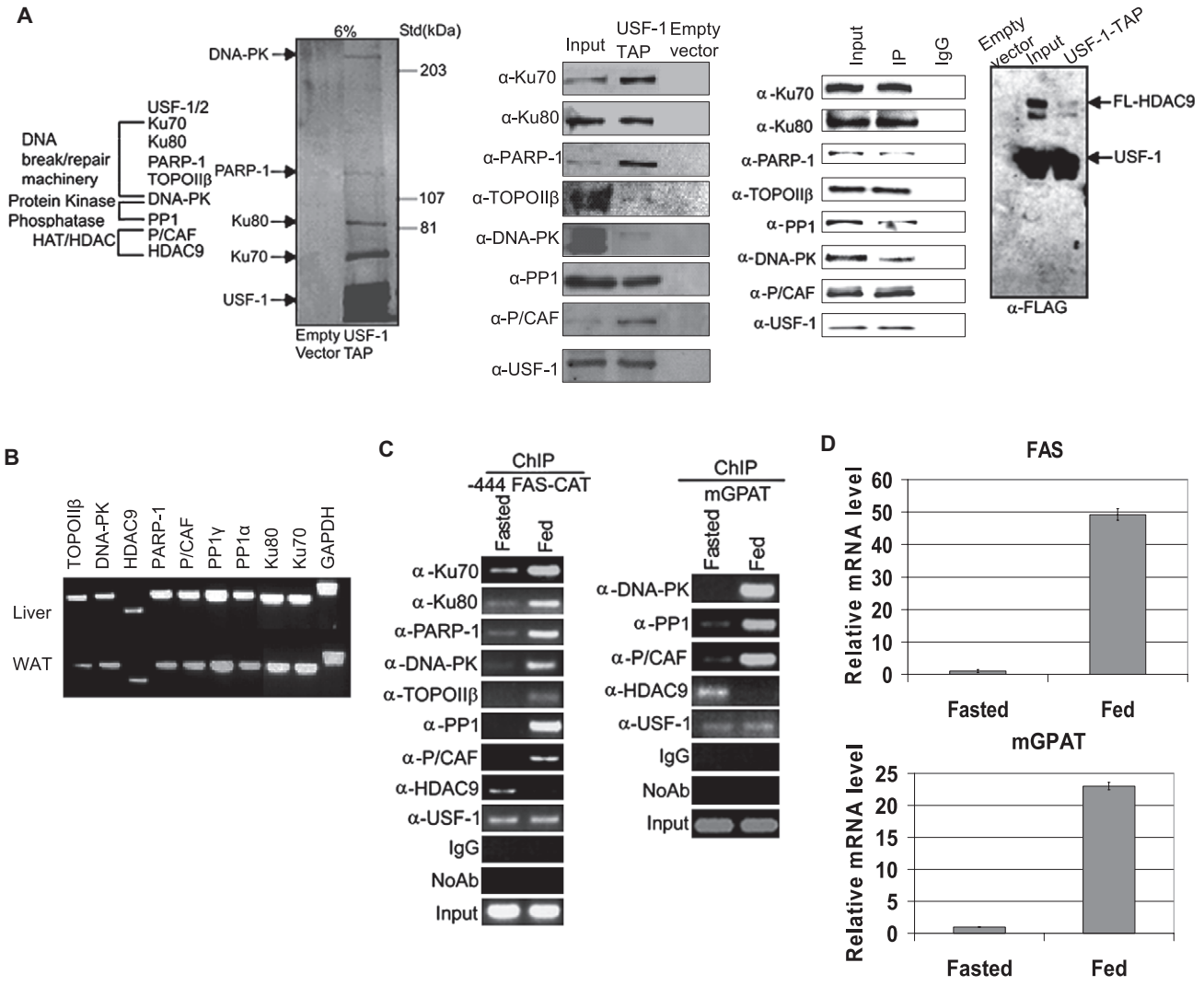
Identification of USF-Interacting Proteins and Their Occupancy on Lipogenic Gene Promoters during Fasting/Feeding

We have previously shown that USF is required for the regulation of FAS promoter activity in fasting/feeding (Wang and Sul, 1995, 1997). However, USF is constitutively bound to the FAS promoter (Griffin et al., 2007; Latasa et al., 2003). We postulated that USF may repress or activate the FAS promoter by recruiting distinct cofactors in fasted and fed conditions. We performed tandem affinity purification (TAP) and mass spectrometry (MS) analysis. The USF-interacting proteins were purified from nuclear extracts prepared from 293 cells overexpressing USF-1 tagged with streptavidin and calmodulin-binding peptides (TAP tagged) as well as a FLAG epitope at its carboxyl terminus. In addition to USF-1 and USF-2, we identified seven polypeptides in the eluates by MS analysis (Figure 1A, left panel and Table S2 available online). These proteins fall into three categories: (1) DNA break/repair components DNA-PK and its regulatory subunits, Ku70, Ku80, as well as poly(ADP-ribose) polymerase-1 (PARP-1) and Topoisomerase IIß (TopoIIβ), (2) protein phosphatase PP1, and (3) P/CAF, which belongs to the histone acetyltransferases (HAT) family. Interestingly, we detected some of the USF-interacting proteins to be poly(ADP-ribosyl)ated (Figure S5E). TAP using cells that were first crosslinked by DSP showed identical USF-1-interacting proteins (data not shown).

We detected at least five of the polypeptides having molecular weights corresponding to the above identified proteins by silver staining of the TAP eluates separated by SDS-PAGE (Figure 1A, second left panel). Blue native (BN) gel electrophoresis of the TAP eluates revealed the presence of a large USF-1-containing complex (Figure S1B). Immunoblotting of the eluates using antibodies against each of the seven polypeptides further confirmed the presence of all seven polypeptides that were copurified with TAP-tagged USF-1 (Figure 1A, third left panel). These identified proteins were specific to USF-1 because none of them were found with the control TAP tag. Confirming USF-1 interaction, coimmunoprecipitation followed by immunoblotting revealed the presence of all interacting proteins in endogenous USF-1 immunoprecipitates (Figure 1A, second right panel). Furthermore, GST pull-down assay showed that DNA-PK and PARP-1, but not TopoIIβ, Ku70/Ku80, and PP1, can directly interact with USF-1 (Figure S1A).

We also attempted to purify and identify USF-interacting proteins by incubating liver nuclear extracts with bacterially expressed TAP-tagged USF immobilized on agarose beads. MS analysis identified an additional USF-interacting protein HDAC9, a transcriptional corepressor that belongs to the class II HDAC family, which was copurified with USF-1 when the nuclear extracts from fasted mice were used (data not shown). The interaction between HDAC9 and USF-1 was confirmed by detection of HDAC9 copurified with USF-1 by TAP in cells overexpressing HDAC9 and USF-1 (Figure 1A, right panel). Overall, except for P/CAF, which has been implicated to function with USF for histone modification in chromosomal silencing (West et al., 2004), none of the above proteins have previously been shown to interact with USF.

All of the USF-interacting proteins were expressed in lipogenic tissues, liver, and white adipose tissue (WAT) (Figure 1B). We next performed ChIP in livers of fasted and fed transgenic mice expressing a CAT reporter gene driven by the −444 FAS promoter, a minimal FAS promoter sufficient for full response to fasting/feeding and diabetes/insulin treatments (Latasa et al., 2000, 2003; Moon et al., 2000). As shown before, we detected binding of USF in both fasted and fed conditions (Figure 1C, left panel). In the fasted state, however, we detected the corepressor HDAC9 bound to the FAS promoter, but not other interacting proteins that we identified by TAP-MS. Upon feeding, HDAC9 was no longer bound to the promoter, but the FAS promoter was now occupied by the coactivator P/CAF, DNA break/repair components that include DNA-PK, Ku70/80, PARP-1, TopoIIβ, as well as PP1 (Figure 1C, left panel). We also performed ChIP analysis of the mGPAT promoter using antibodies against proteins that represent each of the three categories of the USF-interacting proteins. Similar to what we observed with the FAS promoter, USF-1 was bound to the mGPAT promoter in both fasted and fed conditions (Figure 1C, right panel). Furthermore, as seen with the FAS promoter, HDAC9 was bound to the mGPAT promoter only in fasting, whereas DNA-PK, PPI, and P/CAF were bound only in the fed state. We also verified the regulated expression of FAS and mGPAT in these mice. As predicted, FAS and mGPAT mRNA levels were very low in livers of fasted mice, but upon feeding, they were induced drastically to ~50- and 25-fold, respectively (Figure 1D). The similar binding pattern of USF-interacting proteins suggests a common mechanism for lipogenic induction



(*Figure 1 continued*)

