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Both the magnitude and duration of insulin signaling are important in executing its cellular functions. Insulin-induced degradation of insulin receptor substrate 1 (IRS1) represents a key negative feedback loop that restricts insulin signaling. Moreover, high concentrations of fatty acids (FAs) and glucose involved in the etiology of obesity-associated insulin resistance also contribute to the regulation of IRS1 degradation. The scavenger receptor CD36 binds many lipid ligands, and its contribution to insulin resistance has been extensively studied, but the exact regulation of insulin sensitivity by CD36 is highly controversial. Herein, we found that CD36 knockdown in C2C12 myotubes accelerated insulin-stimulated Akt activation, but the activated signaling was sustained for a much shorter period of time as compared with WT cells, leading to exacerbated insulin-induced insulin resistance. This was likely due to enhanced insulin-induced IRS1 degradation after CD36 knockdown. Overexpression of WT CD36, but not a ubiquitination-defective CD36 mutant, delayed IRS1 degradation. We also found that CD36 functioned through ubiquitination-dependent binding to IRS1 and inhibiting its interaction with cullin 7, a key component of the multisubunit cullin-RING E3 ubiquitin ligase complex. Moreover, dissociation of the Src family kinase Fyn from CD36 by free FAs or Fyn knockdown/inhibition accelerated insulin-induced IRS1 degradation, likely due to disrupted IRS1 interaction with CD36 and thus enhanced binding to cullin 7. In summary, we identified a CD36-dependent FA-sensing pathway that plays an important role in negative feedback regulation of insulin activation and may open up strategies for preventing or managing type 2 diabetes mellitus.

Cell signaling is usually initiated by an activating ligand binding to a receptor on the plasma membrane that transmits the signal inside the cell. Distinct cellular responses and outcomes of a signaling pathway are achieved by precise regulation of its duration, magnitude, and subcellular compartmentalization, which is mediated by an integrated network with multiple positive and negative feedback and feedforward loops (1). Insulin plays a critical role in metabolic regulation by promoting glucose uptake and lipid synthesis while inhibiting gluconeogenesis and lipolysis (2). Both magnitude and duration of insulin signaling are important in determining its metabolic functions.

Insulin signaling is initiated by insulin binding to and activation of the insulin receptor (IR)[2] tyrosine kinase, which further induces phosphorylation and recruitment of the insulin receptor substrate (IRS) family of proteins (3). Among the substrates, IRS1 is important in activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (4), and its signaling functions are mediated by phosphorylation and acetylation (5, 6). Ubiquitination of IRS1 following phosphorylation on Ser-307 leads to its proteasome-dependent degradation, which, in conjunction with lysosomal degradation of IR, results in a decrease of insulin sensitivity after long-term stimulation by insulin (7). Insulin-induced IRS1 degradation relies on a proteasome-dependent pathway (8), whereas osmotic stress and oxidative stress enhance IRS1 degradation in a proteasome-independent process (9, 10). Cullin 7 (CUL7) is a key component of the multisubunit cullin-RING E3 ubiquitin ligase complex that targets IRS1 for ubiquitin-dependent degradation, whereas ubiquitin-specific protease 7 can deubiquitinate IRS1, preventing it from proteasomal degradation (11). However, molecular mechanisms regulating stability of IRS1 in response to cellular stress are not well understood.

Cluster of differentiation 36 (CD36) has been shown *in vitro* and *in vivo* to facilitate the transport of many lipid species including FFAs in a variety of cells (12, 13). CD36 is highly expressed in myocytes, adipocytes, and hepatocytes where insulin signaling is a master metabolic regulator (14). Accordingly, potential regulation of insulin signaling by CD36 has been extensively studied. Hepatic CD36 up-regulation is signifi-

[2] The abbreviations used are: IR, insulin receptor; CD36, cluster of differentiation 36; CHO/hIR, CHO cells overexpressing human insulin receptor; CUL7, cullin 7; FA, fatty acid; IRS, insulin receptor substrate; KD, knockdown; K/A, Lys-469 and Lys-472 substituted by alanine; MG53, muscle-specific mitsugumin 53; OA, oleic acid; PA, palmitic acid; PDK, phosphoinositide-dependent kinase; Src-1, Src inhibitor-1; SSO, sulfo-*N*-succinimidyl oleate; FFA, free fatty acid; p-, phospho-; UBA, ubiquitin-associated domain; AMPK, AMP-activated protein kinase; TBST, Tris-buffered saline with Tween-20.

cantly associated with insulin resistance and increased steatosis in patients with non-alcoholic steatohepatitis (15). Unadjusted CD36 mRNA and protein in peripheral blood mononuclear cells were higher among subjects with type 2 diabetes mellitus *versus* control subjects (16), indicating that CD36 could be a negative mediator of insulin sensitivity. However, some other studies suggested the opposite CD36 regulation of insulin sensitivity. CD36 deficiency in spontaneously hypertensive rats underlies insulin resistance and defective FA metabolism and hypertriglyceridemia (17), which are ameliorated by transgenic expression of CD36 (18). Similarly, muscle-specific overexpression of CD36 reverses insulin resistance in a mouse model caused by overexpressing a dominant-negative form of IGF-1 receptor specifically in muscle (MKR mice) (19). Hepatic overexpression of CD36 improved glycogen homeostasis and attenuated high-fat diet–induced hepatic steatosis and insulin resistance (20). The hyperinsulinemic-euglycemic clamp study showed insulin resistance in people with genetic CD36 deficiency, which is common in Asian and African populations (21), but a later study demonstrated that human CD36 deficiency is not necessarily responsible for insulin resistance (22). Collectively, the exact regulation of insulin sensitivity by CD36 is highly controversial, and the underlying mechanisms are still unrevealed.

CD36-null mice clear glucose faster than WT under chow diet. However, after switching to a diet high in fructose, CD36-null mice, but not WT mice, develop hyperinsulinemia, marked glucose intolerance, and decreased muscle glucose uptake (23). Thus, it is likely that insulin signaling is dynamically regulated by CD36, and this regulation may be highly dependent on different nutrient stress and hormone stimulation. Herein, we studied the magnitude and duration of insulin signaling as regulated by CD36 in C2C12 myotubes. Because IRS1 plays a critical role in the insulin signaling pathway and insulin-induced IRS1 degradation is a key negative feedback mechanism to maintain appropriate signal strength and duration, we further examined whether CD36 mediates dynamics of insulin signaling activation by regulating IRS1 stability. We demonstrated that CD36 interacts with IRS1 and reduces its interaction with CUL7, which prevents proteasomal degradation of IRS1 and leads to distinct regulation of insulin signaling before and after chronic insulin treatment.

Results

CD36 sustains insulin signaling and alleviates insulin-induced insulin resistance in C2C12 myotubes

Previous studies suggested that CD36 regulation of insulin signaling may be highly dependent on nutrient and signaling states of the cells. We first investigated the role of CD36 in regulating the dynamic response of Akt activation to insulin stimulation. CD36 was efficiently knocked down in myotubes using an siRNA as we verified previously (24) (Fig. 1A). Control or CD36 knockdown (KD) myotubes were treated with 10 nM insulin, and Akt phosphorylation was monitored for up to 240 min. Phosphorylation of Akt at Thr-308 and at Ser-473 were initially increased but then started to decline, partially due to negative feedback regulation to terminate signaling (Fig. 1, A, B, and C). As compared with control myotubes, insulin-stimu-

lated p-Akt (Thr-308) and GSK3 phosphorylation were increased faster in CD36 KD myotubes but were maintained for a shorter period of time prior to deactivation (Fig. 1, A, B, and D). In contrast, activation and duration of Akt phosphorylation at Ser-473 were similarly regulated by insulin in control and CD36 KD myotubes (Fig. 1, A and C), indicating specific accelerating activation of the p-Akt (Thr-308)/p-GSK3 pathway in response to loss of CD36 in myotubes. Insulin stimulated glycogen storage in both WT and CD36 KD myotubes. Similar to regulation of p-GSK3 by CD36, the increase of glycogen storage immediately after insulin stimulation was faster in CD36 KD myotubes, but this increase was sustained for a shorter period of time, and the glycogen accumulation reached a plateau after 4 h. In contrast, glycogen storage in WT myotubes kept increasing for a longer period of time (Fig. 1E).

