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RESEARCH ARTICLE

Stk40 represses adipogenesis through translational control of CCAAT/enhancer-binding proteins

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ABSTRACT

A better understanding of molecular regulation in adipogenesis might help the development of efficient strategies to cope with obesity-related diseases. Here, we report that CCAAT/enhancer-binding protein (C/EBP) β and C/EBPδ, two crucial pro-adipogenic transcription factors, are controlled at a translational level by serine/threonine kinase 40 (Stk40). Genetic knockout (KO) or knockdown (KD) of *Stk40* leads to increased protein levels of C/EBP proteins and adipocyte differentiation in mouse embryonic fibroblasts (MEFs), fetal liver stromal cells, and mesenchymal stem cells (MSCs). In contrast, overexpression of *Stk40* abolishes the enhanced C/EBP protein translation and adipogenesis observed in *Stk40*-KO and -KD cells. Functionally, knockdown of *C/EBPβ* eliminates the enhanced adipogenic differentiation in *Stk40*-KO and -KD cells substantially. Mechanistically, deletion of *Stk40* enhances phosphorylation of eIF4E-binding protein 1, leading to increased eIF4E-dependent translation of C/EBPβ and C/EBPδ. Knockdown of *eIF4E* in MSCs decreases translation of C/EBP proteins. Moreover, *Stk40*-KO fetal livers display an increased adipogenic program and aberrant lipid and steroid metabolism. Collectively, our study uncovers a new repressor of C/EBP protein translation as well as adipogenesis and provides new insights into the molecular mechanism underpinning the adipogenic program.

KEY WORDS: eIF4E, 4E-BP1, C/EBP, Adipogenesis, Stk40

INTRODUCTION

Adipogenesis is a step-wise process consisting of lineage commitment from multipotent stem cells into preadipocytes and terminal differentiation from the preadipocytes into adipocytes. Adipose tissue plays important roles for energy and metabolism homeostasis, and also serves as an endocrine organ (Shepherd et al., 1993). Aberrant adipogenesis is closely associated with obesity, which can eventually lead to diseases such as type II diabetes, cardiac-metabolic diseases and certain types of cancers (Li et al., 2005). Multiple signaling pathways including those mediated by TGFβ and BMPs (Choy et al., 2000; Tang et al., 2004; Bowers et al., 2006), Wnts (Ross et al., 2000; Kang et al., 2007), MAPKs (Aouadi et al., 2006; Kim et al., 2007; Wang et al., 2009), Shh (Spinella-Jaegle et al., 2001; Suh et al., 2006), insulin and insulin-like growth factors (IGFs) (Smith et al., 1988; Baudry et al., 2006), as well as transcription factors such as those from the CCAAT/enhancer-binding protein family (C/EBPs), act in a temporal pattern to orchestrate adipogenesis (Akira et al., 1990; Chang et al., 1990; Cao et al., 1991; Darlington et al., 1998; Otto and Lane, 2005; Rosen and MacDougald, 2006; Gesta et al., 2007). Among C/EBPs, C/EBPβ is crucial for adipogenic lineage commitment and early differentiation initiation. C/EBPβ can dimerize with C/EBPδ to activate the transcription of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) (Tontonoz et al., 1994; Kawai and Rosen, 2010). PPARγ has two isoforms, PPARγ1 and PPARγ2. PPARγ2 has been reported to be more potent for adipogenesis (Ren et al., 2002; Mueller et al. 2002). C/EBPα and PPARγ then form a self-reinforcing loop and activate the adipogenic program. *C/EBPβ*$^{-/-}$ (*Cebpb*$^{-/-}$) mice have reduced adiposity and *C/EBPβ*$^{-/-}$ MEFs display impaired adipogenesis (Tang et al., 2003), whereas *C/EBPβ*, *C/EBPδ* (*Cebpb*, *Cebpd*) double knockout mice have a further decline in brown adipose tissue and epididymal fat pad mass (Tanaka et al., 1997). C/EBPα is more potent for the adipocyte terminal differentiation and development of adipose tissue (Linhart et al., 2001). So far, regulatory mechanisms for the expression of C/EBPs have not been fully elucidated.

C/EBPs are widely expressed in mammalian organisms and participate in proliferation and differentiation in various cell types, including adipocytes, osteocytes, hematopoietic cells, hepatocytes and neural cells (Chang et al., 1990; Cao et al., 1991; Asimakopoulos et al., 1994; Soriano et al., 1995; Darlington et al., 1998; Seipel et al., 2004; Smink et al., 2009). Besides transcriptional control, the protein levels of C/EBPs are subject to a particular translational control. First, both C/EBPα and C/EBPβ generate multiple isoforms owing to the differential usage of in-frame initiation codons (Calkhoven et al., 2000). C/EBPα has two isoforms, p42 (full length) and p30 (truncation), whereas C/EBPβ has three isoforms, LAP* (full length, 36 kDa), LAP (34 kDa) and LIP (a truncation, 19 kDa). It has been reported that the translation of different C/EBP isoforms is regulated by the activity of translation initiation factors such as eIF2α and eIF4E, which are in turn controlled by eIF2α kinases and mTOR/eIF4E-binding protein 1 (4E-BP1, also known as eIF4EBP1), respectively (Raught et al., 1996; Smink et al., 2009). The phosphorylation state of 4E-BP1 is implicated in the control of eIF4E activities. In addition to the 4E-BP1–eIF4E cascade, several mRNA-binding proteins have been shown to modulate the translation of C/EBPα and/or C/EBPβ through interaction with special motifs or secondary structures located at the corresponding mRNA (Timchenko et al., 2002; Karagiannides et al., 2006; Kawagishi et al., 2008; Haefliger et al., 2011). Nevertheless, the molecular mechanism of C/EBPβ translational control and its physiological impact in adipogenesis remain poorly characterized. The question of whether C/EBPδ is subject to a translational control similar to that for C/EBPβ and C/EBPα has not been answered.

Stk40, a putative serine/threonine kinase, was originally identified as an activator of the Erk1/2 (also known as MAPK3

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and MAPK1, respectively) signaling required for primitive endoderm differentiation from mouse embryonic stem cells, and was later found to be important for mouse fetal lung maturation (Li et al., 2010; Yu et al., 2013). In this study, we report that Stk40 acts as a repressor of adipogenesis through the translational control of C/EBPβ and C/EBPδ. We provide the first experimental evidence that the expression of C/EBPδ is also modulated at a translational level. Moreover, we elucidate that Stk40 modulates C/EBP protein translation through the 4E-BP1–eIF4E cascade. In addition, our microarray analyses reveal that *Stk40* deletion interrupts the global metabolic program in the perinatal fetal liver. Collectively, our study uncovers a new regulator of adipogenesis and provides insights into C/EBP protein translational control and its function in adipogenesis.

RESULTS

Deletion of *Stk40* enhances adipogenesis in MEFs and stromal cells

When *Stk40*$^{-/-}$ (knockout, KO) MEFs were cultured post-confluently without induction, adipocytes containing cytoplasmic accumulation of lipid droplets, indicated by Oil Red O staining, appeared spontaneously (Fig. 1A). In contrast, adipocytes were not observed in wild type (WT) (Fig. 1A) or heterozygous (Het, data not shown) MEFs. When induced to adipogenic differentiation with hormonal cocktails [comprising 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone, or IBMX, dexamethasone and insulin, denoted MDI], KO MEFs exhibited substantially enhanced adipocyte differentiation compared to WT cells (Fig. 1A). At the molecular level, we analyzed the expression of adipocyte markers, including *aP2* (also known as *FABP4*), adipsin, adiponectin (adipoQ) and transcription factors (*C/EBPα* and *PPARγ2*) by quantitative real-time PCR (qRT-PCR) assays. *Stk40*-KO MEF cells expressed significantly higher levels of all these markers than WT cells post induction (Fig. 1B). To test whether there was enhanced adipocyte differentiation from other cell types in *Stk40*-KO mice, we isolated stromal cells from E14.5 fetal livers, which contain fibroblasts, preadipocytes and mesenchymal stem cells (MSCs). Similarly, fetal liver stromal cells from *Stk40*-KO mice readily differentiated into adipocytes after induction, whereas WT cells did not (Fig. 1C). Overexpression of Stk40 markedly abolished the enhanced adipogenesis in *Stk40*-KO MEFs, indicated by both Oil Red O staining and qRT-PCR analyses (Fig. 1D,E; supplementary material Fig. S1A), verifying the specific role of Stk40 in the enhanced adipogenesis. Thus, our results from *Stk40*-KO MEFs and fetal liver stromal cells indicate that Stk40 has a repressive role for adipogenesis.