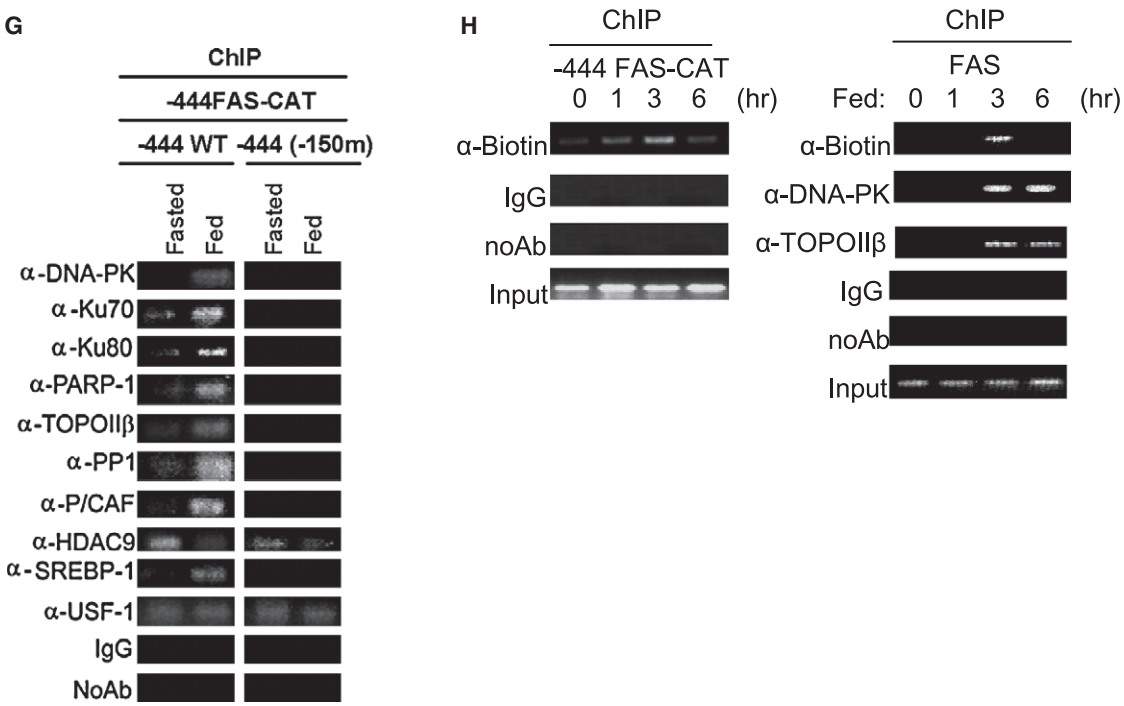


Figure 1. Purification of USF-1-Interacting Proteins

(A) The identities (far left) of USF-1-associated polypeptides. Purified USF-1 eluates on SDS-PAGE by silver staining (second from left). Immunoblotting of TAP eluates (middle). IP of USF-1 (second from right) from 293F cells with monoclonal anti-USF-1 antibodies. TAP eluates from 293F cells were immunoblotted (far right).

(B) RNA from tissues were used for RT-PCR.

(C) ChIP for association of USF-1-interacting proteins to the −444 FAS-CAT promoter (left) in FAS-CAT transgenic mice or the mGPAT promoter (right) in WT mice.

(D) Expression in liver determined by RT-qPCR.

(E) IP of FLAG-tagged USF-1 from HepG2 cells.

(F) ChIP for association of USF-1-interacting proteins to the −444 (−65 m) FAS-CAT (left) promoter or the FAS promoter in HepG2 cells (right). USF-1 protein levels by immunoblotting (bottom right).

(G) ChIP for binding of USF-1-interacting proteins to the −444 FAS-CAT (left) and −444 (−150 m) FAS-CAT (right) promoter.

(H) ChIP analysis for DNA breaks and DNA-PK and TopoIIβ binding to the FAS-CAT (left) or the endogenous FAS promoter (right) in FAS-CAT transgenic or wild-type mice.

Error bars represent ± SEM.

involving USF and its interacting proteins in response to feeding. Overall, USF-1 is constitutively bound to the FAS and other lipogenic promoters in both metabolic states, whereas USF-interacting proteins are bound in a fasting/feeding-dependent manner. We next investigated whether this is due to the differential interaction of USF with these proteins by employing insulin-responsive HepG2 cells overexpressing USF-1. The levels of various USF-interacting proteins in HepG2 cells were similar when cells were cultured in the presence or absence of insulin (Figure S1D). As shown in Figure 1E, in insulin-treated cells, USF-1 preferentially coimmunoprecipitated with those proteins that were found to be bound to the lipogenic promoters in the fed condition, whereas in the absence of insulin, USF-1 preferentially interacted with HDAC9.

To further address whether the binding of the various interacting proteins to the FAS promoter is USF dependent, we performed ChIP in transgenic mice containing CAT driven by the −444 FAS promoter with a specific mutation at the USF-binding site of −65 E box (−444 (−65 m)). We have previously shown that, due to the loss of the critical −65 E box where USF binds, the −444 (−65 m) FAS promoter does not have any activity, although the promoter contains an additional USF-binding site at −332 (Latasa et al., 2003). We did not detect binding of any of the USF-1-interacting proteins to this FAS promoter containing the −65 E box mutation, even though USF-1 was bound to the −332 E box in both fasted and fed states (Figure 1F, left panel). Furthermore, siRNA-mediated knockdown of USF-1 prevented recruitment of the USF-1-interacting proteins to the wild-type FAS promoter (Figure 1F, right panel). Taken together, these data clearly demonstrate the requirement of USF-1 binding to the −65 E box for recruitment of various proteins to the FAS promoter.

Because USF binding to the E box is necessary for SREBP binding to the nearby SRE in lipogenic promoters and USF and SREBP-1 directly interact for promoter activation (Latasa et al., 2003; Griffin et al., 2007), we examined whether the binding of the USF-1-interacting proteins to the FAS promoter is dependent on the SREBP-1 binding to SRE. We performed ChIP in

transgenic mice containing CAT driven by the −444 FAS promoter with a specific mutation at the −150SRE (−444 (−150 m)). As shown in Figure 1G, we could not detect recruitment of the various interacting proteins to the FAS promoter containing the −150 SRE mutation during feeding. Similar results were observed in HepG2 cells when transfected with −444 (−150 m) FAS-Luc or SREBP-1 siRNA (Figures S2A and S2B), correlating with the diminished FAS promoter activation (Figure S2E). As a control, we examined the p53 promoter, which has a proximal E box but does not respond to feeding/insulin (Figures S1C and S2D). Upon insertion of an artificial SRE, the p53 promoter was activated by USF-1 recruiting various interacting proteins in response to insulin (Figures S2D and S2E), demonstrating that nearby SRE is critical for USF-1 to recruit various interacting proteins.

As shown, the components of DNA break/repair machinery were recruited to the FAS promoter in fed state. In this regard, it has recently been reported that a transient DNA break is required for estrogen receptor-regulated transcription (Ju et al., 2006). By end labeling using biotin-UTP and subsequent ChIP, we clearly detected DNA breaks in the −444 FAS-CAT as well as the endogenous FAS promoters after 3 hr of feeding, a time point when binding of DNA-PK and TopoIIβ was detected (Figure 1H). The observed DNA breaks in the FAS promoter region preceded the maximal FAS transcription that occurs 6 hr after the start of feeding (Paulauskis and Sul, 1989).

Feeding-Induced Phosphorylation of USF-1

Constitutive binding of USF-1, despite its differential recruitments during fasting/feeding, prompted us to investigate whether USF-1 is posttranslationally modified. We immunoprecipitated USF-1 from liver nuclear extracts of fasted or fed mice and performed MS analysis. Notably, we detected a phosphoserine residue at the S262 of USF-1 only in nuclear extracts from fed mice. We detected higher S262 phosphorylation of USF-1 in the fed state than in the fasted state (Figure 2A, panel 2) using antibodies against a USF-1 peptide containing phosphorylated S262 (referred to as anti-P-USF-1) that we generated. ChIP analysis of the FAS-CAT promoter using anti-P-USF-1 showed that this specific phosphoUSF-1 occupied the FAS promoter only in the fed state, even though USF-1 occupancy was detected in both fasted and fed conditions (Figure 2B). Similarly, USF-1 bound to the mGPAT promoter was phosphorylated at S262 in fed state (Figure S5D). To test the functional significance of this S262 phosphorylation, we expressed FLAG-tagged-USF-1 containing a mutation at the S262 (S262D or S262A). We detected similar protein levels of transfected S262 mutants and wild-type (WT) USF-1 (Figure 2C, bottom panel). ChIP analysis of the FAS promoter using anti-FLAG antibodies showed no differences in promoter occupancy between WT and FLAG-tagged USF-1 proteins harboring S262 mutation (Figure 2C, top panel). However, the S262D mutant that mimics hyperphosphorylation activated the FAS promoter at a much higher level than WT USF-1, whereas the nonphosphorylatable S262A mutant could no longer activate the FAS promoter (Figure 2C, bottom panel). By immunoblotting lysates from these cells, we also detected changes in FAS protein levels corresponding to the FAS promoter activity (Figure 2C, bottom panel). Taken together, these data suggest that the feeding-dependent phosphorylation of USF-1 at S262 is linked to FAS promoter activation.

Feeding-Induced Acetylation of USF-1

As shown in Figure 1, USF-1-interacting proteins HDAC9 and P/CAF occupied the lipogenic gene promoters in fasted and fed states, respectively. During the MS analysis of USF-1 for posttranslational modification(s), we identified two acetylated lysine residues at K237 and K246 of USF-1. However, when we performed MS analysis of immunoprecipitates from cells cotransfected with USF-1 and P/CAF that interacts with USF in the fed state, we detected acetylation of only K237, but not K246. Therefore, we raised antibodies against USF-1 peptide containing acetylated K237 (anti-Ac-USF-1) and used them to compare acetylation of USF-1 at K237 in fasted and fed states. Indeed, we detected higher K237 acetylation of USF-1 in the fed state (Figure 2D, panel 2) compared to the fasted state. ChIP analysis of the FAS-CAT promoter using anti-Ac-USF-1 showed that the USF-1 bound to the FAS promoter was acetylated at K237 only in the fed state, even though USF-1 was bound to the FAS promoter in both fasted and fed states (Figure 2E). These data indicate that K237 is likely to be a regulatory site of USF-1 during fasting/feeding and that its acetylation might be catalyzed by P/CAF in the fed state.

To test the functional effects of this putative acetylation site, we expressed FLAG-tagged USF-1 with a mutation at the K237 (K237A or K237R) in 293 cells. ChIP analysis of the FAS promoter using anti-FLAG antibodies showed no difference in recruitment among WT USF-1, FLAG-tagged USF-1 with the K237A mutation that mimics hyperacetylation, and the FLAG-tagged USF-1 with nonacetylatable K237R mutation (Figure 2F, top panel). However, in the FAS promoter-reporter assay, cotransfection of the K237A mutant activated the FAS promoter at a much higher level than WT USF-1, whereas 237R mutant could no longer activate the FAS promoter (Figure 2F, bottom panel). These differences in promoter activation were reflected in FAS protein levels upon immunoblotting of cell lysates (Figure 2F, bottom panel). These data suggest that the feeding-dependent acetylation of USF-1 is responsible for FAS promoter activation in the fed condition.