We then set out to compare differential regulation of insulin-induced Akt activation before and after insulin-induced insulin resistance. We examined acute Akt activation induced by insulin in control and CD36 KD myotubes following starvation. Insulin-stimulated phosphorylation of GSK3 and Akt at Thr-308 was significantly higher in CD36 KD myotubes (Fig. 2A, B, and D). In contrast, phosphorylation of Akt at Ser-473 was not affected by CD36 knockdown (Fig. 2, A and C). We next examined whether CD36 is able to protect myotubes from insulin-induced insulin resistance. Myotubes were pretreated with 50 nM insulin for 4 h, washed, and then restimulated with different concentrations of insulin for 5 min (Fig. 2E). Phosphorylations of Akt at Ser-473 and Thr-308 and GSK3 were lower in CD36 KD myotubes than in control myotubes (Fig. 2, F–I), suggesting that CD36 protects myotubes from insulin-stimulated insulin resistance and that its regulation of insulin signaling is dependent on growth conditions.

CD36 protects IRS1 from insulin-induced proteasomal degradation

IRS1 not only plays an important role in relaying insulin stimulation to the activation of the PI3K/Akt pathway but also provides a site of negative feedback regulation to terminate signaling by undergoing insulin-stimulated ubiquitination and proteasomal degradation. We examined whether IRS1 plays a role in the observed protective effect of CD36 on insulin-induced insulin resistance and found that the IRS1 level is higher in the presence of CD36 in myotubes following insulin treatment (Fig. 2, J and K), contributing to higher insulin sensitivity (Fig. 2F). Insulin-induced changes of IRS1 and IRS2 levels in control and CD36 KD myotubes were then followed. The level of IRS1 was gradually reduced after prolonged insulin incubation in control myotubes (Fig. 3A), and the rate of this degradation significantly accelerated after CD36 KD (Fig. 3A). In contrast, the IRS2 level was not decreased. We next examined rates of insulin-stimulated degradation of IRS1 and IRS2 in CHO cells overexpressing human insulin receptor (CHO/hIR) and stably expressing wildtype (CD36 WT) or the ubiquitination-deficient CD36 mutant where C-terminal lysines (Lys-469 and Lys-472) were substituted by alanine (CD36 K/A) as we established previously (25). We observed rapid degradation of IRS1 in CHO/hIR where CD36 is virtually absent. In contrast, the IRS2 level was not decreased by insulin treatment (Fig. 3B).
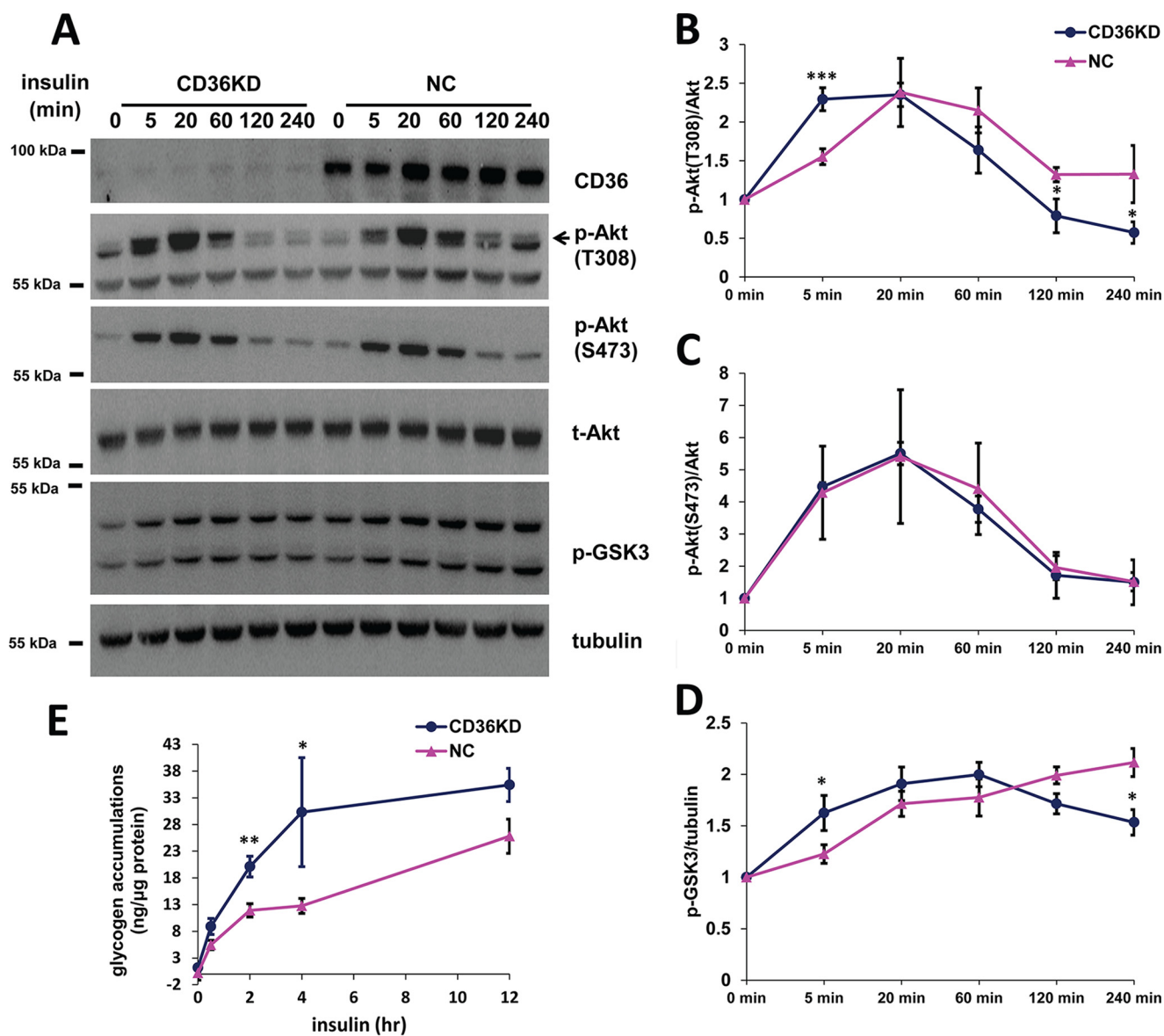
Figure 1. CD36 sustains insulin signaling in myotubes. C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36KD*) as described under "Experimental procedures." *A*, myotubes were starved for about 18 h with low-glucose DMEM containing 0.2% BSA prior to stimulation with 10 nM insulin for the indicated times. Whole-cell lysates were subjected to immunoblot analysis with antibodies against CD36, p-Akt at Thr-308 (*T308*), p-Akt at Ser-473 (*S473*), total Akt (*t-Akt*), p-GSK3, and tubulin. *B*, *C*, and *D*, quantification of the mean of three independent experiments in *A*. *E*, cells were starved for 18 h with low-glucose DMEM containing 0.2% BSA with or without stimulation with 10 nM insulin for the indicated times. Glycogen was extracted and measured using a Glycogen Assay kit according to the manufacturer's instruction. Data are shown as means of three independent experiments. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with negative control.

Overexpression of CD36 WT significantly delayed insulin-stimulated IRS1 degradation, but this effect was not observed in cells overexpressing CD36 K/A (Fig. 3*B*). The expression levels of CD36 WT and CD36 K/A were similar, and the degradation of IR was not obvious (Fig. 3*C*). The insulin-stimulated degradation of IRS1 in CD36 KD myotubes as well as in CHO/hIR cells overexpressing CD36 was completely blocked by MG132 (Fig. S1), suggesting that CD36 regulates proteasomal degradation of IRS1.

CD36 mediates insulin-stimulated phosphorylation and ubiquitination of IRS1

Phosphorylation at serine residues of IRS1 is a major regulator of insulin signaling. Phosphorylation at Ser-307 triggers