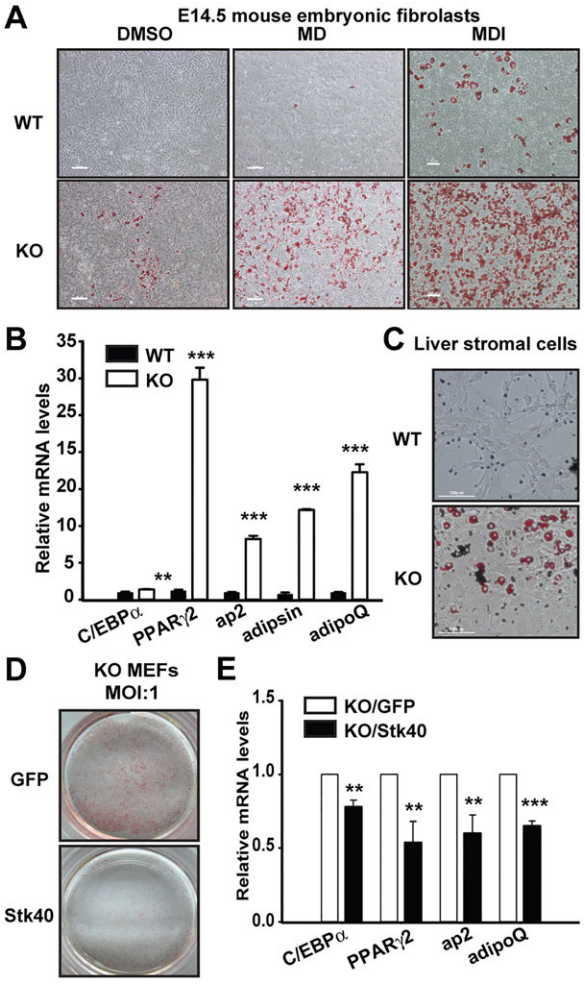

Fig. 1. *Stk40*-KO enhances the adipocyte differentiation of MEFs and fetal liver stromal cells. (A) Oil Red O staining of MEFs (passage <4) derived from E14.5 WT or KO embryos after 8 days of differentiation. DMSO was used as a vehicle; MD, IBMX plus dexmathasone; MDI, IBMX , dexmathasone and insulin. The cells of each genotype were pooled from at least ten embryos. WT, *Stk40* wild type; KO, *Stk40* knockout. Scale bars 100 μm. (B) mRNA levels of adipogenic markers increased *Stk40* in KO MEFs at day 8 after MDI induction. Results are mean±s.d. $^{**}P<0.01$; $^{***}P<0.001$ (Student's *t*-test). (C) Oil Red O staining of fetal liver stromal cells derived from E14.5 WT or KO embryos after 12 days of differentiation. Scale bars: 100 μm. (D) Oil Red O staining of MEFs after 8 days of differentiation. Stk40 overexpression represses adipocyte differentiation in *Stk40*-KO MEFs. MOI, multiplicity of infection. (E) mRNA levels of adipogenic markers decreased in *Stk40-KO* MEFs with Stk40 overexpression (KO/Stk40) after 8 days of differentiation. Results are mean±s.d. $^{**}P<0.01$; $^{***}P<0.001$ (Student's *t*-test).

Knockdown of *Stk40* enhances the adipogenic lineage commitment and differentiation

As MEFs and stromal cells contain MSCs and preadipocytes, their adipogenesis involves both lineage commitment and terminal differentiation (Wang and Sul, 2009). To define at which stage Stk40 functions, we compared the adipogenic function of Stk40 in bone marrow MSCs (BM MSCs), C3H10T1/2 MSCs and 3T3-L1 preadipocytes. Both adipogenic lineage commitment and terminal differentiation can take place in the former two cell types, whereas 3T3-L1 cells serve as a classic model for terminal differentiation of preadipocytes into adipocytes (Tang et al., 2004; Otto and Lane, 2005). When *Stk40* was knocked down (KD) in BM MSCs (Fig. 2A), more adipocytes appeared in KD cells than in control cells (Fig. 2B). Consistently, expression of *aP2*, *C/EBPα* and *PPARγ2* was markedly higher in *Stk40*-KD BM MSCs than in control cells (Fig. 2C). Similarly, *Stk40* KD in C3H10T1/2 MSCs promoted the adipocyte differentiation profoundly (Fig. 2D), although not as efficiently as BMP4, an agent often used to induce the mesoderm lineage commitment (Tang et al., 2004). Notably, *Stk40* mRNA levels declined substantially during BMP4-induced adipogenic commitment in C3H10T1/2 cells (Fig. 2E), implying that downregulation of *Stk40* might contribute to the process of adipogenic commitment. *Stk40* KD and BMP4 treatment promoted the adipocyte differentiation synergistically, as evidenced by both cytoplasmic lipid accumulation and marker gene expression (Fig. 2D,F). However, unlike in BM MSCs and C3H10T1/2 MSCs, *Stk40* KD did not affect adipocyte differentiation in 3T3-L1 preadipocytes (Fig. 2G). At the molecular level, *Stk40* expression decreased during the process of differentiation of 3T3-L1 cells (Fig. 2H). Nevertheless, *Stk40* KD