DNA-PK Mediates Feeding-Dependent Phosphorylation of USF-1

The first step in understanding how the feeding-dependent phosphorylation of USF-1 activates the FAS promoter would be to identify the kinase that catalyzes this S262 phosphorylation. A search of numerous phosphoprotein databases predicted that a member of the PIKK family of kinases likely phosphorylates the S262 site. DNA-PK is a multimeric nuclear serine/threonine protein kinase composed of the DNA-PK catalytic subunit and the Ku70/Ku80 regulatory subunits (Collis et al., 2005). We found all of the DNA-PK subunits to be the USF-1-interacting proteins and bound to the FAS promoter in the fed state. Therefore, to examine whether S262 of USF-1 is a target of DNA-PK, we performed in vitro phosphorylation of bacterially expressed USF-1 by DNA-PK. Indeed, we could easily detect S262 phosphorylation of USF-1 by DNA-PK (Figure 3A, lane 1)

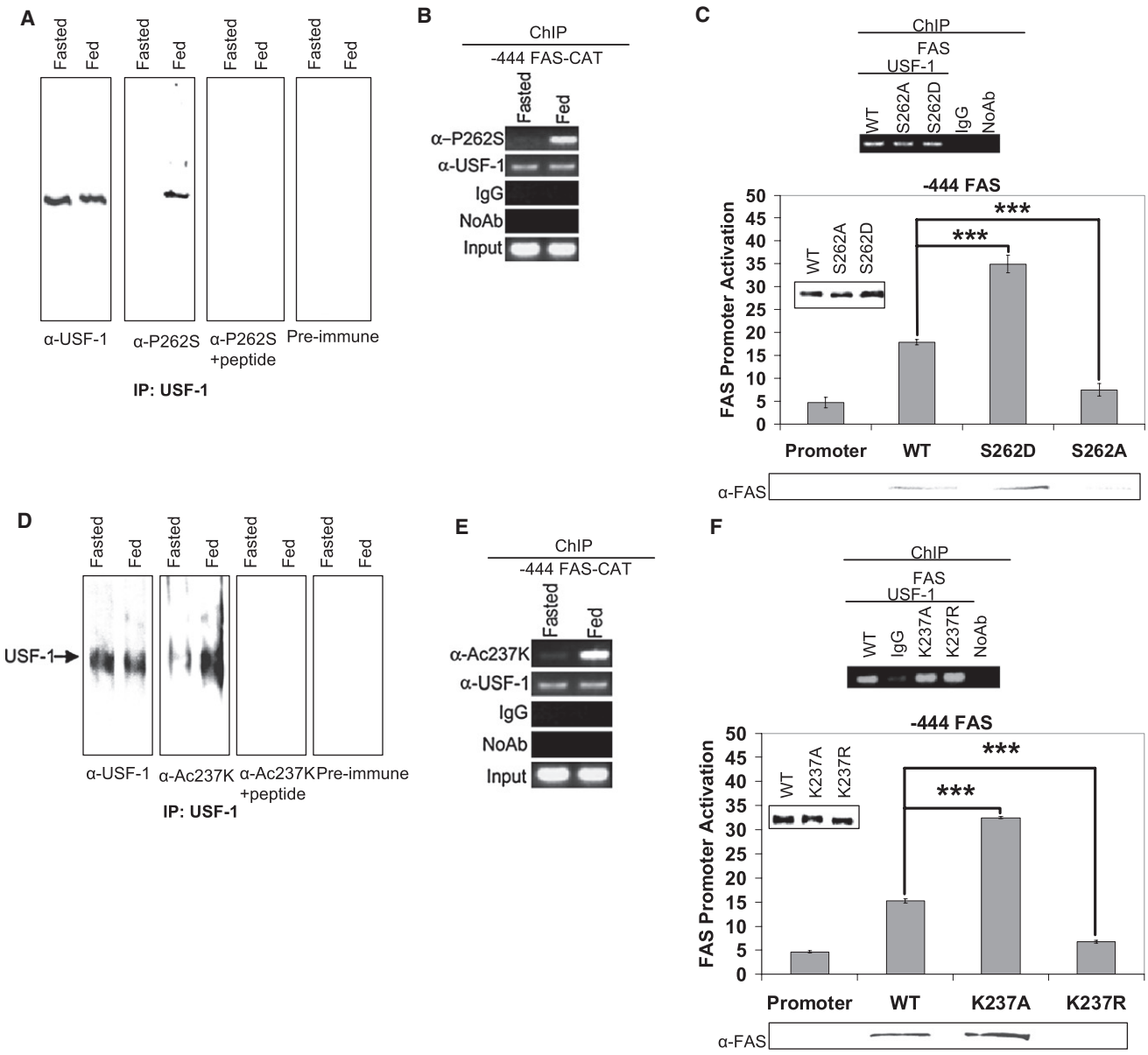


Figure 2. Feeding-Induced S262 Phosphorylation and K237 Acetylation of USF-1

(A) USF-1 immunoprecipitates using monoclonal anti-USF-1 was western blotted with polyclonal anti-USF-1 or anti-P-USF-1. Immunoblotting with anti-P-USF-1 in the presence of peptide or with preimmune serum are shown as controls.

(B) ChIP for indicated proteins binding to the −444 FAS-CAT promoter.

(C) ChIP (top) for WT USF-1 and S262 USF-1 mutant association to the FAS promoter in 293FT cells. The FAS promoter activity (bottom) was monitored. Immunoblotting for protein levels of WT, S262 USF-1 mutants (insert), and FAS are shown.

(D) IP of USF-1.

(E) ChIP for binding of indicated proteins to the −444 FAS-CAT promoter.

(F) ChIP (top) for association of WT USF-1 and K237 USF-1 mutant to the FAS promoter. The promoter activity was measured.

Error bars represent ± SEM.

in vitro, which is DNA-PK concentration dependent (Figure S3A). S262 phosphorylation was abolished when wortmannin was added at a concentration (Hashimoto et al., 2003) effective to inhibit DNA-PK activity (Figure 3A, lane 2). However, we could not detect S262 phosphorylation by PKA or PKC in vitro, nor did we detect changes in phosphorylation upon cotransfection with PKB (Figure S3B). Based on these results and the fact that DNA-PK is associated with USF-1 in the fed state, we conclude that the S262 of USF-1 is a specific target of DNA-PK.

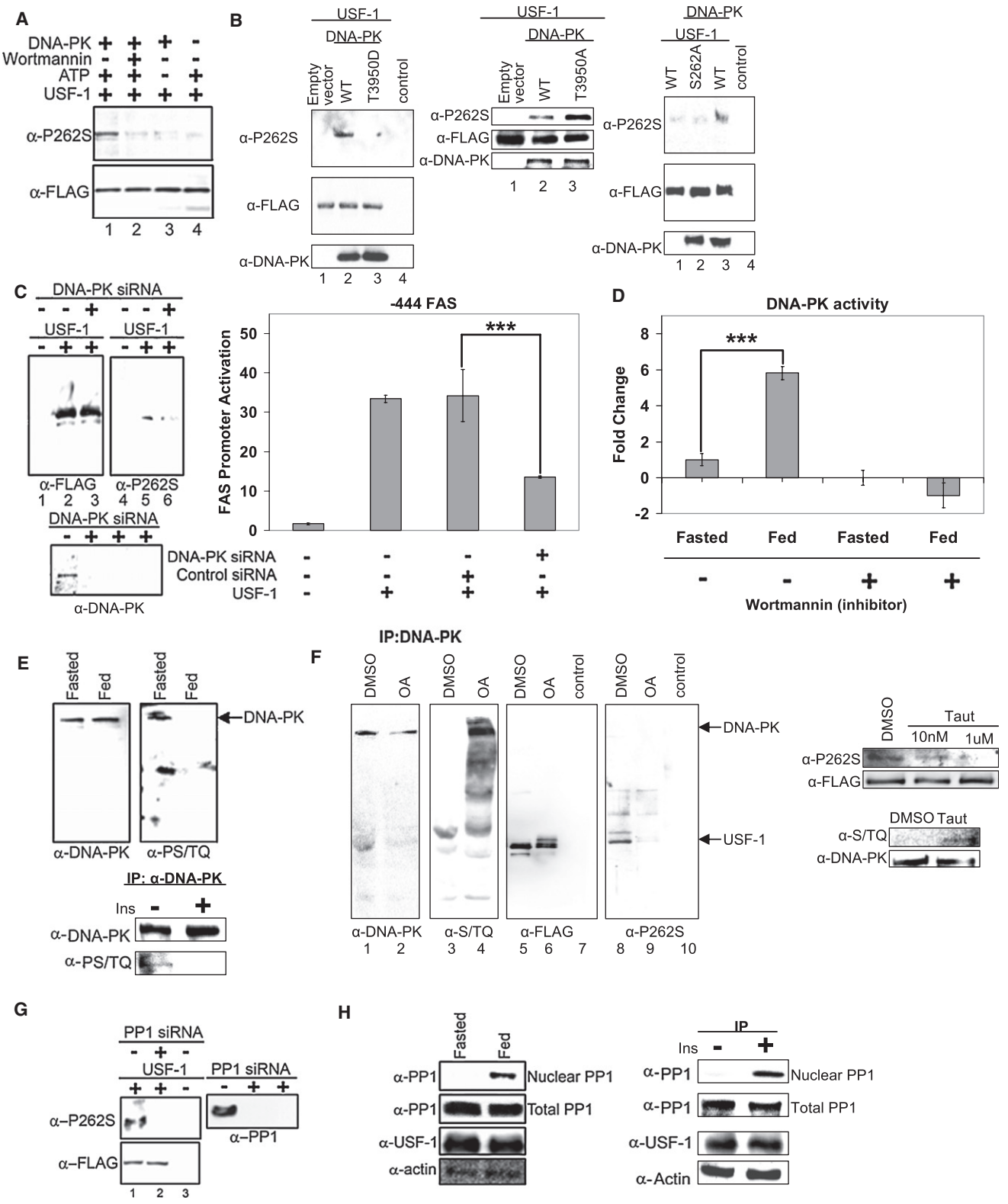


Figure 3. Feeding-Dependent S262 Phosphorylation of USF-1 Is Mediated by DNA-PK that Is Dephosphorylated/Activated in Feeding

(A) USF-1 was incubated with DNA-PK.