IRS1 ubiquitination and degradation, whereas phosphorylation at Ser-612 blocks its interaction with PI3K (5). S307A mutant IRS1 was more resistant to degradation following chronic exposure to insulin in rat hepatoma cells (26) and in myotubes (Fig. 4, *A* and *B*). CD36 interacts with Fyn tyrosine kinase, which binds IRS1 and forms a signaling complex (27–29), suggesting that CD36 may play a role in the IRS1-mediated feedback mechanism of insulin signaling. Control and CD36 KD myotubes were treated with insulin for 15 min, and serine phosphorylation of IRS1 was evaluated. The phosphorylation level of IRS1 at Ser-307 was increased in CD36 KD myotubes as compared with control myotubes (Fig. 4, *C* and *D*), which is consistent with the enhanced insulin-stimulated IRS1 degradation (Fig. 3). In contrast, the phosphorylation level of IRS1 at Ser-
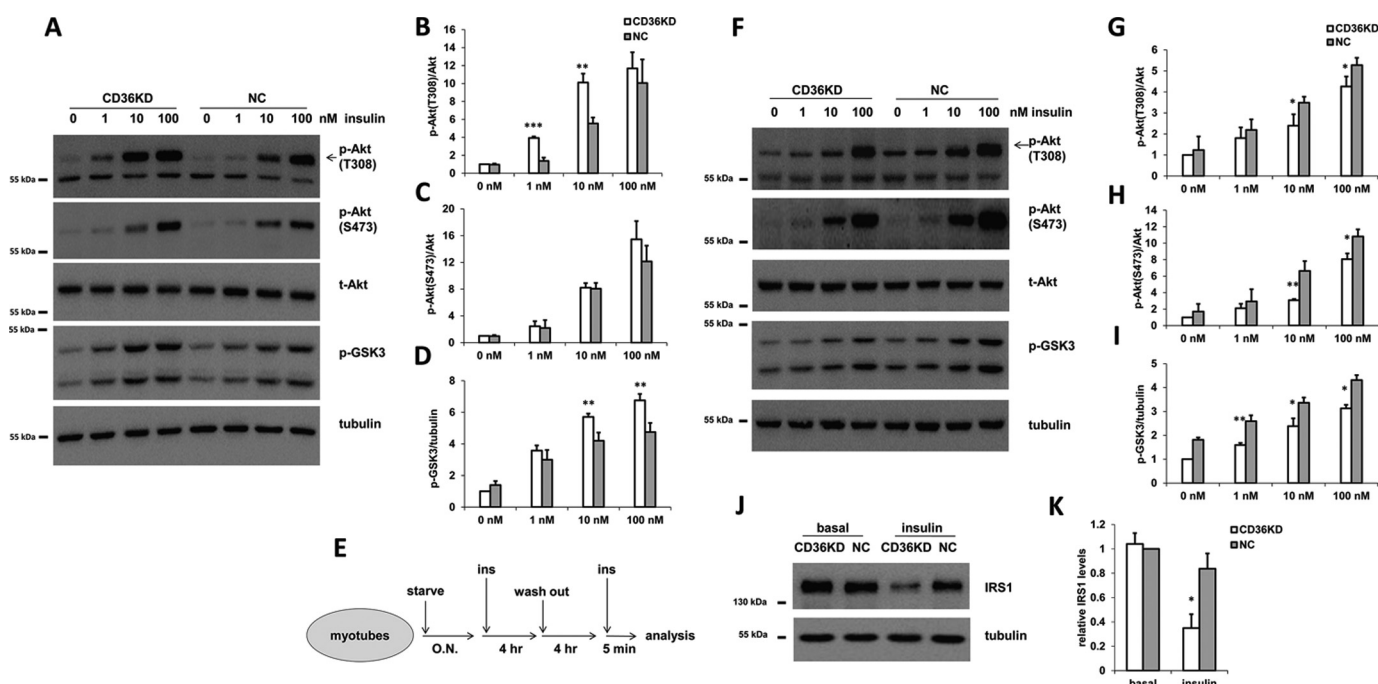
Figure 2. Insulin-induced insulin resistance was exacerbated by CD36 knockdown in myotubes. C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36KD*) as described under "Experimental procedures." *A*, cells were starved for about 18 h with low-glucose DMEM containing 0.2% BSA followed by stimulation with the indicated concentrations of insulin for 5 min. Whole-cell lysates were prepared and subjected to immunoblot analysis using antibodies recognizing p-Akt at Thr-308 (*T308*), p-Akt at Ser-473 (*S473*), total Akt (*t-Akt*), p-GSK3, and tubulin. *B*, *C*, and *D*, quantification of the mean of three independent experiments in *A*. *E*, schematic diagram of the protocol used to study insulin-induced insulin resistance. *F*, myotubes were starved overnight (*O.N.*) with low-glucose DMEM containing 0.2% BSA followed by incubation with 50 nM insulin (*ins*) for 4 h, washed, and serum-starved for an additional 4 h prior to stimulation with the indicated concentrations of insulin for 5 min. Whole-cell lysates were subjected to immunoblot analysis with antibodies as indicated. *G*, *H*, and *I*, quantification of the mean of three independent experiments in *F*. *J*, whole-cell lysates of cells before and after insulin treatment for 4 h were subjected to immunoblot analysis with antibodies as indicated. *K*, quantification of the mean of three independent experiments in *J*. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with negative control.

612 was reduced by CD36 knockdown (Fig. 4, *C* and *E*), which may contribute to the increased Akt activation following acute insulin stimulation (Fig. 2*A*). We used a GST pulldown approach to detect ubiquitinated IRS1 as described (30). Ubiquitinated IRS1 interacted with the GST-UBA fusion protein but not with a mutant version of the UBA domain predicted to abolish ubiquitin binding (GST-UBA mutant). In CD36 KD myotubes, the ubiquitinated IRS1 was higher than that in control myotubes (Fig. 4, *F* and *G*), which is presumably due to increased phosphorylation at Ser-307.

Ubiquitinated CD36 associates with IRS1 and interrupts its interaction with CUL7

We examined interaction of IRS1 with CD36 and how this interaction was affected by insulin in CHO/hIR cell lines. CD36 WT was coimmunoprecipitated with IRS1 using anti-IRS1 antibody (Fig. 5*A*). In contrast, the amount of ubiquitination-defective CD36 K/A mutant coimmunoprecipitated with IRS1 was much lower (Fig. 5*A*). CD36 WT was also able to pull down IRS1, but its K/A mutant was less effective (Fig. 5*B*). Our previous study showed that CD36 associates with the Src family kinase Fyn (31), which binds IRS1 and forms a distinct signaling complex (29). We examined the potential presence of IRS1-Fyn-CD36 complex and its regulation by CD36 ubiquitination and found that Fyn coimmunoprecipitated with IRS1 and CD36 (Fig. 5, *A* and *B*). Our results suggest that CD36 ubiquitination was important in its optimal interaction with IRS1 and in protecting IRS1 from insulin-stimulated degradation (Fig.

3*B*). The proteasomal degradation of IRS1 is regulated by CUL7 E3 ubiquitin ligase, and we next examined whether CD36 competes with CUL7 in interacting with IRS1. Overexpression of CD36 decreased coimmunoprecipitation of CUL7 by anti-IRS1 antibody (Fig. 5*C*). In contrast, the ubiquitination-defective CD36 K/A mutant with weaker interaction with IRS1 was less effective in blocking IRS1-CUL7 interaction (Fig. 5*C*).

We then examined the presence of endogenous IRS1-Fyn-CD36 complex in myotubes. Both CD36 and Fyn coimmunoprecipitated with IRS1, and insulin treatment, which inhibits CD36 ubiquitination (25), decreased its association with IRS1 (Fig. 5*D*). Because Fyn interacts with both IRS1 and CD36, we examined whether Fyn is required for CD36 interaction with IRS1. Fyn was coimmunoprecipitated with IRS1 in the absence of CD36. In contrast, Fyn KD significantly decreased CD36 coimmunoprecipitated with IRS1 (Fig. 5*E*). Disruption of endogenous CD36-IRS1 interaction either directly by CD36 KD or indirectly by Fyn KD enhanced the amounts of CUL7 coimmunoprecipitated with IRS1 (Fig. 5*E*). Inhibition of Fyn by Src inhibitor-1 (Src-1) decreased CD36 and increased CUL7 coimmunoprecipitated with IRS1 (Fig. 5*F*), suggesting a key role of Fyn and its kinase activity in CD36 regulation of IRS1 degradation.