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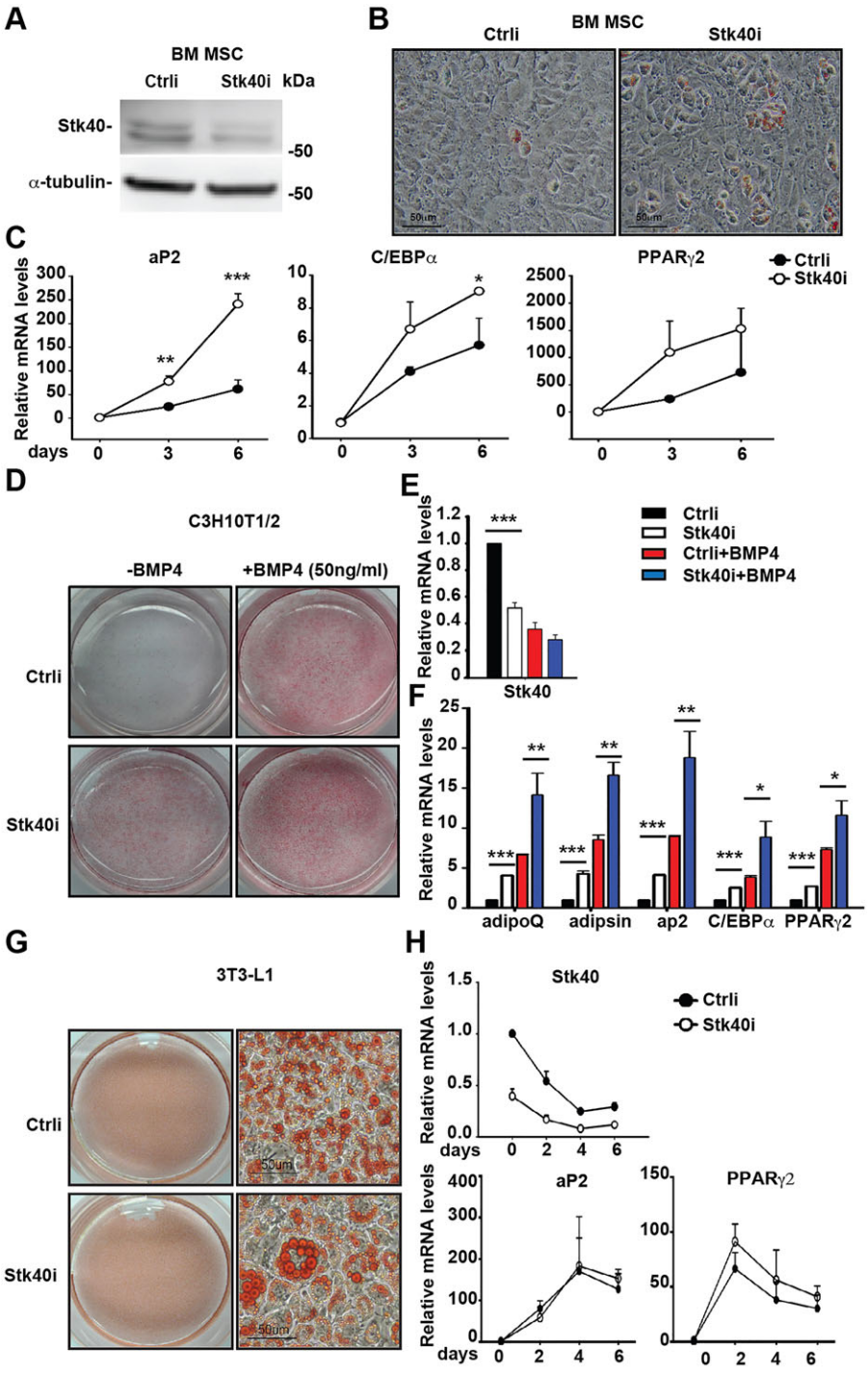

Fig. 2. *Stk40* knockdown enhances adipocyte differentiation in mouse BM MSCs and C3H10T1/2 cells. (A) Knockdown of *Stk40* in BM MSCs. Ctrli, control short hairpin RNA (shRNA); Stk40i, *Stk40* shRNA. α-tubulin was used as a loading control. Positions of protein molecular mass markers are indicated on the right. (B) Oil Red O staining of BM MSCs after 9 days of differentiation. Scale bars: 50 µm. (C) mRNA levels of adipogenic markers were increased in *Stk40*-KD BM MSCs at the indicated time points. Results are mean±s.d. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test). (D) Oil Red O staining of C3H10T1/2 cells after 8 days of differentiation with or without BMP4 pretreatment. (E) The relative mRNA levels of *Stk40* in control or *Stk40*-KD C3H10T1/2 cells treated with or without BMP4 treatment after 8 days of differentiation. Results are mean±s.d. ****P*<0.001 (Student's *t*-test). (F) mRNA levels of adipogenic markers were increased in *Stk40*-KD C3H10T1/2 cells after 8 days of differentiation. Results are mean±s.d. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test). (G) *Stk40* KD did not promote adipocyte differentiation of 3T3-L1 cells. Oil Red O staining was performed after 8 days of differentiation. Scale bars: 50 µm. (H) The relative mRNA levels of *Stk40* and adipogenic markers in 3T3-L1 cells at indicated time points. Results are mean±s.d.

could not enhance the adipogenic program in 3T3-L1 preadipocytes (Fig. 2H). Based on these data, we propose that Stk40 might control adipogenesis primarily through repressing adipogenic commitment, although we do not rule out the possibility that it also plays a role in the terminal differentiation of MSCs.

Increased C/EBPβ protein is responsible for adipogenesis mediated by *Stk40* depletion

To explore the mechanism through which *Stk40* KO and KD promotes adipogenesis, we compared levels of several important adipogenic transcription factors and signaling pathways between WT and *Stk40*-KO MEFs during MDI-induced adipocyte differentiation. Strikingly, the steady-state levels of all three C/EBPβ isofoms and C/EBPδ were obviously higher in *Stk40*-KO cells than in WT cells at all time points examined, without an isoform preference for increased C/EBPβ translation (Fig. 3A). Similar to the early inducers, master genes for late adipogenesis (C/EBPα and PPARγ2) were also substantially higher in *Stk40*-KO MEFs than WT cells (Fig. 3A). Specifically, forced expression of *Stk40* could partially rescue the protein levels of all three C/EBPβ isoforms and C/EBPδ in *Stk40*-KO MEF cells (Fig. 3B), in accordance with our observation that ectopic *Stk40* abolished the enhanced adipogenesis in *Stk40*-KO MEFs (Fig. 1D). In terms of signaling pathways, both Erk1/2 and phosphoinositide 3-kinase (PI3K)–Akt signaling were

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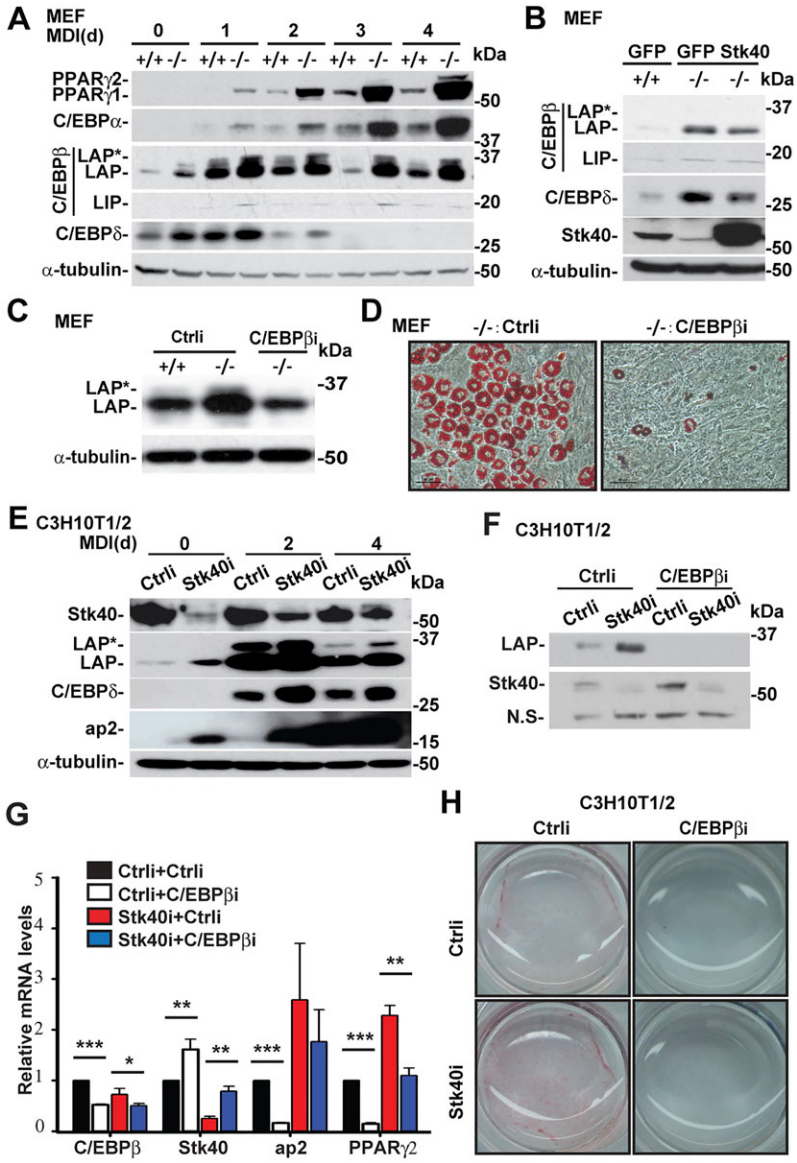