(B) IP of USF-1. Immunoblotting for DNA-PK.

(C) IP (left) of USF-1. The FAS promoter activity was measured (right).

We next tested S262 phosphorylation of USF-1 by DNA-PK in cultured cells. We overexpressed USF-1 along with WT DNA-PK, kinase-dead DNA-PK with a T3950D mutation, or constitutive active DNA-PK with a T3950A mutation. T3950D mutation mimics hyperphosphorylation (Douglas et al., 2007), whereas T3950A mutation mimics dephosphorylation. We detected higher S262 phosphorylation of USF-1 immunoprecipitated from cells overexpressing WT DNA-PK (Figure 3B, left panel, lane 2), but not from cells expressing DNA-PK with T3950D mutation (Figure 3B, lane 3) or control cells (Figure 3B, lane 1). Furthermore, we detected even higher S262 phosphorylation of USF-1 from cells expressing DNA-PK with T3950A mutation compared to WT DNA-PK-expressing cells (Figure 3B, middle panel, lane 3). Next, to investigate whether DNA-PK-mediated phosphorylation of USF-1 is S262 specific, we overexpressed WT USF-1 or the S262A mutant along with DNA-PK. WT USF-1, but not USF-1 containing S262A mutation, was detected to have higher phosphorylation upon cotransfection with DNA-PK (Figure 3B, right panel, lanes 2 and 3). To further verify the role of DNA-PK in S262 phosphorylation, we performed siRNA-mediated knockdown of DNA-PK. We observed low but detectable S262 phosphorylation of USF-1 (Figure 3C, left panel, lane 5). S262 phosphorylation was significantly reduced in the DNA-PK siRNA-transfected cells that had more than an 80% decrease in DNA-PK levels (Figure 3C, lane 6). FAS promoter activity in DNA-PK siRNA-transfected cells was reduced by 65% compared to control siRNA-transfected cells (Figure 3C, right panel), which was similar to that observed upon transfection of nonphosphorylatable S262A USF-1 mutant (Figure 2C). These results demonstrate that S262 phosphorylation of USF-1 is mediated by DNA-PK.

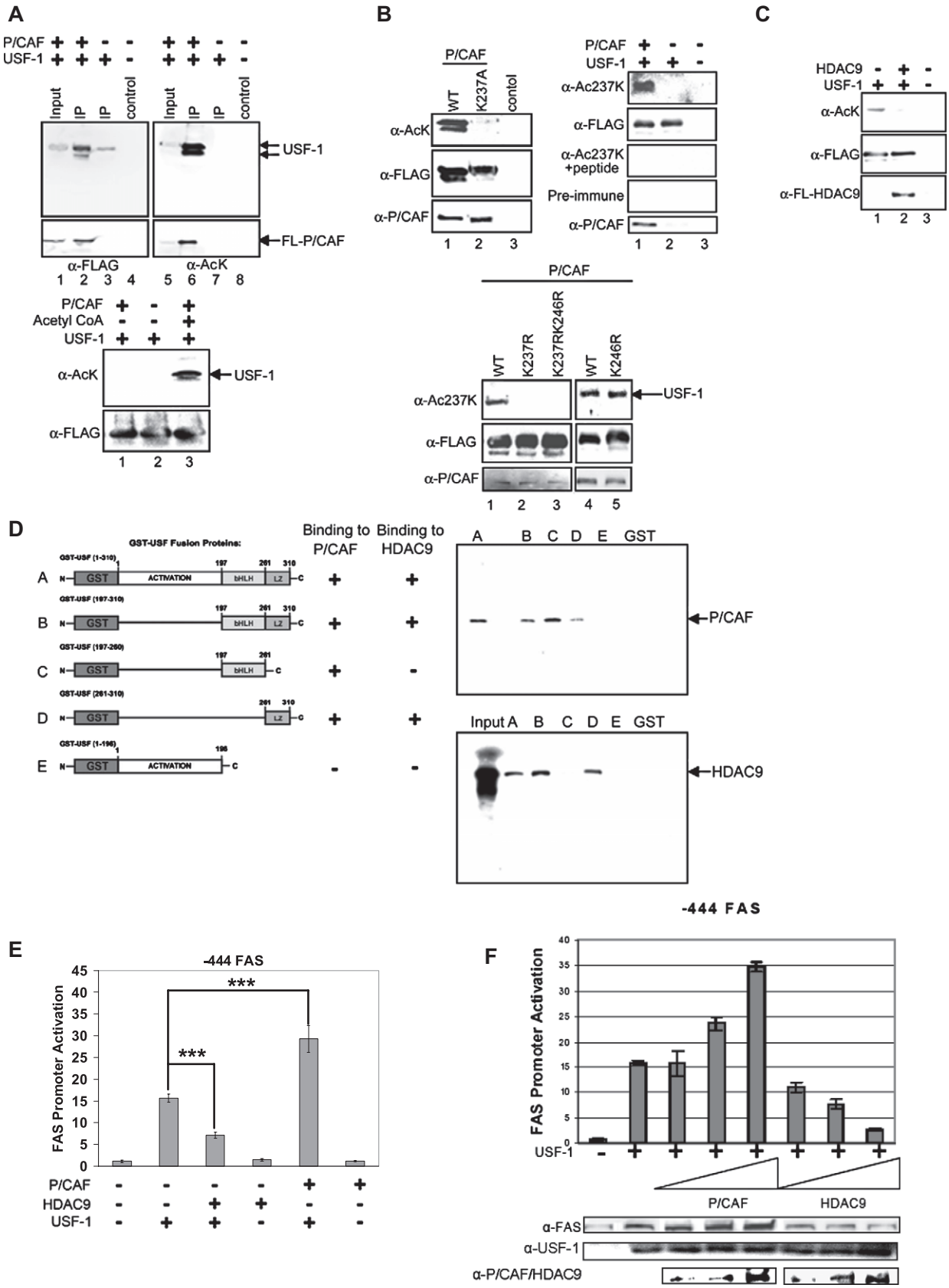
PP1-Mediated Dephosphorylation/Activation of DNA-PK Causes USF-1 Phosphorylation upon Feeding

We found that DNA-PK phosphorylates USF-1 at S262 and that S262 phosphorylation is lower in the fasted state but increases upon feeding. This prompted us to ask whether the changes in DNA-PK activity account for the differences in S262 phosphorylation during fasting/feeding. Using the specific DNA-PK substrate, a biotinylated p53 peptide, we compared DNA-PK activity in liver nuclear extracts of fasted or fed mice (Figure 3D). While total DNA-PK protein levels remained the same (data now shown), DNA-PK activity in the fed state was 6-fold higher than in the fasted state. Wortmannin treatment drastically reduced DNA-PK activity when measured with the DNA-PK-specific peptide as a substrate (Figure 3D). This demonstrates that the kinase activity we detected can be attributed to DNA-PK.

DNA-PK activity is known to be regulated by phosphorylation/dephosphorylation, independent of its activation by DNA. Thus, autophosphorylation of DNA-PK results in a decrease in its kinase activity, whereas dephosphorylation by PP1 activates DNA-PK (Douglas et al., 2001, 2007). Among the PIKK family members, DNA-PK is the only kinase that is activated by dephosphorylation. To examine the involvement of DNA-PK in USF phosphorylation, we first examined the phosphorylation status of DNA-PK in fasted and fed states. DNA-PK phosphorylation was detected using phosphoserine/threonine antibodies that detect autophosphorylation at the S/TQ motifs of DNA-PK. As shown in the top panel of Figure 3E, phosphorylation of DNA-PK was higher in the fasted state than in the fed state, whereas DNA-PK protein levels did not change. We also found that DNA-PK phosphorylation was not detectable in insulin-treated HepG2 cells, whereas phosphorylation was easily detected in noninsulin-treated cells (Figure 3E, bottom panel).

During the examination of the occupancy of USF-interacting proteins, we found that PP1 along with DNA-PK was bound to lipogenic gene promoters in the fed state (Figure 1C) when lipogenesis is induced. It is possible that PP1, which we found to be a USF-interacting protein, mediates the feeding/insulin signal by dephosphorylating DNA-PK. Therefore, we tested the S262 phosphorylation status of USF-1 upon treatment with okadaic acid (OA), which is known to prevent dephosphorylation of DNA-PK (Douglas et al., 2001). As expected, phosphorylation of DNA-PK greatly increased in OA-treated cells (Figure 3F, left panel, lane 4), whereas DNA-PK autophosphorylation was reduced in cells overexpressing PP1γ (Figure S3C). We next examined S262 phosphorylation in OA-treated cells by western blotting of immunoprecipitated USF-1 with anti-FLAG or anti-P-USF-1 antibodies. Compared to a single USF-1 band detected in control DMSO-treated cells, several USF-1 bands were detected in OA-treated cells, suggesting a multisite phosphorylation of USF-1 (Figure 3F, lane 6). However, S262 phosphorylation of USF-1 that was easily detected in control cells was hardly detectable in OA-treated cells (Figure 3F, lane 9). To further test the specificity of PP1 on S262 phosphorylation status, we also used tautomycin (Taut), which is known to more selectively inhibit PP1. As expected, we easily detected phosphorylated DNA-PK in cells treated with Taut at 1 uM, but not in control cells (Figure 3F, right panel). On the other hand, S262 phosphorylation of USF-1 was detected in control cells as expected but was decreased in cells treated with Taut at 10 nM and was hardly detectable at 1 uM (Figure 3F, right panel). We also tested the role of PP1 by using a siRNA approach. S262 phosphorylation of USF-1 did not increase but, rather, greatly decreased in PP1 knockdown cells (Figure 3G, lane 2), indicating that PP1 does not directly dephosphorylate S262 phosphorylation. Furthermore, S262 phosphorylation could be restored upon cotransfection of constitutively active DNA-PK (Figure S3D). This indicates that S262