Fyn participates in CD36 regulation of IRS1 degradation

We set out to examine whether Fyn is involved in CD36 regulation of insulin-stimulated IRS1 degradation. Because the
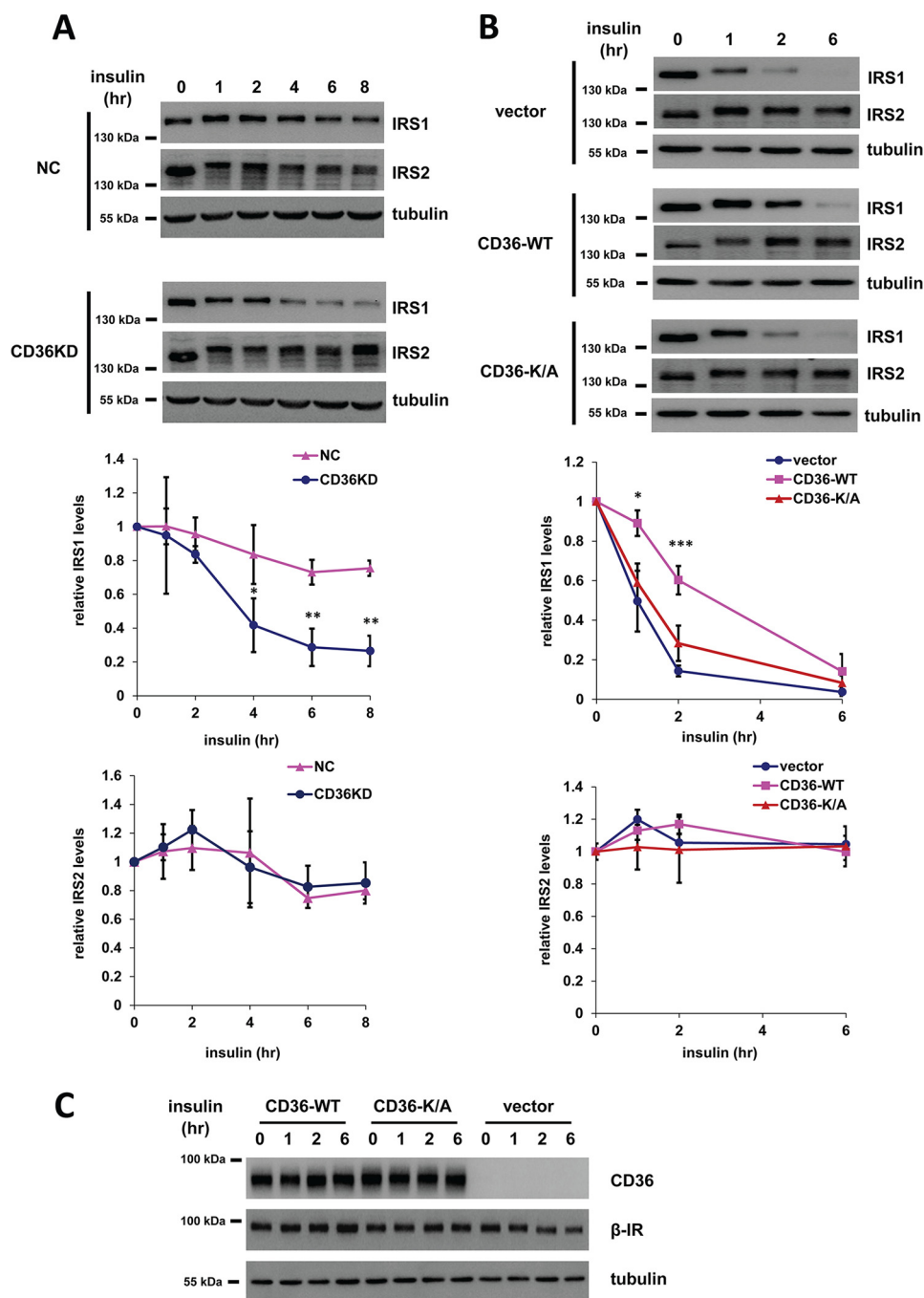
Figure 3. CD36 alleviates insulin-induced degradation of IRS1. *A*, C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36 KD*) as described under "Experimental procedures." Cells were starved overnight with low-glucose DMEM containing 0.2% BSA followed by stimulation with 50 nM insulin for the indicated times. Whole-cell lysates were subjected to immunoblot analysis with antibodies against IRS1, IRS2, and tubulin. Quantification of the mean of three independent experiments is shown. *, $p < 0.05$; **, $p < 0.01$, compared with negative control. *B*, CHO/hIR cells stably expressing empty vector (*vector*), WT CD36 (*CD36-WT*), or mutated CD36 (*CD36-K/A*) were starved for 12 h with F-12 medium containing 0.2% BSA followed by stimulation with 5 nM insulin for the indicated times. Whole-cell lysates were subjected to immunoblot analysis with antibodies recognizing IRS1, IRS2, and tubulin. Quantification of the mean of three independent experiments is shown. *Error bars* represent S.D. *, $p < 0.05$; ***, $p < 0.001$, compared with vector. *C*, whole-cell lysates from *B* were subjected to immunoblot analysis with antibodies recognizing CD36, β subunit of IR (β-*IR*), and tubulin.

enhanced FFA interaction with CD36 promotes Fyn dissociation from the protein complex (31), we studied how FA may interfere with CD36 function in stabilizing IRS1. Myotubes were pretreated with BSA control, palmitic acid (PA), or oleic acid (OA) prior to stimulation with insulin, and the levels of IRS1 were determined. The degradation of IRS1 was significantly accelerated in PA- or OA-pretreated cells (Fig. 6*A*), and

OA was more effective. In CD36 KD myotubes, the degradation of IRS1 could not be further accelerated by PA or OA pretreatment (Fig. 6*A*). Similarly, CD36 inhibition and Fyn dissociation by sulfo-*N*-succinimidyl oleate (SSO) pretreatment enhanced insulin-stimulated degradation of IRS1 (Fig. 6*B*). We next examined the effect of Fyn KD or pharmaceutical Src inhibition. The degradation of IRS1 in Fyn KD myotubes or myotubes

pretreated with Src-1 was significantly accelerated. Fyn KD or Src-1 failed to further expedite the degradation of IRS1 in CD36 KD myotubes (Fig. 6, *C* and *D*). Similar regulation was observed in CHO/hIR cells overexpressing CD36 WT but not in control cells without CD36 or cells overexpressing CD36 K/A (Fig. S2). Therefore, association of Fyn with ubiquitinated CD36 and

Fyn activity may be important in CD36 regulation of IRS1 degradation.