Fig. 3. C/EBPβ is indispensible for *Stk40*-deficiency-caused enhancement of adipogenesis. (A) Protein levels of adipogenic markers increased in *Stk40*-KO MEFs at the indicated time points. LAP*, full-length active C/EBPβ (36 kDa); LAP, active C/EBPβ (34 kDa); LIP, truncated C/EBPβ (19 kDa); +/+, *Stk40* wild type; –/–, *Stk40* knockout; MDI, IBMX+dexmathasone+Insulin. α-tubulin was used as a loading control. Positions of protein molecular mass markers are indicated on the right. (B) Protein levels of C/EBPβ and C/EBPδ decreased in MEFs with Stk40 overexpression. α-tubulin was used as a loading control. (C) Knockdown of C/EBPβ in *Stk40*-KO MEFs by lentiviral-mediated *C/EBPβ* short hairpin RNA (C/EBPβi). α-tubulin was used as a loading control. (D) Oil Red O staining of *Stk40*-KO MEFs after 8 days of differentiation. *C/EBPβ* knockdown abrogated increased adipocyte differentiation in *Stk40-KO* MEFs. –/–, *Stk40* knockout; Ctrli, control shRNA; C/EBPβi, *C/EBPβ* shRNA. Scale bars, 50 µm. (E) Protein levels of adipogenic markers increased in *Stk40*-KD C3H10T1/2 cells at indicated time points. Ctrli, control shRNA; Stk40i, *Stk40* shRNA. (F) Knockdown of C/EBPβ in C3H10T1/2 cells rescued Stk40 protein expression. N.S, non-specific band recognized by Stk40 antibody as a sample loading control. Ctrli, Control shRNA; Stk40i, *Stk40* shRNA; C/EBPβi, *C/EBPβ* shRNA. (G) mRNA levels of adipogenic genes decreased in *C/EBPβ*-KD C3H10T1/2 cells after 8 days of differentiation. *C/EBPβ* KD abolished increased adipocyte differentiation in *Stk40*-KD C3H10T1/2 cells. Ctrli, control shRNA; Stk40i, *Stk40* shRNA; C/EBPβi, *C/EBPβ* shRNA. Results are mean±s.d. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test). (H) Oil Red O staining of C3H10T1/2 cells treated as in G after 8 days of differentiation. Ctrli, control shRNA; Stk40i, *Stk40* shRNA; C/EBPβi, *C/EBPβ* shRNA.

activated by MDI induction, but consistently attenuated in KO MEFs compared to WT MEFs (supplementary material Fig. S1B), as previously reported (Li et al., 2010; Yu et al., 2013). Thus, Stk40 deficiency prompts a potent adipocyte differentiation preference in MEFs even under a condition of attenuated Erk1/2 and PI3K–Akt adipogenic signals.

Between C/EBPβ and C/EBPδ, C/EBPβ is more potent for the induction of lineage commitment and differentiation, whereas C/EBPδ can potentiate the function of C/EBPβ (Cao et al., 1991). Therefore, we tested whether the elevated C/EBPβ protein levels could account for the enhanced adipogenesis in *Stk40*-KO MEFs through specific KD of *C/EBPβ*. Silencing of *C/EBPβ* efficiently abrogated the enhanced adipocyte differentiation in *Stk40*-KO MEFs (Fig. 3C,D), suggesting that an essential role of *C/EBPβ* in *Stk40* KO caused the enhancement of adipocyte differentiation. In a similar pattern, *Stk40* KD in MSC lines, including C3H10T1/2 and BM MSCs, substantially increased the protein levels of C/EBPβ and C/EBPδ as well as adipocyte differentiation (Fig. 3E; supplementary material Fig. S2A). However, *C/EBPβ* KD abrogated the increased adipocyte differentiation in *Stk40*-KD C3H10T1/2 cells efficiently (Fig. 3F–H). Interestingly, we noticed elevated levels of Stk40 transcripts and proteins in *C/EBPβ*-KD cells, hinting at the existence of an inhibitory feedback loop between C/EBPβ and Stk40 (Fig. 3F,G). These results support the notion that enhanced adipogenesis in *Stk40*-KO MEFs or *Stk40*-KD MSCs is most likely due to the elevated levels of C/EBPβ and C/EBPδ.

The elevation in C/EBP protein levels is not due to impaired protein degradation

To understand why the steady-state level of C/EBP proteins was markedly elevated in *Stk40*-KO and -KD cells, we first examined their mRNA levels. Surprisingly, the mRNA levels of both C/EBPβ and C/EBPδ were lower in *Stk40*-KO MEF cells than in WT cells in the first few hours after MDI induction, opposite to their protein levels (Fig. 4A,B). Similarly, the mRNA level of C/EBPβ was also lower in *Stk40*-KD BM MSCs than in control cells (supplementary material Fig. S2B). Consistently, the mRNA levels of C/EBPβ and C/EBPδ were also lower in *Stk40*-KD C3H10T1/2 cells than in control cells (supplementary material Fig. S2C). This finding excluded the possibility that the increased C/EBP proteins were the results of higher transcription. As the steady-state level of a protein in cells is determined by the balance between protein synthesis and

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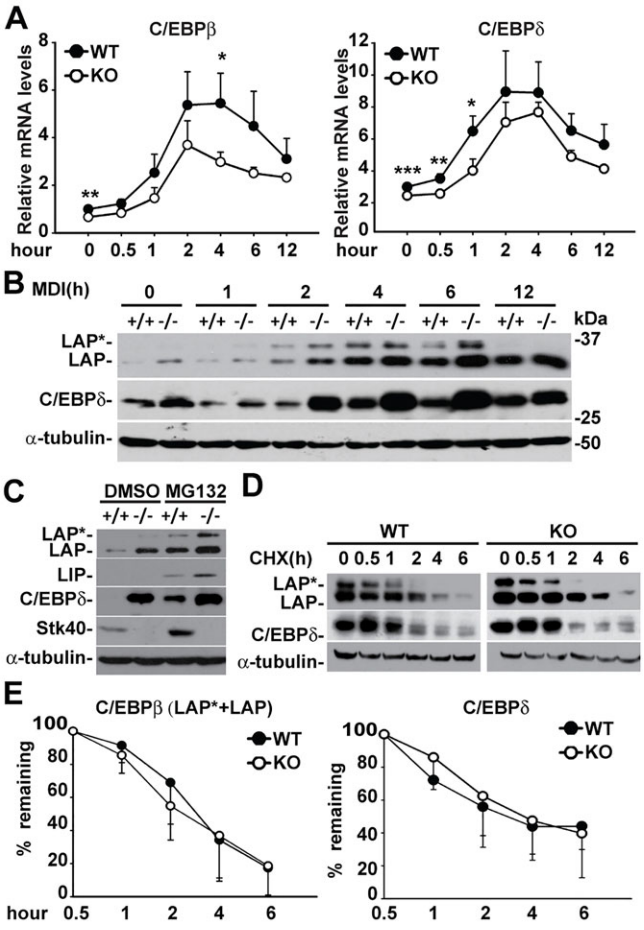