(D) DNA-PK activity was assayed.
(E) IP of DNA-PK.
(F) IP of USF-1-FLAG. Total and phosphorylated DNA-PK by western blotting.
(G) IP of USF-1. PP1 protein levels by western blotting.
(H) IP of PP1 from nuclear extracts or total lysates. USF-1 and β-actin protein levels by western blotting.
Error bars represent ± SEM.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HDAC9
USF-1
α-AcK
α-FLAG
α-FL-HDAC9
1 2 3
D
GST-USF Fusion Proteins:
Binding to P/CAF
Binding to HDAC9
A
B
C
D
E
GST
ACTIVATION
bHLH
LZ
A B C D E GST
P/CAF
Input A B C D E GST
HDAC9
E
-444 FAS
FAS Promoter Activation
***
***
P/CAF
HDAC9
USF-1
F
-444 FAS
FAS Promoter Activation
USF-1
P/CAF
HDAC9
α-FAS
α-USF-1
α-P/CAF/HDAC9

phosphorylation is through DNA-PK that is first dephosphorylated/activated by PP1. When we compared the abundance of PP1 in liver nuclear extracts, we detected higher levels of PP1 in the nucleus in the fed state than in the fasted state, whereas PP1 protein levels in total cell lysates as well as PP1 gene expression levels did not change (Figure 3H, left panel and Figure S3E). Similarly, PP1 was not detected in nuclear extracts from control HepG2 cells but was increased upon insulin treatment (Figure 3H, right panel). Overall, we conclude that the feeding-dependent S262 phosphorylation of USF-1 is mediated by DNA-PK. But first, DNA-PK is dephosphorylated/activated by PP1 whose level in nucleus increases in response to feeding/insulin.

P/CAF-Mediated Acetylation of USF-1 Activates the FAS Promoter, whereas HDAC9-Mediated Deacetylation Causes Promoter Inactivation

HDAC9 and P/CAF are recruited by and interact with USF-1 in a fasting/feeding-dependent manner. Therefore, we next examined whether acetylation and deacetylation of USF-1 is through P/CAF and HDAC9, respectively. When we cotransfected USF-1 and P/CAF, by using pan-acetyl lysine antibodies, we detected higher acetylation of USF-1 (Figure 4A, top panel, lane 6). As shown in the bottom panel of Figure 4A, USF-1 was acetylated in vitro by P/CAF (lane 3), and acetylation was not detected in the absence of P/CAF or acetyl CoA (lane 1 and 2). MS analysis of USF-1 in cells overexpressing P/CAF revealed a regulatory site at K237, the residue that was acetylated upon feeding (Figure 2). To examine whether this site was a target of P/CAF, we overexpressed FLAG-tagged WT USF-1 or USF-1 mutated at K237 along with P/CAF. As detected by pan-acetyl lysine antibodies, only WT USF-1 was efficiently acetylated by P/CAF (Figure 4B, top-left panel, lane 1), but the K237A USF-1 mutant was not (Figure 4B, top-left panel, lane 2). We next employed anti-Ac-USF-1 antibodies specific for USF-1 acetylated at K237, and we detected higher K237 acetylation in cells overexpressing P/CAF (Figure 4B, top right, lane 1). To further investigate whether P/CAF-mediated acetylation of USF-1 is K237 specific, we overexpressed WT USF-1 and various (K237 and K246) USF-1 mutants along with P/CAF. WT and K246R (Figure 4B, bottom panel, lanes 1, 4, and 5), but not K237R or K237R/K246R (Figure 4B, bottom panel, lanes 2 and 3), of USF-1 were found to be acetylated upon cotransfection with P/CAF, demonstrating that acetylation of K237, but not K246, is mediated by P/CAF.

With the binding of HDAC9 to the lipogenic promoters only in the fasted state, we speculated that HDAC9 would be an ideal candidate to remove the P/CAF-mediated acetylation of USF-1 in the fed state. We transfected USF-1 and P/CAF along with HDAC9 or a control empty vector into 293 cells. We detected a decrease in P/CAF-catalyzed acetylation of USF-1 in cells cotransfected with HDAC9 (Figure 4C, lane 2). Furthermore, we detected significant HDAC9 protein levels in liver nuclear extracts from fasted, but not fed, mice or in nuclear extracts of HepG2 cells cultured in the absence, but not presence, of insulin (Figure S4A), whereas its expression did not change in various conditions (Figure S4B). These experiments indicate that, in the fasted state, nuclear HDAC9 is in higher abundance and is recruited to the FAS promoter to deacetylate USF-1.

We found by GST pull-down that USF-1 can directly interact with HDAC9 and P/CAF (but not p300) (Figure S4C). Therefore, we dissected the domains of USF-1 required for interaction with P/CAF and HDAC9. As shown in Figure 4D, the bHLH domain of USF-1, the domain containing K237 that is acetylated by P/CAF, was sufficient for the interaction with P/CAF, although the leucine zipper (LZ) domain could weakly interact with P/CAF. On the other hand, for the USF-1 interaction with HDAC9, the LZ domain of USF-1 was sufficient for its interaction with HDAC9. Thus, the domains of USF-1 required for interaction are in proximity to K237, the residue modified by these HAT/HDAC.

Cotransfection of USF-1 together with HDAC9 resulted in a 50% decrease in FAS promoter activity in a fashion similar to that detected upon cotransfection of USF-1 containing a K237R mutation (Figures 2F and 4E). In contrast, the expression of USF-1 with P/CAF resulted in a 2-fold higher promoter activity in a manner similar to that observed upon cotransfection of USF-1 containing the K237A mutation (Figures 2F and 4E). Furthermore, cotransfection of P/CAF enhanced, while cotransfection of HDAC9 suppressed, USF-1 activation of the FAS promoter in a dose-dependent manner (Figure 4F). We detected changes in FAS protein levels parallel to the FAS promoter activity. In addition, cotransfecting P/CAF or HDAC9 with USF-1 containing K237A or K237R mutation did not change the FAS promoter activity or FAS protein levels (Figure S4E). These data indicate that acetylation and deacetylation of USF-1 catalyzed by P/CAF and HDAC9, respectively, function as a dynamic switch for the transition between fasting/feeding in FAS promoter regulation.

Phosphorylation-Dependent Acetylation of USF-1

Since USF-1 is both phosphorylated and acetylated at nearby sites and these posttranslational modifications are critical for USF-1 function in FAS promoter activation, we tested whether an increase in S262 phosphorylation of USF-1 could affect K237 acetylation. We cotransfected USF-1 and DNA-PK and examined S262 phosphorylation and K237 acetylation of USF-1. If S262 phosphorylation affects acetylation, cotransfection of DNA-PK would cause not only S262 phosphorylation of USF-1,

Figure 4. Acetylation of K237 of USF-1 by P/CAF and Deacetylation by HDAC9

(A) IP of USF-1 (top). USF-1 was in vitro acetylated with P/CAF (bottom).

(B) IP of USF-1. P/CAF protein levels by immunoblotting.

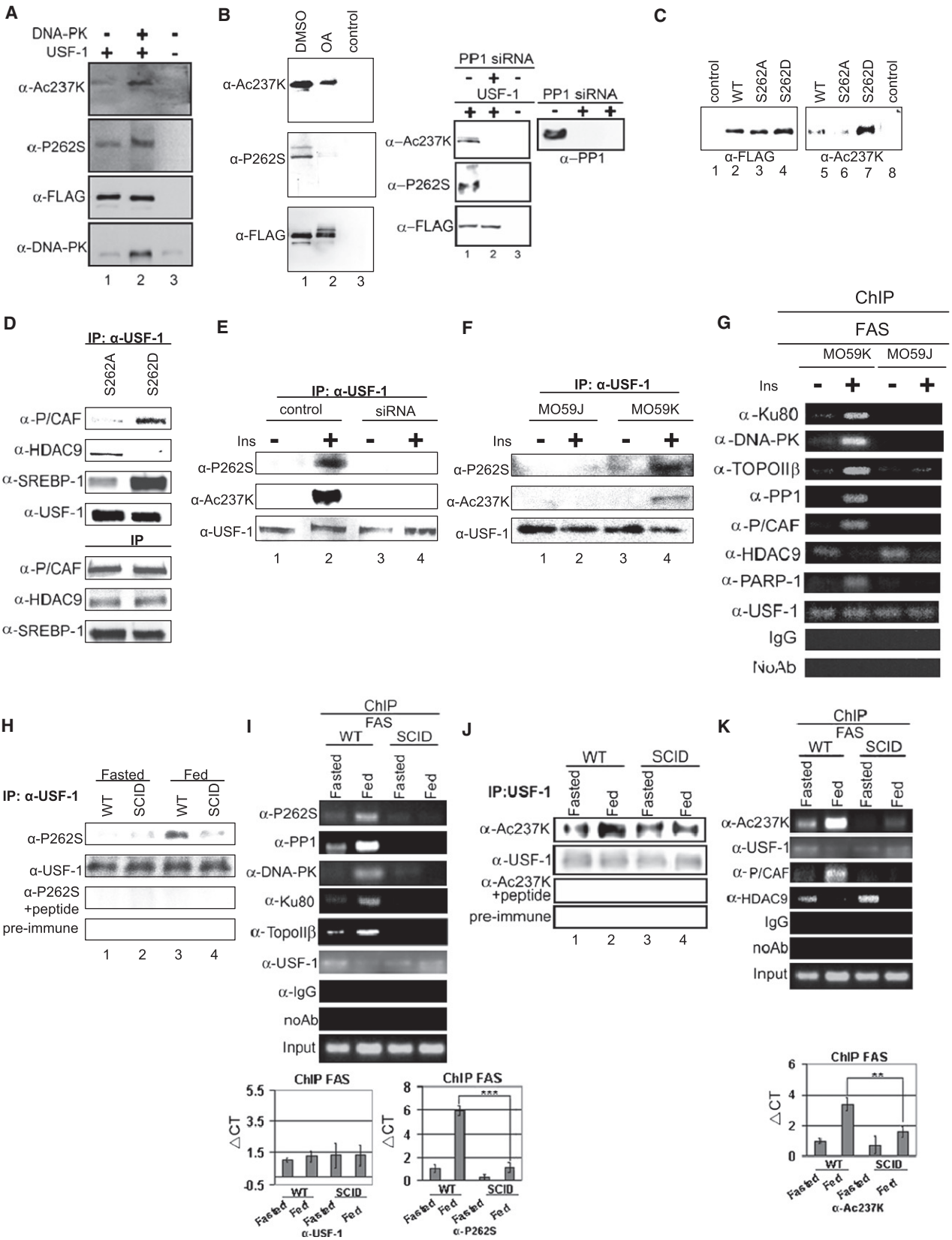
(C) IP of USF-1.