Discussion

The PI3K/Akt pathway conveys signals from receptor tyrosine kinases to regulate cell metabolism, proliferation, and sur-
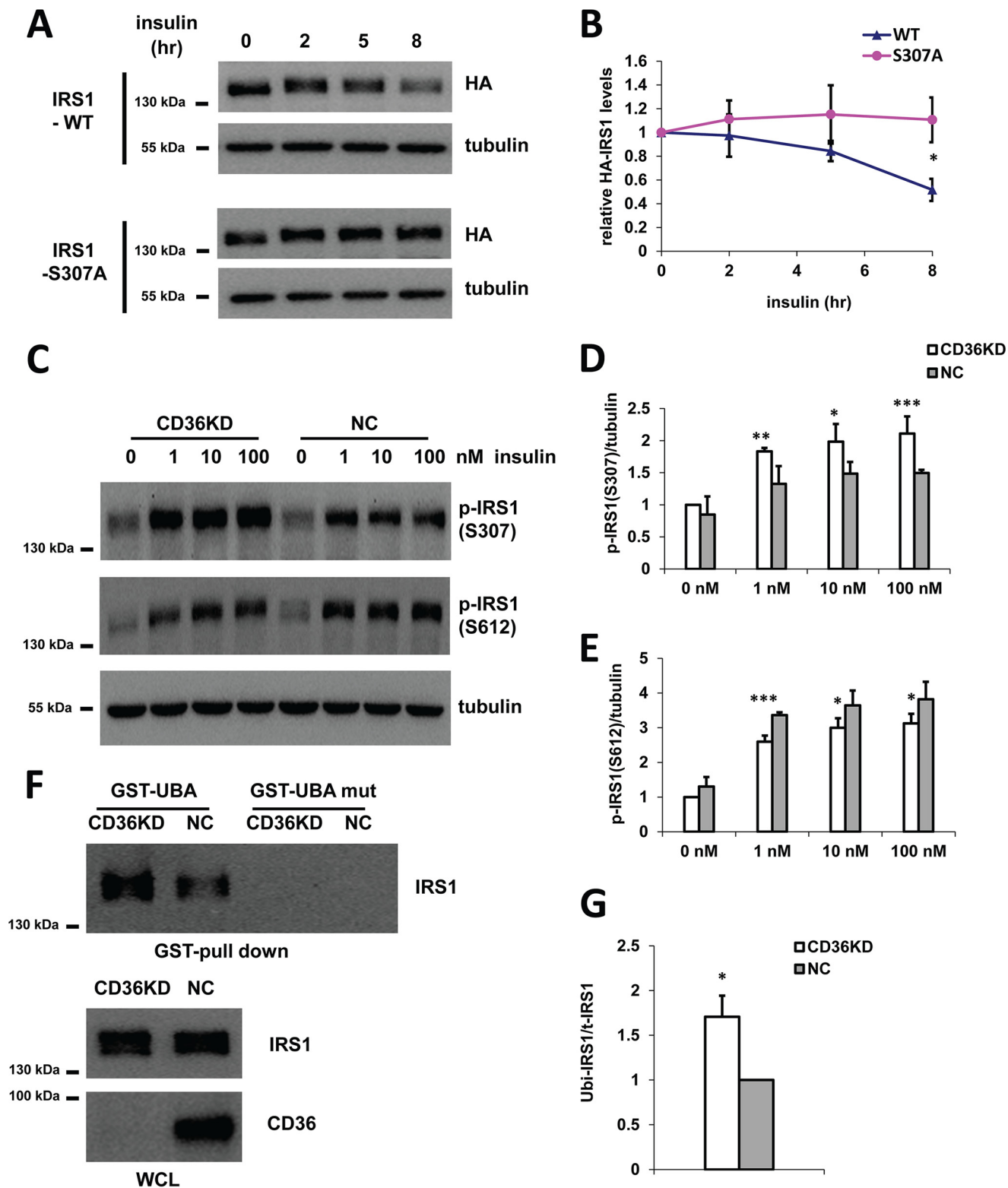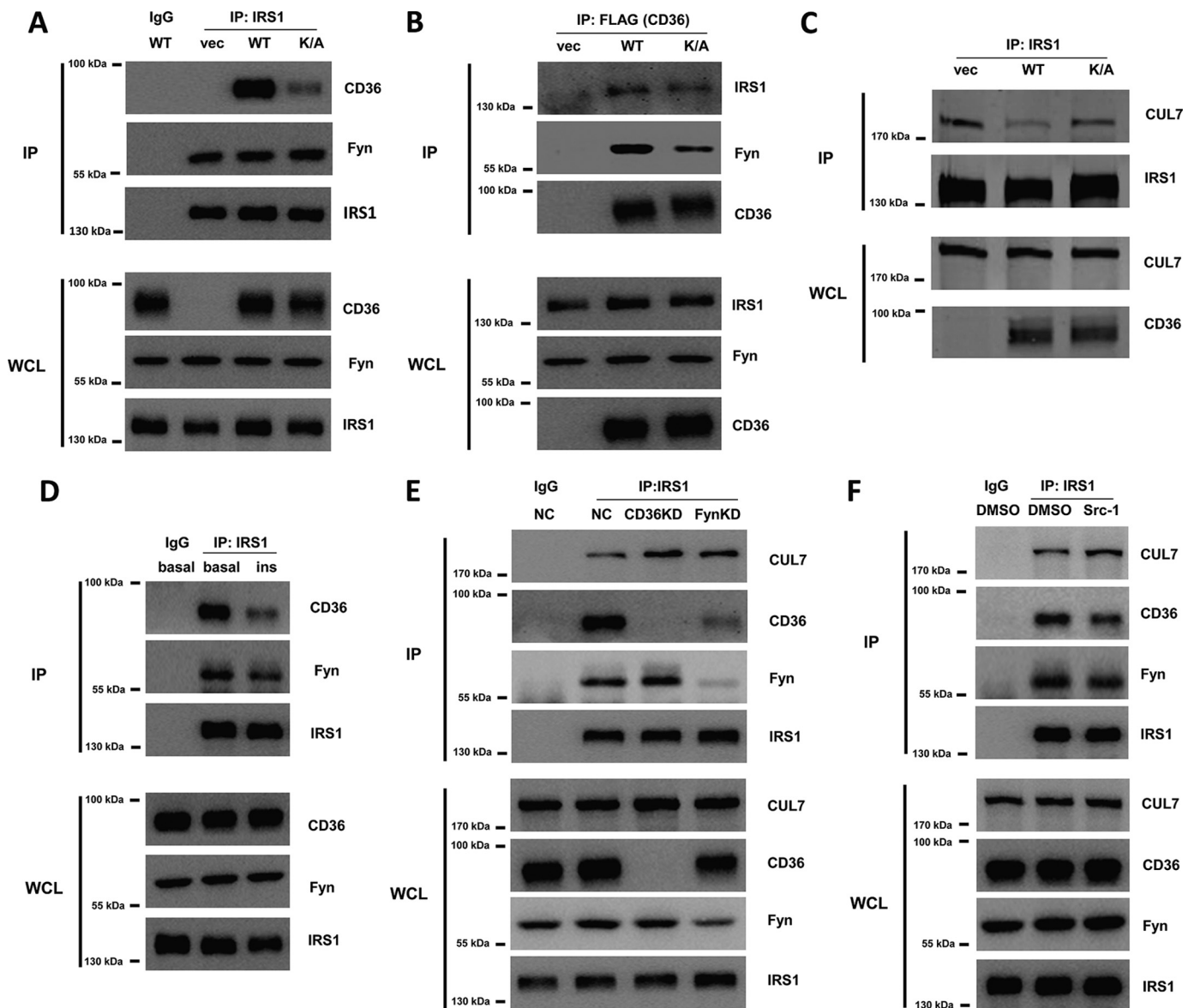
Figure 5. CD36 associates with IRS1 and interrupts its interaction with CUL7. *A*, CHO/hIR cells expressing empty vector (*Vec*), CD36 WT (*WT*), or CD36 K/A (*K/A*) were lysed for immunoprecipitation (*IP*) with IRS1 antibody or control IgG as indicated. The immunoprecipitates were subjected to immunoblot analysis using antibodies recognizing IRS1, CD36, and Fyn. *B*, CHO/hIR cells were lysed for immunoprecipitation with FLAG affinity gel followed by immunoblot analysis with antibodies against IRS1, CD36, and Fyn. *C*, CHO/hIR cells were transfected with HA-CUL7 and IRS1. The cell lysates were prepared for immunoprecipitation with antibody against IRS1, and the precipitates were subjected to immunoblot analysis using antibodies recognizing IRS1 and CUL7. *D*, C2C12 myotubes were untreated (*basal*) or starved for 18 h followed by incubation with 50 nM insulin (*ins*) for 10 min. Cells were lysed for immunoprecipitation with IRS1 antibody or control IgG. The immunoprecipitates were subjected to immunoblot analysis using antibodies recognizing IRS1, CD36, and Fyn. *E*, C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36KD*) or Fyn (*FynKD*) as described under "Experimental procedures." Cells were lysed for immunoprecipitation with IRS1 antibody or control IgG. The immunoprecipitates were subjected to immunoblot analysis using antibodies recognizing IRS1, CD36, Fyn, and CUL7. *F*, C2C12 myotubes were treated with 10 μM Src-1 or DMSO for 30 min. Cells were lysed for immunoprecipitation with IRS1 antibody or control IgG. The immunoprecipitates were subjected to immunoblot analysis using antibodies recognizing IRS1, CD36, Fyn, and CUL7. The experiment was repeated three times with similar results. *WCL*, whole-cell lysates.