Fig. 4. C/EBPβ and C/EBPδ are post-transcriptionally regulated and their degradation is not impaired in *Stk40*-null MEFs. (A) mRNA levels of *C/EBPβ* and *C/EBPδ* decreased in *Stk40*-KO MEFs at the indicated time points after MDI induction. Results are mean±s.d. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test). (B) Protein levels of C/EBPβ and C/EBPδ increased in *Stk40*-KO MEFs at the indicated time points after MDI induction. LAP*, full-length active C/EBPβ (36 kDa); LAP, active C/EBPβ (34 kDa); +/+, *Stk40* wild type; –/–, *Stk40*-KO; MDI, IBMX+dexmathasone+insulin. α-tubulin was used as a loading control. Positions of protein molecular mass markers are indicated on the right. (C) C/EBPβ and C/EBPδ were degraded through 26S proteasome pathway. MEFs were treated with 30 μM MG132 for 4 h before harvest. +/+, *Stk40* wild type; –/–, *Stk40* knockout; DMSO was used as a vehicle. α-tubulin was used as a loading control. (D) The degradation of C/EBPβ and C/EBPδ was comparable in wild-type and *Stk40-KO* MEFs. MEFs were treated with 100 μM CHX for indicated time before harvest. CHX, cyclohexamide. (E) The half-life of C/EBPβ and C/EBPδ proteins was comparable in wild type and *Stk40-KO* MEFs as measured from blots for the experiments shown in D. The gray density of blots was measured by software ImageJ. The levels of C/EBPβ and C/EBPδ at 0.5 h after cyclohexamide treatment were set as 100. LAP*, full length active C/EBPβ (36 kDa); LAP, active C/EBPβ (34 kDa). Results are mean±s.d.

degradation, we then examined the degradation of C/EBPβ and C/EBPδ. C/EBPβ and C/EBPδ could be turned over through the 26S proteasome pathway, as a 26S proteasome inhibitor (MG132) but not a lysosome inhibitor (chloroquine, CQ) increased the steady-state levels of C/EBPδ and all three isoforms of C/EBPβ proteins in both WT and KO MEFs (Fig. 4C; supplementary material Fig. S2D). However, the protein levels of C/EBPs remained higher in *Stk40*-KO cells than in WT cells when the 26S proteasome pathway was inhibited, suggesting that the differential protein level of the C/EBPs was not due to the reduced protein degradation in *Stk40*-KO cells. To further support the conclusion, we evaluated the turnover rate of C/EBPβ and C/EBPδ proteins after treatment with the protein synthesis inhibitor cyclohexamide (CHX). C/EBP proteins degraded quickly and the half-life was comparable in WT and KO MEFs (Fig. 4D,E). Therefore, higher C/EBP protein levels in *Stk40*-KO or -KD cells might result from the enhanced protein synthesis, rather than changes in their transcription or protein degradation.

An increase in eIF4E-mediated translation of C/EBPβ might account for the enhanced adipogenesis in *Stk40*-deficient cells

Protein synthesis is subject to multifaceted controls. Because microRNAs have been reported to regulate mRNA translation (Lee et al., 1993), we performed a genome-wide microRNA array to determine whether microRNAs took part in the enhanced C/EBP protein synthesis in *Stk40-KO* MEFs. However, none of microRNAs reported or predicted to be associated with *C/EBPβ* or *C/EBPδ* mRNA was enriched in *Stk40-KO* MEFs (supplementary material Table S1). We then turned our attention to RNA-binding proteins, including calreticulin and protein disulfide isomerase family A, member 3, which have been previously reported to regulate C/EBPα and C/EBPβ mRNA translation (Timchenko et al., 2002; Haefliger et al., 2011). Our western blot analyses did not reveal obvious differences in the levels of these two proteins between *Stk40-KO* and WT MEFs or *Stk40*-KD and control C3H10T1/2 cells (supplementary material Fig. S3A).

One of the key steps in the eukaryotic mRNA translation is the recognition of the cap structure by the cap binding protein complex, eukaryotic translation initiation factor 4F (eIF4F), which contains three subunits: eIF4E, eIF4A and eIF4G. The function of eIF4E is tightly controlled by eIF4E-binding protein 1 (4E-BP1). Hypo-phosphorylated 4E-BP1 strongly interacts with eIF4E to inhibit the translation of mRNAs having a 5′-cap structure, whereas 4E-BP1 dissociates from eIF4E upon hyper-phosphorylation, in turn facilitating the translation. The major signaling pathway modulating the phosphorylation of 4E-BP1 is the mTOR pathway (von Manteuffel et al., 1996). As *C/EBP* mRNAs contain the cap structure, we hypothesized that Stk40 might regulate the phosphorylation of 4E-BP1 to control the translation of C/EBPs. Indeed, the levels of intermediate- and hyper-phosphorylated 4E-BP1 proteins (β and γ forms) were markedly higher in *Stk40*-KO MEFs than in WT cells. The increase of 4E-BP1 phosphorylation was further verified by antibodies specifically against phosphorylation at sites of Thr37 and Thr46, Ser65 and Thr70, respectively (Fig. 5A). In contrast, eIF4E protein levels were not markedly different between WT and *Stk40*-KO MEFs. A higher phosphorylation level of p70S6K1 (also known as RPS6KB1 or S6K1), another downstream target of mTOR signaling, is also detected in Stk40-KO MEFs. The findings suggested that the mTOR activity was increased, which could in turn lead to an increase in S6K1 mediated protein synthesis as well as eIF4E-mediated cap-dependent translation in *Stk40*-KO MEFs. Similar to MEFs, *Stk40*-KD C3H10T1/2 cells had enhanced phosphorylation of both 4E-BP1 and S6K1 (Fig. 5B). However, it is worth mentioning that overexpression of Stk40 in *Stk40*-KO and -KD cells rescued the protein levels of C/EBPβ and C/EBPδ as well as the phosphorylation level of 4E-BP1, but not S6K1, implying that Stk40 might control 4E-BP1 phosphorylation independently of mTOR signaling (Fig. 5C,D). To further clarify how Stk40 controlled 4E-BP1 phosphorylation, an inhibitor of mTOR (rapamycin) was employed. Rapamycin completely blocked S6K1

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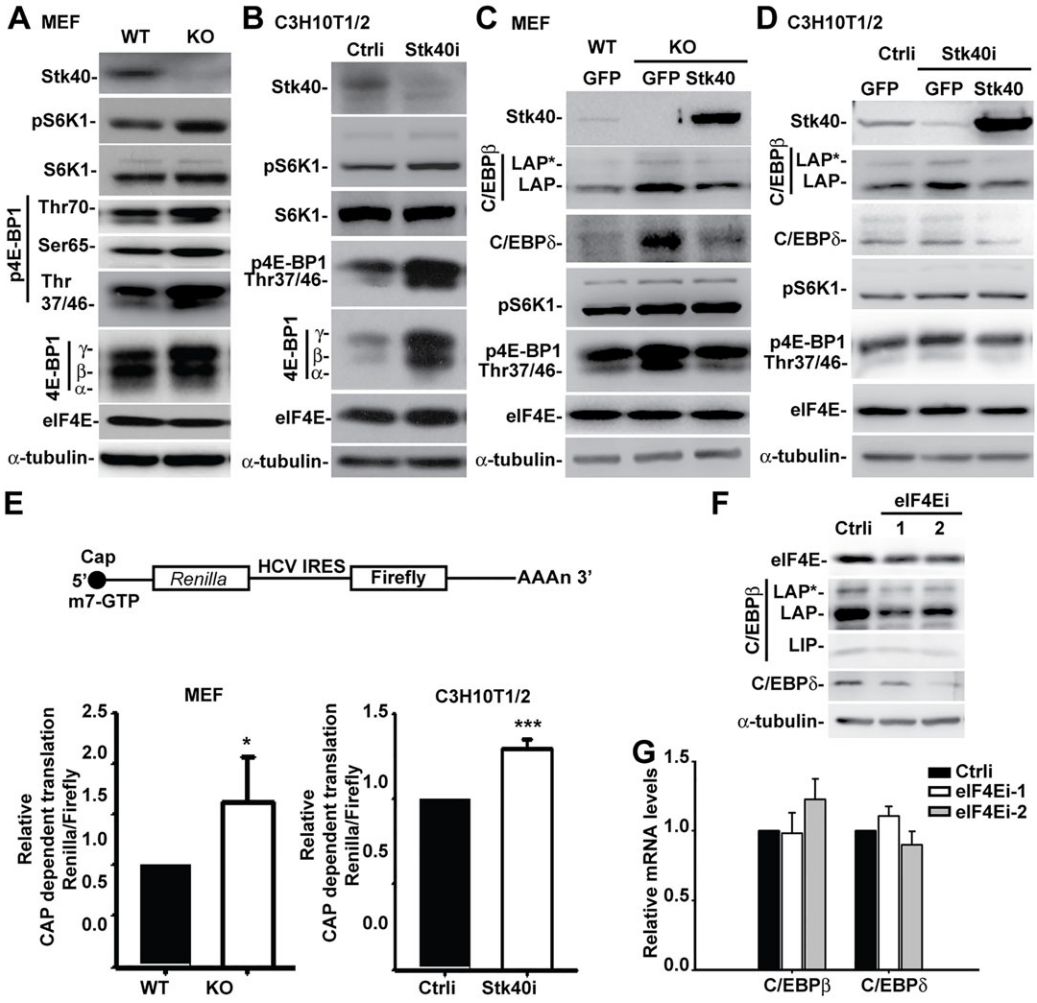