(D) USF-1 was incubated with in vitro translated ^{35}S-labeled proteins before subjecting to GST pull-down. GST was used as a control.

(E) The −444 FAS-Luc promoter activity was measured.

(F) The −444 FAS-Luc promoter activity was measured. Total cell lysates were immunoblotted.

Error bars represent ± SEM.


A
DNA-PK
USF-1
α-Ac237K
α-P262S
α-FLAG
α-DNA-PK
1 2 3
B
DMSO
OA
control
α-Ac237K
α-P262S
α-FLAG
1 2 3
PP1 siRNA
USF-1
PP1 siRNA
α-Ac237K
α-P262S
α-FLAG
α-PP1
1 2 3
C
control
WT
S262A
S262D
WT
S262A
S262D
control
α-FLAG
α-Ac237K
1 2 3 4 5 6 7 8
D
IP: α-USF-1
S262A
S262D
α-P/CAF
α-HDAC9
α-SREBP-1
α-USF-1
IP
α-P/CAF
α-HDAC9
α-SREBP-1
E
IP: α-USF-1
control
siRNA
Ins
α-P262S
α-Ac237K
α-USF-1
1 2 3 4
F
IP: α-USF-1
MO59J
MO59K
Ins
α-P262S
α-Ac237K
α-USF-1
1 2 3 4
G
ChIP
FAS
MO59K
MO59J
Ins
α-Ku80
α-DNA-PK
α-TOPOIIβ
α-PP1
α-P/CAF
α-HDAC9
α-PARP-1
α-USF-1
IgG
NoAb
H
IP: α-USF-1
Fasted
Fed
WT
SCID
WT
SCID
α-P262S
α-USF-1
α-P262S +peptide
pre-immune
1 2 3 4
I
ChIP
FAS
WT
SCID
Fasted
Fed
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Fed
α-P262S
α-PP1
α-DNA-PK
α-Ku80
α-TopoIIβ
α-USF-1
α-IgG
noAb
Input
ChIP FAS
△CT
5.5
3.5
1.5
-0.5
WT
SCID
Fasted
Fed
Fasted
Fed
α-USF-1
ChIP FAS
△CT
8
6
4
2
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***
WT
SCID
Fasted
Fed
Fasted
Fed
α-P262S
J
IP:USF-1
WT
SCID
Fasted
Fed
Fasted
Fed
α-Ac237K
α-USF-1
α-Ac237K +peptide
pre-immune
1 2 3 4
K
ChIP
FAS
WT
SCID
Fasted
Fed
Fasted
Fed
α-Ac237K
α-USF-1
α-P/CAF
α-HDAC9
IgG
noAb
Input
ChIP FAS
△CT
6
4
2
0
**
WT
SCID
Fasted
Fed
Fasted
Fed
α-Ac237K

but also K237 acetylation. Indeed, S262 phosphorylation of USF-1 upon DNA-PK transfection strongly enhanced USF-1 acetylation at K237 (Figure 5A, lane 2). Conversely, we detected a significant level of K237 acetylation of USF-1 in control cells, which was reduced in OA-treated cells (Figure 5B, left panel, lane 2). Likewise, K237 acetylation of USF was high in control cells but was reduced to an undetectable level in PP1 siRNA-transfected cells (Figure 5B, right panel, lane 1). Inactivation of PP1 by OA treatment or siRNA-mediated knockdown of PP1 caused phosphorylation/inactivation of DNA-PK resulting in reduced S262 phosphorylation of USF-1. This suggests that S262 phosphorylation brings about K237 acetylation. We then asked whether phosphorylation of USF-1 at S262 could affect USF-1 acetylation status by transfecting FLAG-tagged WT USF-1 or S262 mutants and examining the K237 acetylation status of the various USF-1 forms. We found that the S262A mutant had the lowest K237 acetylation among the three USF-1 forms (Figure 5C, lane 6), whereas the S262D mutant displayed the highest acetylation to a level significantly higher than WT USF-1 (Figure 5C, lane 7). Overall, these results demonstrate phosphorylation-dependent acetylation of USF-1.

The simplest hypothesis underlying S262 phosphorylation-dependent acetylation of USF-1 would be that S262 phosphorylation/dephosphorylation affects recruitment of P/CAF and HDAC9, causing acetylation and deacetylation of K237 and USF-1, respectively. Coimmunoprecipitation assay showed that the S262D mutant preferentially interacted with P/CAF in comparison to the S262A mutant (Figure 5D). On the other hand, compared to the S262D mutant, the S262A mutant preferentially interacted with HDAC9, although the signal was low probably due to the low HDAC9 levels in the nucleus. We next examined whether S262 mutation of the USF-1 affects interaction of USF with SREBP-1 that we previously reported. We found that the S262D USF mutant, as compared to S262A mutant, preferentially interacted with SREBP-1. Taken together, these results show that the phosphorylation-dependent acetylation of USF-1 functions as a sensitive molecular switch, detecting nutritional status during the transition between fasting/feeding.

Feeding/Insulin-Dependent Phosphorylation/Acetylation of USF-1 Are Diminished in DNA-PK Deficiency

To further demonstrate the requirement of DNA-PK in mediating the feeding/insulin-dependent phosphorylation/acetylation of USF-1, we transfected DNA-PK siRNA into HepG2 cells. Insulin treatment of these cells markedly increased S262 phosphorylation as well as K237 acetylation in control siRNA-transfected cells, whereas USF-1 levels remained the same (Figure 5E, lanes 1 and 2). In contrast, insulin-mediated S262 phosphorylation/K237 acetylation of USF-1 in cells transfected with DNA-PK siRNA was markedly reduced and undetectable (Figure 5E, lanes 3 and 4). We next compared the human glioblastoma cell line, M059J, which lacks DNA-PKcs and DNA-PK activity, and the related M059K cells containing WT DNA-PK (Feng et al., 2004) as a control. Treatment of M059K cells with insulin increased S262 phosphorylation and K237 acetylation of USF-1 (Figure 5F, lanes 3 and 4), whereas insulin treatment of M059J cells did not result in any significant increase in USF modifications (Figure 5F, lanes 1 and 2). These data demonstrate that DNA-PK is required not only for S262 phosphorylation, but also for K237 acetylation of USF-1 upon insulin treatment.

By ChIP, we also tested whether recruitment of various proteins to FAS promoter by USF is dependent on DNA-PK (Figure 5G). Those proteins that were found to be bound to the lipogenic gene promoters in the fed condition were recruited by USF in insulin-treated M059K cells, but not in the DNA-PK deficient M059J cells. In the absence of insulin, HDAC9 was recruited by USF in both M059J and M059K cells, most likely because cytoplasmic export of HDAC9 was not affected by DNA-PK. Similarly, coimmunoprecipitation showed that USF-1 can interact better with various partners in insulin-treated M059K, but not in M059J cells (Figure S5A). Furthermore, USF-1 interaction and recruitment of various proteins were abolished in 293 cells upon treatment with Taut that inhibits DNA-PK activity (Figures S5B and S5C). Overall, these results show that the recruitment of various proteins by USF-1 in feeding/insulin treatment is dependent on DNA-PK and DNA-PK-mediated S262 USF-1 phosphorylation.

We next examined in vivo the DNA-PK-mediated and feeding-dependent S262 phosphorylation/K237 acetylation of USF-1 by employing DNA-PK-deficient SCID (severe combined immune deficiency) mice. A spontaneous mutation in the *DNA-PK* gene causes a 90% reduction of the protein in SCID mice (Danska et al., 1996), producing a phenotype highly reminiscent of DNA-PK null mice. Indeed, feeding-induced phosphorylation of USF-1 at S262 was greatly reduced in SCID mice compared to that observed in WT mice (Figure 5H, lanes 4 and 3). ChIP analysis showed that the USF-1 detected on the FAS promoter in SCID mice in the fed state was not phosphorylated at S262 compared to the phosphoUSF-1 detected on the promoter in WT mice (Figure 5I). Similarly, USF-1 bound to the mGPAT promoter was not phosphorylated at S262 in SCID mice in the fed state (Figure S5D). Furthermore, we could not detect occupancy by DNA-PK, Ku80, TopoIIβ, and PP1 on the FAS promoter in SCID mice upon feeding (Figure 5I). Because K237 acetylation of USF-1 is dependent on S262 phosphorylation as shown

Figure 5. Feeding/Insulin-Induced Phosphorylation and Acetylation of USF-1 Are Greatly Reduced in DNA-PK Deficiency

(A–C) IP of FLAG-tagged USF-1. Nuclear extracts from nontransfected cells were used as a control.

(D) IP of USF-1-FLAG from HepG2 cells (top). Total protein levels by immunoblotting (bottom).

(E and F) IP of USF-1 from HepG2 cells (E) or from M059J or M059K cells (F).

(G) ChIP for binding of indicated proteins to the FAS promoter.

(H) IP of USF-1.