Figure 4. CD36 mediates insulin-stimulated phosphorylation and ubiquitination of IRS1. *A*, C2C12 myotubes were infected with adenovirus containing HA-tagged full-length WT mouse IRS1 (*IRS1-WT*) or its S307A mutant (*IRS1-S307A*). Cells were induced to differentiate, starved overnight, and stimulated with 50 nM insulin for the indicated times. Whole-cell lysates were subjected to immunoblot analysis with antibodies against HA and tubulin. *B*, quantification of the mean of three independent experiments in *A* is shown. *, $p < 0.05$, compared with IRS1 WT. *C*, C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36KD*) as described under "Experimental procedures." Cells were starved for about 18 h with low-glucose DMEM containing 0.2% BSA followed by incubation with the indicated concentrations of insulin for 15 min. Protein samples were subjected to immunoblot analysis using antibodies recognizing p-IRS1 at Ser-307 (*S307*), p-IRS1 at Ser-612 (*S612*), and tubulin. *D* and *E*, quantification of the mean of three independent experiments in *C*. *F*, myotubes were serum-starved for 16 h with low-glucose DMEM containing 0.2% BSA and then treated with 20 μM MG132 for 30 min before incubation with 50 nM insulin for 4 h. The ubiquitinated IRS1 were detected as described under "Experimental procedures." *G*, the relative levels of ubiquitinated IRS1 were quantified with ImageJ software. The data represent the mean of three independent experiments. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with negative control.
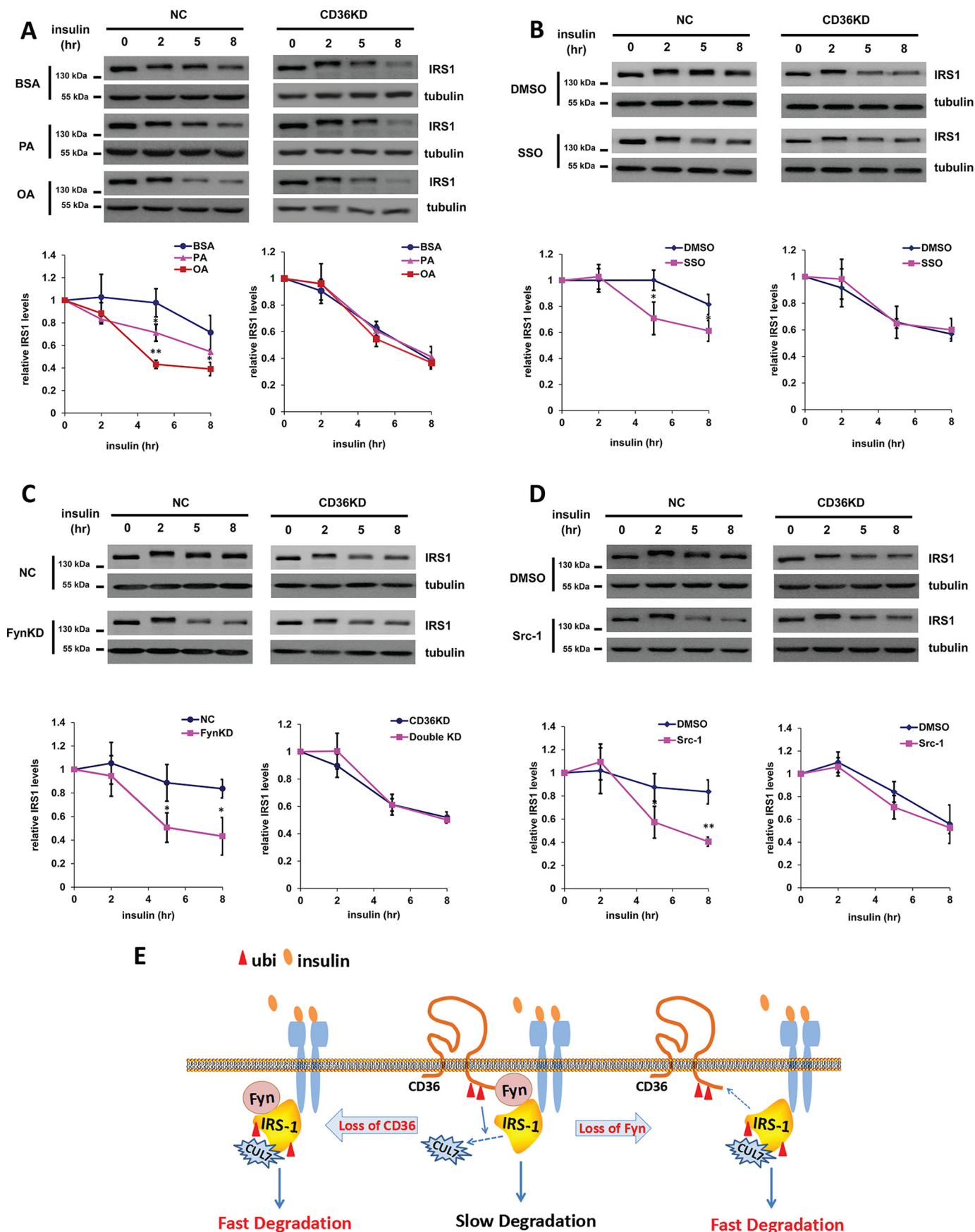
vival. The spatial, temporal, or strength-controlling regulation of PI3K/Akt signaling determines its functional specificity. Sustained and periodic Akt signaling has an integral role in regulating T cell longevity, and antigen-activated CD4^{+} cells without Akt activity maintained over time are short-lived (32). In Chinese hamster embryonic fibroblasts, the sustained β-arrestin1–independent Akt activity, but not the rapid β-arrestin1–dependent signaling, prevents G_1 phase progression (33). Sustained inhibitory phosphorylation of GSK3 mediated by PI3K/Akt limits nerve regeneration after peripheral injury (34). Although the signaling duration of PI3K/Akt pathway and its importance have been studied in several *in vitro* and *in vivo* systems, the molecular mechanisms underlying this dynamic regulation are still unclear. Herein, we demonstrated an important role of CD36, a FA-transporting and -sensing protein, in regulating the rate and duration of insulin-stimulated Akt activation by mediating IRS1 stability.

Insulin-activated IR can tyrosine-phosphorylate IRS proteins, which recruit and activate PI3K. The activity of Akt is markedly stimulated in a PI3K-dependent manner, which predominantly relies on the phosphorylation of Akt on Thr-308 in the activation loop of the kinase catalytic domain and Ser-473 in the "hydrophobic motif" C-terminal domain. The protein kinase responsible for phosphorylating Akt on Thr-308 is phosphoinositide-dependent kinase (PDK) and mTOR (mechanistic target of rapamycin) complexed to RICTOR is the Ser-473 kinase (35). The phosphorylation on Thr-308 is associated with glycogen synthesis, whereas that on Ser-473 site stimulates GLUT4 trafficking to the plasma membrane and glucose uptake (36, 37). Our current study demonstrated that insulin-stimulated p-Akt (Thr-308) and GSK3 phosphorylation were increased faster in CD36 KD myotubes but maintained for a shorter period of time prior to deactivation. In contrast, Ser-473 phosphorylation was not affected by CD36 KD. Moreover, IRS1-independent ERK activation is not affected (data not shown). Loss of CD36 in myotubes leads to AMPK activation (31), and the PDK1/atypical protein kinase C pathway is involved in AMPK-stimulated increases in glucose transport (38). It is possible that AMPK activation by loss of CD36 may enhance PDK1 activity and determine the specific faster phosphorylation of Akt on Thr-308, leading to an acute increase of glycogen storage.

Insulin-stimulated IRS1 degradation plays a role in both the feedback inhibition of the insulin signal and cellular insulin resistance. CD36 deficiency renders the cells more sensitive to insulin-induced insulin resistance by destabilizing IRS1 protein without affecting the IRS1 mRNA level (data not shown). This dynamic regulation of insulin sensitivity by CD36 is consistent with the increased insulin sensitivity in CD36-null mice under chow diet but marked glucose intolerance in high-fructose diet–induced hyperinsulinemia (23). IRS1 can be degraded in lysosomes or in proteasomes, and loss of CD36 enhances insulin-stimulated IRS1 degradation by the ubiquitin proteasome pathway, which is sensitive to MG132. CD36 regulation of IRS1 ubiquitination is achieved by its competitive interaction to decrease IRS1 interaction with E3 ligase CUL7. We provided several lines of evidence to support the requirement of ubiquitination in CD36 regulation of IRS1 degradation. CD36 interaction with IRS1 is decreased by insulin, which inhibits CD36 ubiquitination (25). Moreover, CD36 K/A without ubiquitination has a much weaker interaction with IRS1. Consistent with this, CD36 WT, but not K/A mutant, inhibits IRS1 interaction with CUL7 and promotes insulin-stimulated IRS1 degradation. A recent study showed that muscle-specific mitsugumin 53 (MG53) can also act as an E3 ligase and mediates the degradation of both IR and IRS1 (39). Because CD36 does not affect IR degradation, it may have minimal effect on MG53 activity. However, whether CD36 plays a role in MG53-mediated IRS1 degradation requires further study.

IRS1 can be phosphorylated at multiple serine residues, and this phosphorylation has a dual role either to enhance or to terminate the insulin effects (5). Phosphorylation at Ser-307 enhances IRS1 ubiquitination and degradation, and S307A mutant IRS1 is more resistant to degradation following chronic exposure to insulin, whereas phosphorylation at Ser-612 decreases its interaction with PI3K (40), demonstrating separable mechanisms for IRS1 inactivation by serine phosphorylation. Moreover, feedback of IRS1 phosphorylation on some Ser/Thr sites can strengthen the output of insulin signaling by reducing tyrosine dephosphorylation and inhibitory Ser/Thr phosphorylation at other sites (41). Recent *in vivo* studies demonstrated a much more complex regulation of insulin signaling by serine phosphorylation of IRS1. Transgenic mice with muscle-specific IRS1 Ser-302, Ser-307, and Ser-612 mutated to alanine are protected from fat-induced insulin resistance in skeletal muscle (42). Surprisingly, given the sensitizing effect of the S307A IRS1 mutation in cell-based assays, homozygous transgenic mice with S307A IRS1 show increased fasting insulin *versus* control mice as well as very mild glucose intolerance (43).

Our results showed that the phosphorylation at Ser-307 was enhanced after CD36 knockdown, consistent with increased ubiquitination of IRS1. In contrast, phosphorylation at Ser-612 was decreased by CD36 knockdown, which may contribute to faster up-regulation of PI3K/Akt activity prior to insulin-in-

Figure 6. FAs or Fyn inhibition enhances CD36 regulation of IRS1 degradation. C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36KD*) and starved overnight with low-glucose DMEM medium containing 0.2% BSA as described under "Experimental procedures." *A*, cells were incubated with 200 μM PA, OA, or equivoluminal 20% BSA for 15 min and stimulated with 50 nM insulin for the indicated times after removal of FA. *B*, cells were treated with 10 μM SSO or DMSO for 30 min and stimulated with 50 nM insulin for the indicated times. *C*, cells cotransfected with siRNA targeting mouse Fyn (*FynKD*) were stimulated with 50 nM insulin for the indicated times. *D*, cells were treated with 10 μM Src-1 or DMSO for 30 min and stimulated with 50 nM insulin for the indicated times. Whole-cell lysates were subjected to immunoblot analysis using antibodies recognizing IRS1 and tubulin. Quantification of the mean of three independent experiments is shown. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$, compared with BSA (*A*), DMSO treatment (*B* and *D*) or negative control cells (*C*). *E*, schematic model of CD36 regulation of insulin-induced IRS1 degradation. Ubiquitinated CD36 interacts with IRS1 and prevents its interaction with CUL7. Insulin stimulation partially inhibits IRS1-CD36 association via decrease of CD36 ubiquitination, which leads to enhanced IRS1-CUL7 interaction and IRS1 degradation. In the absence of CD36 or expression of CD36 K/A, which does not bind to IRS1 well, IRS1 interaction with CUL7 was enhanced, leading to accelerated degradation. FA-induced dissociation of Fyn from CD36, Fyn KD, or pharmacological inhibition of Fyn activity in CD36-expressing cells also blocks CD36 interaction with IRS1 and its effect on IRS1 stability. *ubi*, ubiquitin.