Fig. 5. Cap-dependent translation of C/EBPs is enhanced in *Stk40*-KO and -KD cells. (A) The levels of proteins involved in protein translation in *Stk40*-KO MEFs. Phosphorylation of 4E-BP1 and S6K1 was increased in *Stk40*-KO MEFs. α–γ isoforms represent the phosphorylation status of 4E-BP1; α, hypo-phosphorylated isoform; β, intermediate-phosphorylated isoform; γ, hyper-phosphorylated isoform. Thr37/46, Ser65 and Thr70, antibodies against 4E-BP1 phosphorylated at indicated sites. α-tubulin was used as a loading control. (B) The levels of proteins involved in protein translation in *Stk40*-KD C3H10T1/2 cells. Phosphorylation of 4E-BP1 and S6K1 was increased in *Stk40*-KD (Stk40i) C3H10T1/2 cells compared with control (Ctrli, control shRNA). α-tubulin was used as a loading control. (C) Stk40 overexpression abolished the increased phosphorylation of 4E-BP1 in *Stk40*-KO MEFs. *GFP* or *Stk40* was delivered by retroviral vectors. α-tubulin was used as a loading control. (D) Stk40 overexpression abolished the increased phosphorylation of 4E-BP1 in *Stk40*-KD C3H10T1/2 cells. *GFP* or *Stk40* was transduced by retroviral vectors. α-tubulin was used as a loading control. (E) Cap-dependent translation assays were conducted with a dual *Renilla* and firefly luciferase system with the human hepatitis C virus IRES driving firefly luciferase expression. Cap-dependent translation was increased in *Stk40*-KO MEFs (lower left) and *Stk40*-KD C3H10T1/2 cells (lower right). Cassettes of the dual *Renilla* and firefly luciferase plasmid were indicated in schema (upper). Results are mean±s.d. **P*<0.05; ****P*<0.001 (Student's *t*-test). (F) Protein levels of C/EBPβ and C/EBPδ decreased after *eIF4E* KD in C3H10T1/2 cells. α-tubulin was used as a loading control. (G) mRNA levels of C/EBPβ and C/EBPδ did not alter after *eIF4E* KD in C3H10T1/2 cells. Results are mean±s.d.

activation, whereas phosphorylation of 4E-BP1 in *Stk40*-KO MEFs remained increased at all rapamycin dosages tested (supplementary material Fig. S3B). These results favor a notion that Stk40 represses phosphorylation of 4E-BP1 independently of mTOR.

To evaluate the impact of the increased phosphorylation of 4E-BP1 and in turn the higher eIF4E activity in the cap-dependent translation, a dual luciferase reporter system that could distinguish cap-dependent versus IRES-directed translation was used. Reporter assays revealed that cap-dependent translation increased moderately but significantly in both *Stk40*-KO MEFs and *Stk40*-KD C3H10T1/2 cells (Fig. 5E). Therefore, *Stk40* deficiency promoted the cap-dependent translation as compared to control cells. We then assessed the specific impact of eIF4E on C/EBPβ and C/EBPδ protein translation. Silencing of eIF4E in C3H10T1/2 cells decreased the protein levels of all three isoforms of C/EBPβ and C/EBPδ dramatically without significant changes in their mRNA levels (Fig. 5F,G), indicating that the activity of eIF4E was required for appropriate translation of C/EBPs. Taken together, Stk40 deficiency elicited an increased phosphorylation of 4E-BP1, promoting the eIF4E-dependent C/EBP protein translation and adipogenesis.

Fetal organs of *Stk40-KO* mice display enhanced adipogenic gene expression and 4E-BP1 phosphorylation

Death of *Stk40*-KO mice at birth prevented us examining the *in vivo* function of Stk40 during adipogenesis. To surmount this problem, we looked at global gene expression profiling between *Stk40*-KO and WT fetal livers at E18.5. A total of 2140 differentially expressed probes (fold changes >1.5) were identified between *Stk40-KO* and WT livers. Although fetal liver possesses residual hemapoietic activity at this stage, it is also starting the hepatic metabolism at this time. The Gene Ontology (GO) analyses of differentially expressed genes (DEGs) in livers showed enriched genes were associated with

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oxidation and reduction, the immune and inflammatory response and a large proportion that were involved in the metabolic process of steroid, glucose, hexose, monosaccharide and cholesterol (supplementary material Fig. S4). We also comparatively analyzed the 2140 differentially expressed probes in the liver with previously published 734 differentially expressed probes (FC>1.5, $P<0.05$) between *Stk40*-KO and WT lungs (Yu et al., 2013). Of these differentially expressed probes, there were 197 probes (166 genes) that were shared by the liver and lung, with 81 genes upregulated and 56 genes downregulated in both organs (Fig. 6A). These genes might reflect the general physiological impact of Stk40 on cellular functions independently of its specific role in particular organs. GO analyses of the common DEGs revealed that a large proportion of genes was involved in white and brown fat cell differentiation, lipid transport and lipid localization (Fig. 6B, indicated by asterisk *). The expression of several important genes participating in adipogenesis, like those encoding *aP2*, adipsin, adiponectin and *PPARγ2*, which was significantly higher in *Stk40*-KO livers than in WT or heterozygous livers was further validated by qRT-PCR (Fig. 6C). These data indicate that Stk40 might have a general role for regulating expression of genes involved in adipogenesis not only in cultured cell *in vitro* but also in fetal organs *in vivo*.

Given that increased phosphorylation of 4E-BP1 caused by *Stk40* KO in MEFs and MSCs, we anticipated that the activation of 4E-BP1- and eIF4E-dependent translation might contribute to the altered expression of genes associated with adipogenesis and metabolism in *Stk40*-KO fetal organs. Indeed, similar to in cultured cells, phosphorylation of 4E-BP1 and S6K1 was substantially increased in *Stk40*-KO livers ($n>10$ for each genotype) (Fig. 6D,E). Moreover, the protein levels of aP2 were significantly higher in *Stk40*-KO livers than in WT or heterozygous livers (Fig. 6D,E). However, unlike in cell culture, proteins of C/EBPs were hardly detectable in livers at this stage. Collectively, the activity of protein translation machinery appeared increased in *Stk40-KO* livers, possibly leading to aberrant expression of the metabolism and adipogenesis markers. The key factor responsible for the altered gene expression in the *Stk40-KO* livers needs to be identified by further study.