(I) ChIP for indicated protein association to the FAS promoter. ChIP samples were analyzed by semiquantitative PCR (top) or qPCR (bottom).

(J) IP of USF-1.

(K) ChIP for indicated protein association to the FAS promoter.

Error bars represent ± SEM.

above, we investigated whether K237 acetylation was also reduced in SCID mice. We found that K237 acetylation upon feeding was greatly reduced in SCID mice compared to that detected in WT mice (Figure 5J, lanes 4 and 2). The acetylated USF-1 bound to the FAS promoter in the fed state also was greatly reduced in SCID mice in ChIP analysis (Figure 5K). This decrease in acetylated USF-1 bound to the FAS promoter could be explained by the decreased recruitment of P/CAF by USF-1 (Figure 5K). HDAC9 binding was not different between WT and SCID mice probably because cytoplasmic export of HDAC9 was not affected in SCID mice. Overall, these results show in vivo the requirement of DNA-PK for S262 phosphorylation of USF-1 and for P/CAF-mediated K237 acetylation leading to transactivation of the FAS promoter.

Feeding-Dependent Activation of the FAS Gene and De Novo Lipogenesis Are Diminished in DNA-PK-Deficient SCID Mice

Because phosphorylation/acetylation of USF-1 for FAS promoter activation is through the PP1/DNA-PK-mediated signaling pathway, we assessed the transcriptional activation of the FAS gene in DNA-PK-deficient SCID mice during fasting/feeding. We first measured the nascent FAS RNA levels in liver nuclei from WT or SCID mice that were either fasted or fed (Figure 6A) by RT-PCR. In WT mice, the FAS nascent RNA was not detectable in fasting but increased drastically upon feeding. On the other hand, the nascent FAS RNA was barely detectable in either fasted or fed SCID mice. RT-qPCR analysis indicated a 50-fold increase in FAS nascent transcript in WT mice upon feeding, whereas in SCID mice, the increase was 20-fold, representing approximately a 50%–60% decrease (Figure 6B). We next performed nuclear run-on assays using nuclei from WT and SCID mice upon feeding at various time points. The rate of transcription measured by RT-qPCR of the newly extended nascent transcripts increased up to 10-fold in WT mice 6 hr after feeding, a result consistent with our previously published study. However, FAS transcription in SCID mice increased only by 6-fold, a 40% reduction compared to WT mice (Figure 6C).

Because we observed transient DNA breaks in the FAS promoter region that preceded transcriptional activation upon feeding (Figure 1I), we next examined whether the DNA break occurs in the FAS promoter region in SCID mice, but we could not detect transient DNA breaks, which we clearly detected in WT mice after 3 hr of feeding (Figure 6D). Furthermore, in contrast to WT mice, ChIP analysis did not show binding of DNA-PK or TopoIIβ to the FAS promoter region in SCID mice. Because TopoIIβ catalyzes DNA breaks, the absence of DNA breaks in the FAS promoter region in SCID mice can be attributed to the impaired TopoIIβ recruitment that is dependent on the DNA-PK-catalyzed phosphorylation of USF-1. Thus, not only the diminished acetylation of USF-1, but also the impaired recruitment of the DNA break/repair components, which is dependent on USF-1 phosphorylation, probably contributed to the attenuated feeding-dependent transcriptional activation of the FAS gene in SCID mice. Overall, these results clearly show in vivo the critical role of DNA-PK in activation of FAS transcription by feeding.

We examined in vivo hepatic de novo lipogenesis in WT and SCID mice using a stable isotope method. Fractional de novo lipogenesis was hardly detected in fasting but was increased drastically during a 24 hr period of feeding in WT mice (Figure 6E). However, feeding-induced fractional de novo lipogenesis was 60% lower in SCID mice after 24 hr of feeding compared to WT mice. To confirm that the decrease in de novo lipogenesis in SCID mice was due to a decrease in FAS induction, we examined the FAS protein levels in livers of WT and SCID mice after 24 hr of feeding. Indeed, FAS protein levels in SCID mice were significantly lower compared to WT mice (Figure 6F). The hepatic triglyceride levels after 24 hr feeding were approximately 30% lower in SCID mice compared to WT mice; serum triglyceride levels were also significantly lower in SCID mice (Figure 6G). Thus, impairment of feeding-dependent activation of FAS transcription in SCID mice leads to blunted induction of de novo lipogenesis, resulting in lower hepatic as well as—probably reflecting decreased VLDL secretion—serum triglyceride levels. In this regard, SCID mice also had a lower adipose tissue mass, indicative of a long-term defect in feeding induced lipogenesis (Table S1).

DISCUSSION

FAS levels in the liver change drastically during varying nutritional states, correlating with circulating insulin/glucagon levels. During fasting, fatty acid synthesis is virtually absent. However, upon feeding, accompanying insulin secretion, fatty acid synthesis is induced drastically. While many metabolic effects of insulin are mediated through protein phosphorylation by the activation of the well-characterized PI3K cascade, insulin can also exert metabolic effects through dephosphorylation catalyzed mainly by PP1. A central issue in metabolic regulation is to define coordinated molecular strategies that underlie the transition from fasting to feeding, such as the transcriptional activation of lipogenesis along specific transduction pathways. Here, we report a novel pathway that underlies the feeding/insulin response, which is based on posttranslational modifications of a key transcription factor, USF-1, by an atypical kinase, DNA-PK.

Differential Binding of USF-1-Interacting Proteins to Lipogenic Gene Promoters in Fasted and Fed States

Our study shows that USF recruits three different coregulator classes to lipogenic gene promoters. They are (1) the DNA break/repair machinery, (2) kinase/phosphatase, and (3) HAT/HDAC family. The distinct binding pattern of USF-interacting proteins on the FAS promoter in response to feeding/fasting is correlated with lipogenic gene activation/repression, which involve molecular events that require the presence of specific coactivators/corepressors, respectively.

FAS and other lipogenic enzymes such as mGPAT are coordinately regulated by feeding/insulin involving USF and SREBP-1c binding to the closely spaced E box and SRE, respectively. We show here that the USF-1 bound to the −65 E box recruits various USF-1-interacting proteins as well as SREBP-1c to bind SRE. Herein, we address the molecular function of various USF-1-interacting proteins and USF-1 modifications required

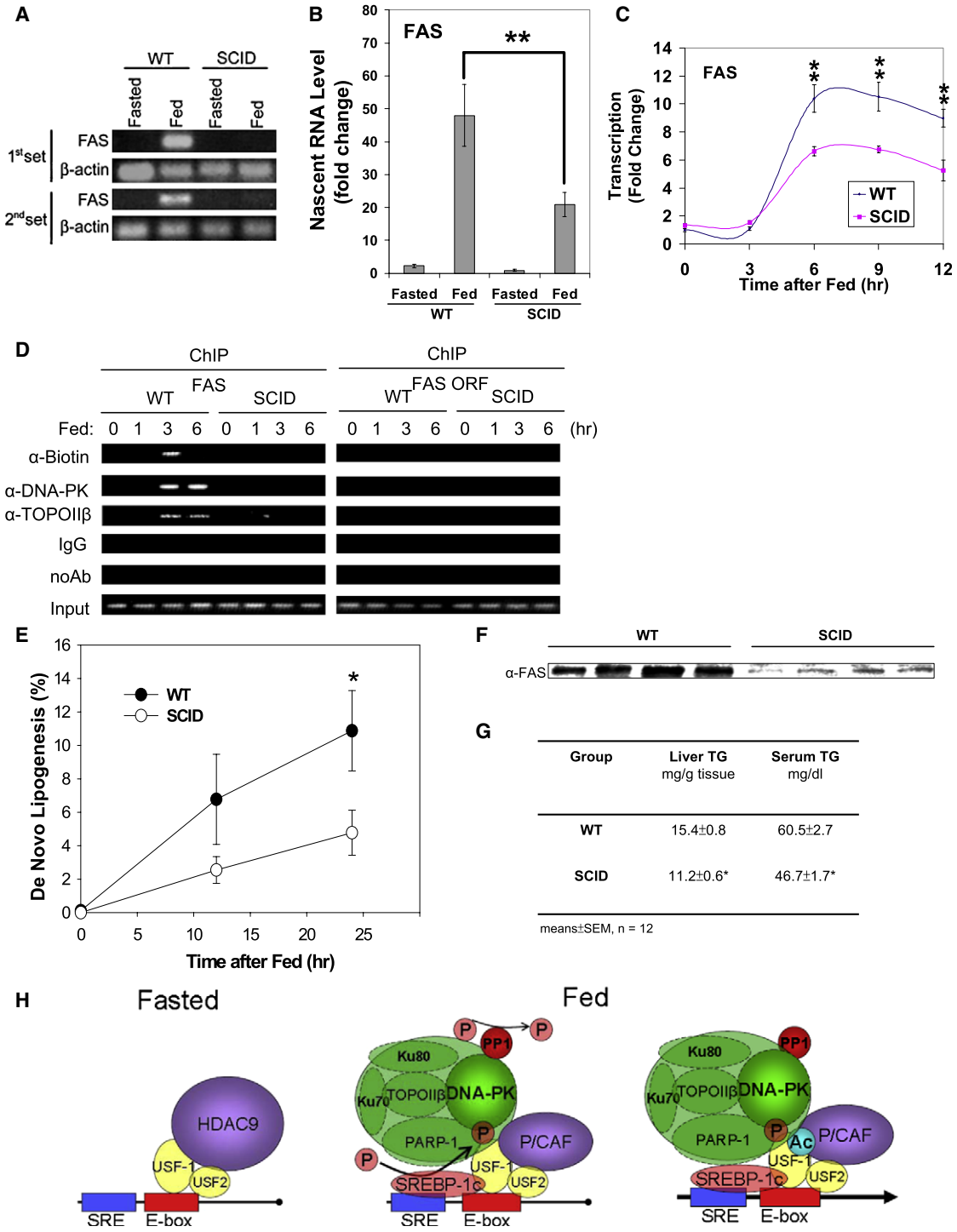

Figure 6. Diminished FAS Induction Leading to Blunted De Novo Lipogenesis and Decreased Triglyceride Levels in Liver and Serum

(A and B) Nascent RNA were used for (A) RT-PCR or (B) RT-qPCR. Fold induction normalized by β-actin.