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duced IRS1 degradation. CD36 functions as a receptor recognizing a variety of lipid and non-lipid ligands. In addition to cargo transportation, it also initiates signaling pathways, and a common theme in CD36 signal transduction is activation of Src family kinases (44). FA interaction with CD36 dissociates Fyn from the protein complex, allowing AMPK activation (31). Our data suggested that Fyn is required for CD36 interaction with IRS1, and at least part of CD36 is present in the endogenous CD36-Fyn-IRS1 complex (Fig. 5). Dissociation of Fyn from CD36 by FFA, Fyn KD, or pharmaceutical inhibition of Fyn diminishes the stabilizing effect of CD36, suggesting that CD36 regulation of IRS1 degradation requires Fyn and its kinase activity (Fig. 6*E*).

Recent studies demonstrated frequent association of CD36 function with cancer development and metastasis. A subpopulation of cancer cells with high CD36 expression has unique metastasis-initiating potential, highlighting a key role of CD36-regulated lipid metabolism and signaling in metastatic colonization (45). Lysophosphatidic acid/protein kinase D1/CD36 signaling is a *bona fide* breast cancer promoter via stimulating microvascular remodeling in chronic diet-induced obesity (46). CD36-positive B lymphoblasts predict poor outcome in children with B lymphoblastic leukemia (47). There is increasing evidence that IRS1 is an important growth-regulatory adaptor molecule that plays a role in cell proliferation. Prolylcarboxypeptidase- and prolylendopeptidase-mediated stabilization of IRS1 is critical for PI3K/Akt signaling and is associated with development and clinical aggressiveness of pancreatic cancer (48). Moreover, IRS1 is highly expressed in localized breast tumors and regulates the sensitivity of breast cancer cells to chemotherapy (49). The pathway of CD36-mediated IRS1 stability as identified in this study may contribute to CD36 function in regulating cancer development and metastasis, which could provide a pharmaceutical approach to treat cancers that are dependent on the IRS1/PI3K pathway.

In summary, this study presents a novel dynamic regulatory pathway of insulin signaling in myotubes. Ubiquitinated CD36 delays, but sustains, insulin signaling by interfering with IRS1 interaction with CUL7 and blocking its degradation. Identification of CD36 as a critical regulator of IRS1/PI3K/Akt signaling in this study may help design more effective therapies for metabolic dysfunctions of muscle.

Experimental procedures

Materials

Horse serum and FBS were purchased from Invitrogen (Grand Island, NY). siRNA targeting mouse CD36 (5′-AACGACATGATTAATGGCACA-3′) (24), mouse Fyn (5′-CCTGTATGGAAGGTTCACAAT-3′) (50) and scrambled siRNA were purchased from Life technologies (Foster City, CA). SSO was synthesized and utilized as we described previously (24). Full-length HA-tagged CUL7 plasmid was kindly provided by Dr. James DeCaprio (Dana-Farber Cancer Institute) and FLAG-tagged IRS1 plasmid was from Dr. Richard Roth (Stanford University). Information of antibodies was provided in Table S1. Other reagents were from Sigma-Aldrich (St. Louis, MO).

Cell culture

C2C12 murine myoblasts (from American Type Culture Collection) were maintained in high-glucose DMEM supplemented with 10% FBS, 200 units/ml penicillin, and 50 μg/ml streptomycin and were differentiated in high-glucose DMEM containing 2% horse serum for 6–7 days. CHO cells were maintained in Ham's F-12 medium containing 10% FBS, 200 units/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained at 37 °C with 5% CO_2. C2C12 myotubes or CHO cells were serum-starved for the indicated times in low-glucose DMEM or Ham's F-12 with 0.2% BSA and then treated as indicated.

siRNA and plasmid transfection

Transfection of siRNAs was carried out using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions on day 3 after differentiation of C2C12 myoblasts with the indicated siRNAs (10 nM final concentration). Experiments were performed 3 days after transfection. FLAG-tagged IRS1 and HA-tagged CUL7 (2 μg/ml final concentration) were transfected using Lipofectamine™ 2000 (Invitrogen) in CHO cells following the manufacturer's instructions.

Protein extraction and Western blotting

Cells were washed thrice with ice-cold PBS and lysed at 4 °C for 30 min with a lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10% glycerol, 1 mM EDTA, 1 mM EGTA) containing a protease inhibitor mixture (Sigma-Aldrich), then the cell lysates were clarified by centrifugation (12,000 × *g*, 10 min, 4 °C), and supernatant was collected and stored at −80 °C or used for immunoblotting as described (51). Protein samples were separated by SDS-PAGE after quantification with BCA kits (Thermo Scientific) and transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 (TBST) or 5% BSA in TBST for 1 h at room temperature and then incubated with primary antibodies in 2% BSA in TBST overnight at 4 °C followed by incubation with horseradish peroxidase-conjugated secondary antibodies and analysis by chemiluminescence. The Western blotting results were quantified using ImageJ software.

Coimmunoprecipitation

Cells were lysed in a lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA) containing a protease inhibitor mixture (Sigma-Aldrich). The cell lysates were clarified by centrifugation (12,000 × *g*, 10 min, 4 °C), and the clarified lysates were incubated with primary antibodies against IRS1 (overnight at 4 °C). Immune complexes were then incubated with 20 μl of recombinant Protein G-Sepharose® 4B (GE Healthcare) for 4 h at 4 °C, and the beads were washed five times with lysis buffer/PBS (1:1). For immunoprecipitation of FLAG, the clarified lysates were incubated with Red Anti-FLAG M2 affinity gel beads (Sigma-Aldrich) overnight at 4 °C and then washed five times as described above. Proteins were eluted by boiling (5 min) in

50 μl of SDS loading buffer. All of the samples were separated by 6–10% gradient SDS-PAGE.

GST fusion pulldowns

Expression of GST-UBA and GST-UBA mutant in *Escherichia coli* (BL21-DE3, TransGen Biotech, Beijing) was induced by isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich), and the GST fusion proteins were purified with Glutathione-Sepharose™ 4B (GE Healthcare) as described (30). The beads were then incubated with cell lysates overnight at 4 °C and then washed three times. Proteins were eluted by boiling (5 min) in 50 μl of SDS loading buffer prior to separation by SDS-PAGE.

Glycogen measurement

The cellular glycogen concentrations were measured using a Glycogen Assay kit (Sigma-Aldrich) according to the manufacturer's instruction.

Statistical analysis

The data are presented as mean $\pm$ S.D. Statistically significant differences between mean values were determined using unpaired Student's *t* test or one-way analysis of variance. In all cases, a significant result was defined as $p < 0.05$.

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Supplemental Data:

Ubiquitinated CD36 sustains insulin-stimulated Akt activation by stabilizing insulin receptor substrate 1
in myotubes

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Supplemental Figure S1. MG132 blocks insulin-induced IRS1 degradation.