DISCUSSION

In this study, we show that Stk40 is a new repressor of adipogenesis, acting through 4E-BP1- and eIF4E-mediated translational control of the key early pro-adipogenic transcription factors, particularly C/EBPβ and C/EBPδ. Several lines of experimental evidence obtained in this study support this conclusion. First, KO or KD of *Stk40* leads to increased adipogenesis in mouse MEFs, fetal liver stromal cells and MSCs. Second, protein levels of C/EBPβ and C/EBPδ substantially increase in *Stk40*-KO MEFs or *Stk40*-KD MSCs, whereas knockdown of C/EBPβ abolished the enhanced adipogenic potential of *Stk40*-KO MEFs and *Stk40*-KD MSCs. Third, levels of C/EBPβ and C/EBPδ increase through an enhancement of cap-dependent translation, rather than by transcriptional or degradation regulation. In *Stk40*-KO MEFs

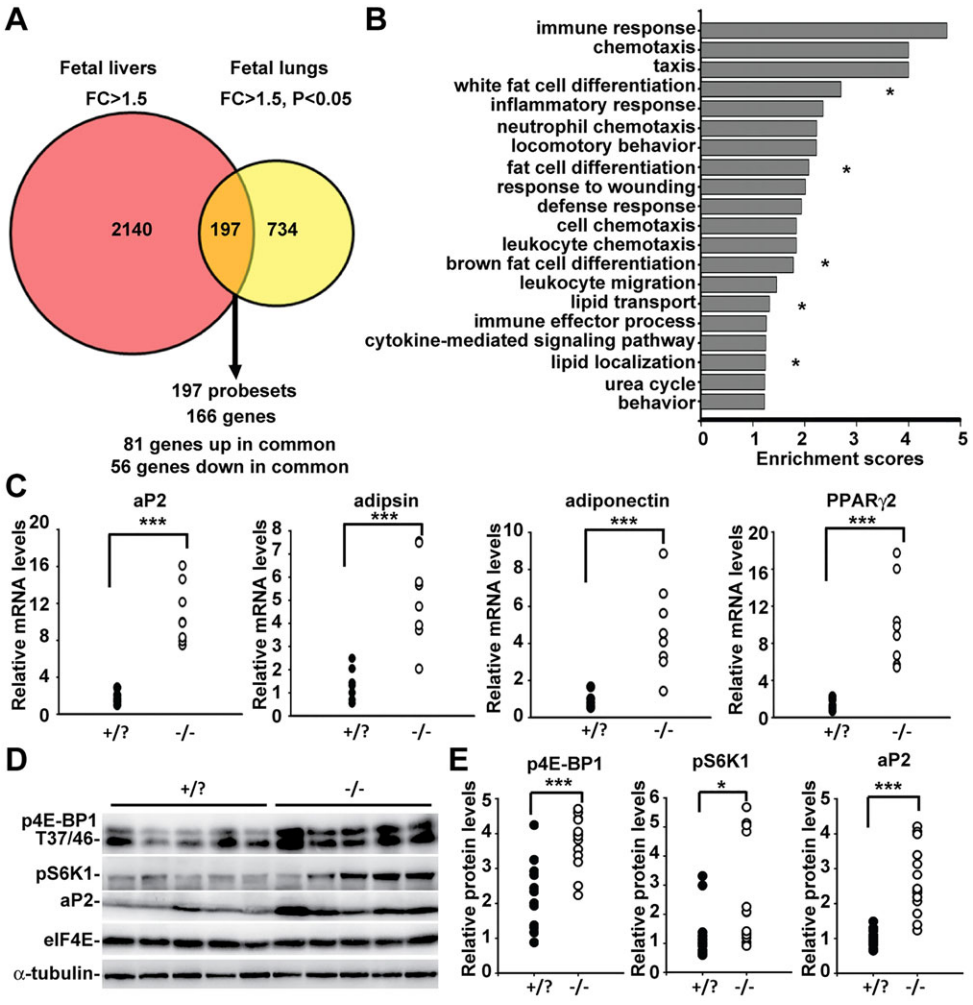

Fig. 6. Fetal organs of *Stk40*-KO mice display enhanced adipogenic gene expression and 4E-BP1 phosphorylation. (A) Venn diagram of differentially expressed genes (DEGs) in *Stk40*-KO lungs and in *Stk40*-KO livers at E18.5. FC, fold change. (B) Gene ontology (GO) analyses of DEGs from a ranking by enrichment scores. Adipogenesis- or lipid-related terms are indicated by asterisks. Enrichment scores were calculated as $-\log_{10}$ (*P*-value). (C) mRNA levels of adipogenic markers in *Stk40*-KO livers were increased. +/?, *Stk40* wild-type or heterozygous, $n=8$; –/–, *Stk40* knockout, $n=8$. Results are mean±s.d. ***$P<0.001$ (Student's *t*-test). (D) The protein levels of 4E-BP1 and S6K1 in livers at E18.5. Adipocyte marker aP2 as well as phosphorylation of 4E-BP1 (p4E-BP1) and S6K1 (pS6K1) increased in *Stk40*-KO livers. α-tubulin was used as a loading control. (E) Relative protein levels of p4E-BP1, pS6K1 and aP2 increased in *Stk40*-KO livers. +/?, *Stk40* wild type or heterozygous, $n=15$; –/–, *Stk40* knockout, $n=14$. Results are mean±s.d. *$P<0.05$; ***$P<0.001$ (Student's *t*-test).

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and *Stk40*-KD MSCs, phosphorylation of 4E-BP1 increases, releasing more eIF4E for cap-dependent translation initiation. Fourth, knockdown of *eIF4E* in MSCs decreases C/EBP protein translation. Fifth, forced expression of Stk40 abrogates the increased phosphorylation of 4E-BP1, decreases the translation of C/EBPβ and C/EBPδ, and blocks the adipogenesis. Finally, *Stk40*-KO fetal livers display an increased adipogenic program, and aberrant lipid and steroid metabolism globally. This study provides new insights into how C/EBP proteins are controlled at a translational level and reveals important function of this regulation in adipogenesis.

Interestingly, the inhibitory effect of Stk40 on adipocyte differentiation was observed in cell types with the potential for adipogenic lineage commitment, but not in specified 3T3-L1 preadipocytes. This phenomenon argues that its primary role is for the adipocyte lineage commitment. Although the expression of Stk40 declined quickly after induction of adipocyte differentiation in 3T3-L1 cells, *Stk40* KD did not promote the adipocyte differentiation program in 3T3-L1 cells. This might be explained by a low level of Stk40 or a lack of special functional context for Stk40 in 3T3-L1 cells. Thus, Stk40 might function predominantly in the adipogenic commitment of mesenchymal cells.

Stk40 can activate the Erk1/2 pathway (Li et al., 2010; Yu et al., 2013), which is known to be essential for early pro-adiogenic factor transcription, such as *C/EBPβ* and *C/EBPδ* (Wang et al., 2009). Therefore, the reduction in transcriptional levels of *C/EBPβ* and *C/EBPδ* in *Stk40*-KO and -KD cells could be attributed to the attenuated activation of Erk1/2 signaling. In spite of lower transcriptional levels of *C/EBPβ* and *C/EBPδ*, their translation was increased in *Stk40*-KO and -KD cells. Translational control of C/EBPα and C/EBPβ has previously been reported (Timchenko et al., 2002; Karagiannides et al., 2006; Kawagishi et al., 2008; Haefliger et al., 2011), although the detailed mechanism and its physiological impact on adipogenesis are not fully elucidated. Our data support the notion that Stk40 represses the translation of at least three members of the C/EBP family, including α, β and δ. Translation of C/EBPβ has been shown to be dependent on eIF4E activity, either in an isoform-selective fashion through different translation initiation sites (Lin et al., 1994; Pause et al., 1994) or in an isoform nonselective manner (Li et al., 2011). Our results suggest that Stk40 controlled translation of C/EBPβ without an isoform preference, as levels of all three C/EBPβ isoforms altered when *Stk40* was deleted. To elucidate how Stk40 modulated the C/EBP translation, we examined the activity of mTOR, a 'sensor' of the synthesis of proteins, and a protein which is essential for cell proliferation, differentiation and survival (Heitman et al., 1991; Brown et al., 1995; Khaleghpour et al., 1999; Carnevalli et al., 2010). 4E-BP1 and S6K1 are downstream targets of mTOR. Usually, mTOR activation leads to the phosphorylation of S6K1 and 4E-BP1 in order to mediate an increase in protein synthesis and cap-dependent translation, respectively (Khaleghpour et al., 1999). Deletion of *S6K1* diminishes adipogenic lineage commitment and early adipogenesis (Carnevalli et al., 2010), whereas knockout of *4E-BP1* results in a reduction in the amount of adipose tissue due to the transition of white adipocytes into brown-like adipocytes, and increased energy consumption (Tsukiyama-Kohara et al., 2001). Moreover, *4E-BP1*, *4E-BP2* double KO mice suffer from severe high-fat-diet-induced obesity and insulin resistance, which could be explained partially by increased C/EBP and PPARγ transcription (Le Bacquer et al., 2007). In addition, there are various cross-talks and feedback regulation within the mTOR cascade or between mTOR and other signaling pathways (von Manteuffel et al., 1996; Le Bacquer et al., 2007; Laplante and Sabatini, 2009). Our data indicate that Stk40 might repress 4E-BP1 phosphorylation through inhibiting a specific kinase or activating a phosphatase of 4E-BP1 independently of mTOR. Therefore, Stk40 inhibited the phosphorylation of 4E-BP1, leading to reduced activity of eIF4E and translation of C/EBPβ and C/EBPδ, thus repressing adipogenic commitment and differentiation. Currently, the factor linking Stk40 and the phosphorylation of 4E-BP1 is still missing.