(C) Run-ons of labeled nascent transcripts were analyzed by RT-qPCR.

(D) ChIP for DNA breaks and indicated protein binding to the FAS promoter.

(E) Newly synthesized labeled fatty acids in livers from 9-week-old mice were measured. Values are means ± SEM. n = 12.

(F) Immunoblotting of equal amounts of liver extracts from 9-week-old mice after 24 hr of feeding.

(G) Hepatic and serum triglyceride levels were measured in 9-week-old fed mice.

(H) Schematic representation of USF-1 and its interacting partners and their effects on lipogenic gene transcription in fasting/feeding.

Error bars represent ± SEM.

for FAS promoter activation. Furthermore, FAS and mGPAT have the same differential recruitment of distinct USF-interacting proteins, indicating a common key mechanism in the induction of lipogenic gene transcription in response to fasting/feeding.

Phosphorylation-Dependent Acetylation of USF-1 Functions as a Sensor for Nutritional Status

Because USF-1 levels and its binding to the E box are unaltered between fasting/feeding, it can be predicted that USF-1 is regulated posttranslationally. Even though the changes in phosphorylation states of metabolic enzymes during the transition between fasting/feeding are common and well understood, the posttranslational modifications of transcription factors in these metabolic states are not well studied. We show here that S262 and the nearby K237 of USF-1 are modified in response to fasting/feeding. The S262 of USF-1 as well as nearby residues are conserved among mammalian species but are not found in USF-2 even though there is a 44% overall homology between USF-1 and USF-2 (Corre and Galibert, 2005). Activation of the FAS gene by feeding has been shown to be impaired by 80% in either USF-1 or USF-2 knockout mice (Casado et al., 1999). Thus, USF functions as a heterodimer, and both USF-1 and USF-2 were found to bind the FAS promoter (Wang and Sul, 1995, 1997). However, the unique S262 of USF-1 points toward its pivotal role as a sensor for lipogenic gene transcription.

There is increasing evidence for acetylation of some transcription factors in addition to the well-recognized histone acetylation (Gu and Roeder, 1997), and reversible acetylation may be critical in regulation of transcription factor activity in response to different stimuli. However, USF acetylation has never been reported. Here, we have addressed USF-1 as a primary substrate for HAT/HDAC. The functional significance of acetylation of transcription factors appears to be varied. In the case of p53, acetylation results in stimulation of DNA binding, whereas acetylation of E2F may change protein stability (Martinez-Balbas et al., 2000). The fact that USF levels do not change during fasting/feeding and that USF acetylation does not affect DNA binding but affects FAS promoter activation suggests transactivation results from USF acetylation, and our study demonstrates that acetylation of USF-1 at K237 increases FAS promoter activity. Further studies are needed to clarify the exact functional consequence of USF acetylation. Deacetylation is mainly mediated by HDACs that generally function as transcriptional repressors. HDAC9 is recruited to the FAS promoter in the fasted state to deacetylate USF-1. Although HDAC9 has been shown to associate with transcription factors to repress transcription (Mejat et al., 2005), to our knowledge, HDAC9 deacetylation of USF-1 that we report here is the first nonhistone substrate of HDAC9.

Crosstalk between acetylation and phosphorylation is well recognized. In our present study, K237 acetylation is dependent on S262 phosphorylation in response to feeding/insulin by preferential interaction with P/CAF rather than HDAC9. Thus, the phosphorylation-dependent acetylation of USF-1 functions as a dynamic molecular switch in sensing the nutritional transition from fasting to feeding. Such a multistep switch provides a way to fine-tune transcription of lipogenic genes in response to different nutritional states.

PP1-Mediated Dephosphorylation of DNA-PK Is Critical for Feeding-Dependent Lipogenic Gene Transcription

It has been well established that PI3K pathway mainly mediates insulin signaling for metabolic regulation (Engelman et al., 2006). Our in vitro phosphorylation studies and the fact that S262 phosphorylation is abolished in DNA-PK-deficient mice point to the notion that DNA-PK is the kinase for the S262 phosphorylation occurring in the fed condition. However, DNA-PK is not known to be a component in the PI3K pathway or in the insulin-signaling pathway. Although DNA-PK was previously implicated in phosphorylation of S473 of PKB/Akt (Feng et al., 2004), recent research indicates that mTORC2, another member of PIKK, is the authentic kinase that phosphorylates this critical site of PKB/Akt (Sarbassov et al., 2005). However, our present study shows a link between DNA-PK and insulin-signaling pathway.

Although the molecular mechanism is complex, the stimulation of PP1 by insulin has been well documented. For example, insulin inhibits breakdown and promotes synthesis of glycogen by activating primarily PP1. PP1 is compartmentalized in cells by discrete targeting subunits, and several proteins called "protein targeting to glycogen (PTG) can target PP1 to the glycogen particle where PP1 dephosphorylates enzymes in glycogen metabolism (Printen et al., 1997). Recent studies indicate that PP1 can rapidly move between subcellular compartments with the aid of targeting units. PNUT, a PP1 associated cofactor, may act as a nuclear targeting subunit of PP1 (Allen et al., 1998). We postulate that feeding/insulin might regulate PNUT-mediated nuclear translocation of PP1 into the nucleus to activate DNA-PK. Thus, PP1-mediated dephosphorylation of DNA-PK is critical in transmitting the feeding/insulin signal to regulate lipogenic genes.

Among USF-interacting proteins, DNA-PK, along with Ku70, Ku80, PARP-1, and TopoIIß, are identified. These proteins are known to function in double-strand DNA break/repair, and it has recently been shown that a transient double-strand DNA break is required for estrogen receptor-dependent transcription. Although Ku70, Ku80, and DNA-PK are in the same complex with PARP-1 and TopoIIß, their function in DNA break for transcriptional activation has not been reported. Here, we identified all components of DNA break/repair machinery for transcriptional activation of the FAS promoter by fasting/feeding, and we observed transient DNA breaks that preceded transcriptional activation.

We show here a unique function of DNA-PK as a signaling molecule in response to feeding/insulin. DNA-PK is required for USF-1 complex assembly and recruitment of its interacting proteins. Therefore, DNA-PK-mediated USF-1 phosphorylation governs interaction between USF-1 and its partners. SREBP-1 interacts more efficiently with the phosphorylated USF-1, which, in turn, enhances the interaction between USF-1 and DNA-PK, leading to USF-1 phosphorylation, an indication of positive feed-forward regulation. Thus, impaired transcriptional activation of lipogenic genes in DNA-PK-deficient SCID mice is probably due to the dual effects of DNA-PK on USF-1 phosphorylation for feeding/insulin signaling and the transient DNA

breaks required for transcriptional activation. In SCID mice, the absence of the feeding-induced transient DNA breaks in the FAS promoter could be attributed to the impairment of feeding/insulin-induced USF phosphorylation by DNA-PK, which results in a failure to recruit various USF-1-interacting proteins, including those for transient DNA breaks such as TopoIIβ.

Taken together, we propose the following model for the mechanism underlying USF function in the transcriptional regulation of lipogenic genes during fasting/feeding (Figure 6H). In the fasted state, USF-1 recruits HDAC9, which deacetylates USF-1 to repress transcription despite its binding to the E box (Figure 6H, left panel). Upon feeding, DNA-PK, which is dephosphorylated/activated by PP1, phosphorylates USF-1, which then recruits SREBP-1 and other USF-1-interacting proteins. Thus, DNA-PK-catalyzed phosphorylation of USF-1 allows P/CAF recruitment and subsequent acetylation of USF-1 (Figure 6H, right panel). As a result, FAS transcription is activated by USF-1 in a reversible manner in response to nutritional status.

EXPERIMENTAL PROCEDURES

Supplemental Experimental Procedures are available in the Supplemental Data.

Purification of USF-1-Interacting Proteins and Preparation of Nuclear Extracts

TAP was performed as described previously (Griffin et al., 2007). Purified protein mixture was subjected to mass spectrometry. Liver nuclear extracts were prepared by centrifugation through sucrose cushion in the presence of NaF.

Chromatin Immunoprecipitation

Livers from fasted or fed mice were fixed with DSG at 2 mM for 45 min at RT before formaldehyde crosslinking. ChIP was performed as described previously (Latasa et al., 2003).

In Vitro Phosphorylation, Acetylation, and DNA-PK Kinase Assay

In vitro phosphorylation and acetylation were performed using recombinant/purified enzymes. DNA-PK kinase assay was performed with nuclear extracts pretreated with or without wortmannin using SignaTect DNA-PK assay system (Promega) and γ^{32}P-ATP (Roche).

Nuclear Run-On Assay and Preparation of Nascent RNA

Nuclei were isolated as described previously (Paulauskis and Sul, 1989) for nascent RNA and nuclear run-on assay (See the Supplemental Experimental Procedures for further details).

Immunoprecipitation, GST Pull-Down, Luciferase Reporter Assays

Immunoprecipitation from nuclear extracts was performed under standard procedures. GST pull-down was performed as described previously (Griffin et al., 2007). Luciferase assays were performed in 293FT cells using Dual-Luc reagent (Promega).

RT-PCR Analysis

RNA was isolated and reverse transcribed for PCR or qPCR.

Measurement for Metabolite and Hormone Levels

Insulin, glucose, NEFA, and triglycerides were measured by ELISA (Crystal), glucometer (Roche), NEFA C kit (Wako), and Infinity kit (Thermo), respectively.

De Novo Lipogenesis (DNL)

Fatty acids formed during a 4 hr ^2H_2O body water labeling (see the Supplemental Experimental Procedures for further details).

Statistical Analysis

The data are expressed as the means ± SE of the means. Student's t test was used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.0001$).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00003-8.

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