Supplemental Figure S2. FAs and Fyn inhibition enhance insulin-induced IRS1 degradation in CHO/hIR cells in the presence of wild type CD36.
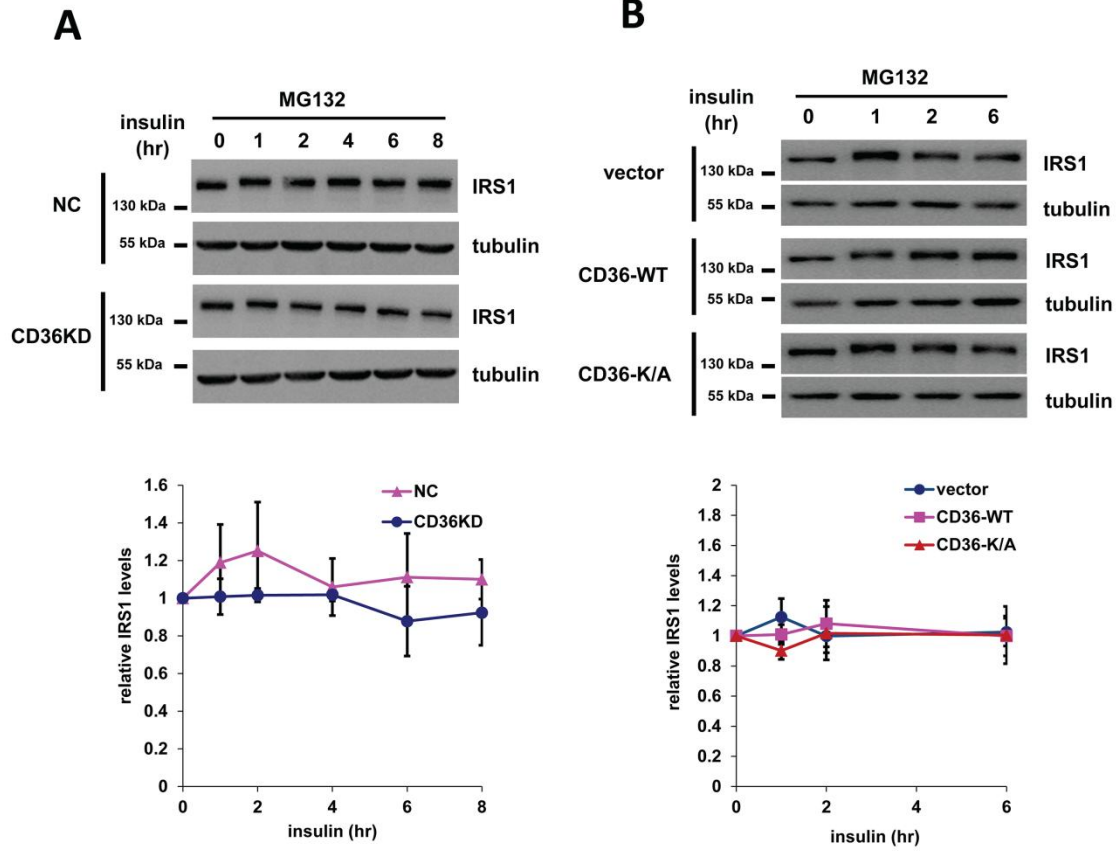
Supplemental Table S1. Antibody information.

Supplemental Figure Legends:

Supplemental Figure S1. MG132 blocks insulin-induced IRS1 degradation. *A*, C2C12 myotubes were treated with a scrambled negative control (*NC*) siRNA or siRNA targeting mouse CD36 (*CD36KD*) as described under "Experimental Procedures." Cells were starved with DMEM containing 0.2% BSA overnight, and treated with 20 µM MG132 for 30 min followed by stimulation with 50 nM insulin for indicated time. The whole cell lysates were subjected to immunoblot analysis with antibodies against IRS1 and tubulin. *B*, CHO/hIR cells expressing empty vector, CD36-WT, or CD36-K/A were starved with F12 containing 0.2% BSA overnight, then pretreated with 20 µM MG132 for 30 minutes and stimulated with 5 nM insulin for indicated time. Cell extracts were subjected to immunoblotting with antibodies against IRS1 and tubulin. Quantification of the mean ± SD of three independent experiments were shown.

Supplemental Figure S2. FAs and Fyn inhibition enhance insulin-induced IRS1 degradation in CHO/hIR in the presence of wild type CD36. CHO/hIR cells expressing empty vector *(A)*, CD36-WT *(B)*, or CD36-K/A *(C)* were starved with F12 containing 0.2% BSA overnight. Cells were incubated with 200 µM Palmitic acid (*PA*), oleic acid (*OA*), equivoluminal 20% BSA for 15 min or with 10 µM Src-1 (Src inhibitor-1) or DMSO for 30 min. After removal of FA, cells were stimulated with 5 nM insulin for indicated times. Whole cell lysates were subjected to immunoblot analysis with antibodies against IRS1 and tubulin. The data shown are representative of at least two experiments.

Supplemental Fig. S1

Supplemental Fig. S2

A vector

B CD36-WT

C CD36-K/A
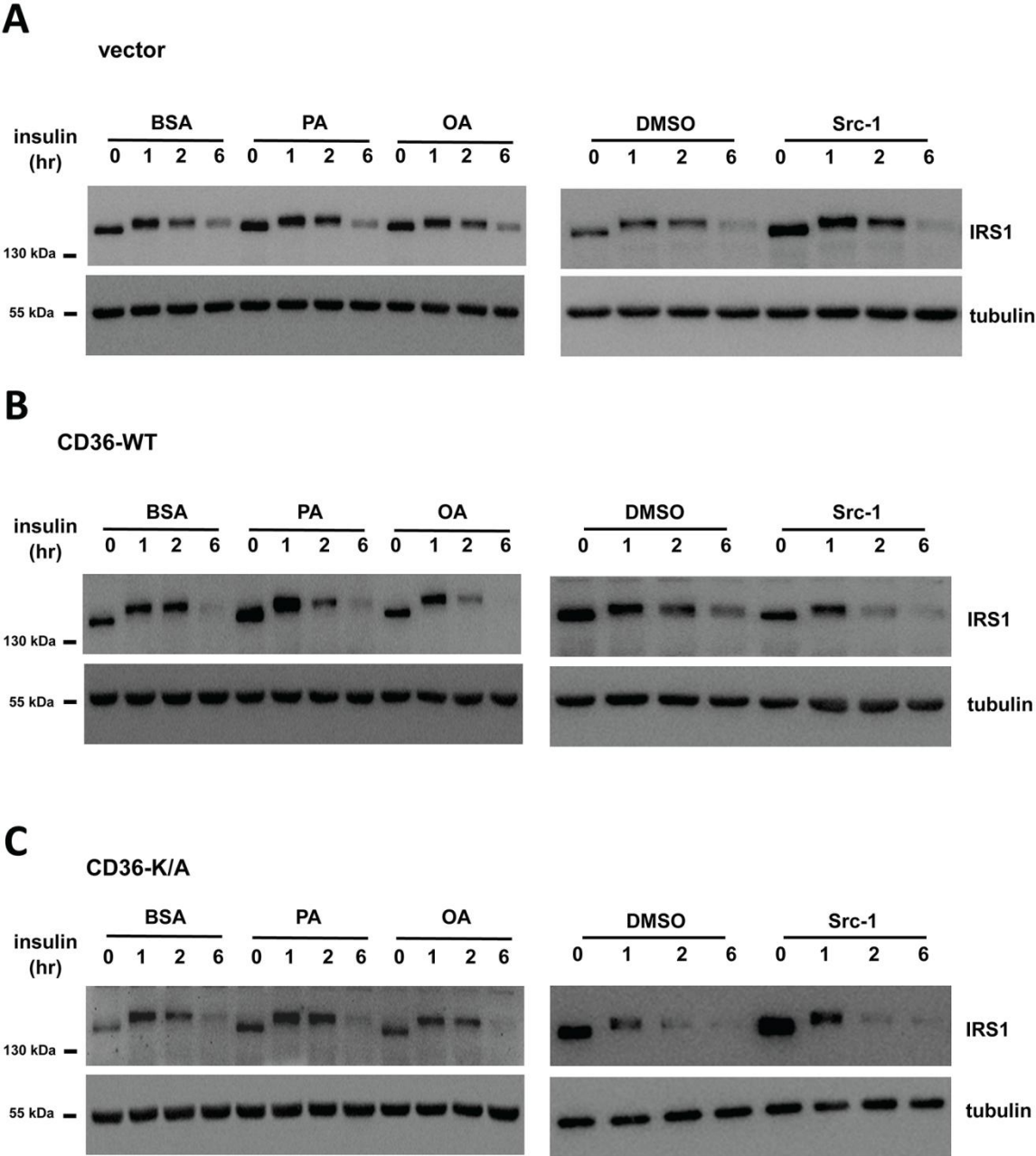
Supplemental Table S1. Antibody information

Name	Company	Catalog Number
human CD36	R&D Systems	AF1955
mouse CD36	R&D Systems	AF2519
Insulin Receptor β	Cell Signaling Technology	3025
IRS1	Cell Signaling Technology	2382
IRS2	Cell Signaling Technology	3089
Phospho-IRS1 (Ser307)	Cell Signaling Technology	2381
Phospho-IRS1 (Ser612)	Cell Signaling Technology	2386
Phospho-AKT (Thr308)	Cell Signaling Technology	2965
Phospho-AKT (Ser473)	Cell Signaling Technology	4058
AKT	Cell Signaling Technology	9272
Phospho-GSK3α/β (Ser21/9)	Cell Signaling Technology	8566
Fyn	Cell Signaling Technology	4023
Cullin 7	Sigma-Aldrich	C1743
α-tubulin	Sigma-Aldrich	T6199

Ubiquitinated CD36 sustains insulin-stimulated Akt activation by stabilizing insulin receptor substrate 1 in myotubes

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