Our data show that, by controlling C/EBP protein translation through the 4E-BP1–eIF4E cascade, Stk40 incorporates translational regulation of the key early pro-adipogenic factors into the chorus of the adipogenic program. Hence, the study elucidates a new function of Stk40 in adipogenesis and fetal liver metabolism. Finally, as C/EBP proteins are implicated in the function of various cell types, investigation of how Stk40 controls translation of C/EBPs will provide new insights into the differentiation of adipocytes and related diseases as well as other C/EBP-expressing cell types.

METHODS AND MATERIALS

Isolation of MEFs and fetal liver stromal cells

All animals were raised in the specific pathogen-free facility, and procedures were performed according to the guidelines approved by the Shanghai Jiao Tong University School of Medicine. Founder mice were firstly generated from embryonic stem cells of the 129 mouse strain and had been backcrossed with C57BL/6 for at least 10 generations. Genotypes of mice were determined as previously described (Yu et al., 2013). Primers for genotyping are provided in supplementary material Table S2. MEFs were generated from E14.5 mouse embryos. Fetal liver stromal cells were isolated from E14.5 livers. Single cells of the fetal liver were prepared and seeded in a semi-solid methylcellulose medium (Methocult GM3434, Stemcell) on ultra-low-attachment dishes for 1 week. After removal of hematopoietic cells, the attached fibroblast-like cells were designated as fetal liver stromal cells.

Cell culture and adipocyte differentiation

MEFs, 3T3-L1 and C3H10T1/2 cells were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum (FBS) (3T3-L1 and C3H10T1/2 cells were gifts from Guang Ning, Ruijin Hospital, Shanghai). Mouse bone marrow MSCs were grown in DMEM (low glucose) with 10% FBS, 1% sodium pyruvate, 1% NEAA and 1% L-glutamine (the bone marrow MSC line was a gift from Yufang Shi, Institute of Health Sciences, Shanghai, China).

To induce MEFs to differentiate into adipocytes, 2 days after confluence, the cells were treated with a culture medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 1 µM dexamethasone (Sigma) and 10 mg/l insulin for 96 h, and then in a maintaining medium containing 10 mg/l insulin for additional 4 days. Media were replenished every other day. 3T3-L1 cells were induced to differentiate by adding 0.5 mM IBMX, 1 µM dexamethasone and 1 mg/l insulin for 48 h and then switching to the maintaining medium with 1 mg/l insulin. C3H10T1/2 cells and fetal liver stromal cells were induced using the same procedure with 3T3-L1 cells but with an insulin concentration of 10 mg/l. Bone marrow MSCs were induced at confluence with 0.5 mM IBMX, 0.1 µM dexamethasone, 60 µM indomethasome and 10 mg/l insulin. Media were replenished every 3 days.

The presence of lipid droplets in adipocytes was verified by staining for triglycerides with Oil Red O (Sigma).

Virus package and transduction

For retrovirus and lentivirus production, viral particles were prepared and used as previously described (Yu et al., 2013). Mouse *Stk40* cDNA was cloned into pMIG vector for overexpression. The small RNA interference sequences for retroviral vector pSIREN were: control, 5′-GTGCGCTGCTGGTGCCAAC-3′; and Stk40, 5′-GGACCCATCGGATAACTAT-3′. The small RNA interference sequences for lentiviral vector pLKO.1 were: *C/EBPβ*, 5′-ACAAGCTGAGCGACGAGTACA-3′; *eIF4E-1*, 5′-GGTGGTCACTTCTGTGCAAAT-3′; and *eIF4E-2*, 5′-GCTGGAACCCTGCTATAAAGC-3′.

Protein preparation and western blotting

Total proteins in the lysis buffer (2 mM EDTA, 0.5% NP-40, 50 mM Tris-HCl 7.5, 150 mM NaCl with protease inhibitor phenylmethanesulfonyl fluoride and phosphatase inhibitors, sodium fluoride and sodium orthovanadate) were collected and quantified using the BCA kit (Pierce). Western blotting analysis was conducted by chemiluminescence (Pierce) and in at least three different experiments. Representative data are shown. The software ImageJ was used for quantitating western blots. For protein degradation assays, cells were treated with MG132 (30 μM) or chloroquine (100 μM) for 4–6 h before harvest.

Antibodies against specific antigens are provided in supplementary material Table S2.

RNA extraction and qRT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. Reverse transcription was performed with a Fastquant reverse kit (Tiangen). Quantitative real-time PCR (qRT-PCR) was carried out on ABI 7900 using the FastStart Universal SYBR Green Master (Roche). Primers used in this study are provided in supplementary material Table S2.

RNA microarray analyses

Total fetal liver RNA was isolated as described above. Each sample contained pooled RNA from six livers at E18.5. Two biological replicates for each genotype were then prepared and hybridized to the Affymatrix mouse 430 2.0 array by the Shanghai Biochip Company (SBC). Gene Ontology clustering was analyzed by online DAVID Bioinformatics Resources (Huang et al., 2009).

Dual luciferase reporter assays

For luciferase assays, activities of both Firefly and *Renilla* luciferase in cell lysates were measured using a dual-luciferase reporter assay system according to the manufacturer's recommendations (Promega). For cap-dependent translation analysis, MEFs or C3H10T1/2 cells were seeded the day before transfection at a density of 1×10^5 cells per well on 12-well plates. Using transfection reagent Xtreme HP (Roche), cells were transfected with 1 μg of the pRhcvF bicistronic vectors (a gift from Anne Willis, University of Leicester, UK) (Stoneley et al., 2000). Cells were harvested and luciferase activities were measured 48 h later. Cap-dependent translation levels in cells were calculated by normalizing *Renilla* luciferase levels to those of Firefly luciferase (human hepatitis C virus IRES-directed).

Statistical analysis

All results were analyzed with SigmaPlot version 10.0. Data are presented as the mean±s.d. Two-tailed Student's *t*-tests were used to compare the differences between two groups with at least three independent experiments or samples. Significance is indicated as $*P<0.05$; $**P<0.01$; $***P<0.001$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conception, design and analysis was performed by H.Y., K.H. and Y.J. Experiments were performed by H.Y., K.H., L.W., J.H., J.G., C.Z., R.L., J.G. Manuscript writing was undertaken by H.Y. and Y.J.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.170282/-/DC1

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