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

Hongyao Yu†, Ke He†, Lina Wang1, Jing Hu1, Junjie Gu1, Chenlin Zhou2, Rui Lu2#, Ying Jin1,2*

1Laboratory of Molecular Developmental Biology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China
2Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China

†Hongyao Yu and Ke He contributed equally to this study.
#Current address: UNC Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA

*To whom correspondence should be addressed:
Ying Jin, M.D., Ph.D.
Laboratory of Molecular Developmental Biology
Shanghai Jiao Tong University School of Medicine
280 South Chongqing Road
Shanghai 200025, China
Abstract
A better understanding of molecular regulation in adipogenesis may help develop efficient strategies to cope with obesity-related diseases. Here, we report CCAAT/enhancer binding protein (C/EBP) β and δ, two critical 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/KD cells. Functionally, knockdown of C/EBPβ eliminates the enhanced adipogenic differentiation in Stk40-KO/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/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.

Keywords: 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 serve as an endocrine organ (Shepherd et al., 1993). Aberrant adipogenesis is closely associated with obesity, which may 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 TGFβ/BMPs (Choy et al., 2000; Tang et al., 2004; Bowers et al., 2006), Wnt (Ross et al., 2000; Kang et al., 2007), MAPK (Aouadi et al., 2006; Kim et al., 2007; Wang et al., 2009), Shh (Spinella-Jaegle et al., 2001; Suh et al., 2006), Insulin and IGFs (Smith et al., 1988; Baudry et al., 2006), as well as transcription factors such as 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 gamma (PPARγ) (Tontonoz et al., 1994; Kawai and Rosen, 2010). The latter two master factors then form a self-reinforce loop and activate the adipogenic program. C/EBPβ−/− mice have reduced adiposity and C/EBPβ−/− MEFs display impaired adipogenesis (Tang et al., 2003), while C/EBPβ/C/EBPδ double knockout mice have a further decline in brown adipose tissue and epidydimal 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 the 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 subjected to a particular translational control. Firstly, both C/EBPα and C/EBPβ generate multiple isoforms due to the differential usage of in-frame initiation codons (Calkhoven et al., 2000). C/EBPα has two isoforms, p42 (full length) and p30 (truncation), while C/EBPβ has three isoforms, LAP* (full length, 36 kDa), LAP (34 kDa) and LIP (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), 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 subjected to a translational control as similar as C/EBPβ and C/EBPα has not been answered.

Stk40, a putative serine/threonine kinase, was originally identified as an activator of MAPK/Erk1/2 signaling required for primitive endoderm differentiation from mouse embryonic stem cells, and later found 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 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\(^{+/−}\) (KO) MEFs were cultured post-conflually without induction, adipocytes containing cytoplasmic accumulation of lipid droplets indicated by Oil Red O (O.R.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 the hormonal cocktails (M, 3-isobutyl-1-methylxanthine; D, dexamethasone; I, insulin), KO MEFs exhibited substantially enhanced adipocyte differentiation compared to WT cells (Fig. 1A). At the molecular levels, we analyzed the expression of adipocyte markers, including aP2, adipin, adiponectin (adipoQ) and transcription factors (C/EBP\(\alpha\) and PPAR\(\gamma\)) by RT-qPCR assays. Stk40 KO MEF cells expressed significantly higher levels of all these markers than WT cells post induction (Fig. 1B). To test whether there would be enhanced adipocyte differentiation from other cell types of Stk40 KO mice, we isolated stromal cells from E14.5 fetal livers, which contained fibroblasts, preadipocytes and 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 O.R.O staining and RT-qPCR analyses (Fig 1D, E and see Fig. S1A in supplementary material), 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.
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 functioned, 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 could take place in the former two cell types, while 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 evidently during BMP4-induced adipogenic commitment in C3H10T1/2 cells (Fig. 2E), implicating that down-regulation 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 could not enhance the adipogenic program in 3T3-L1 preadipocytes (Fig. 2H). Based on these data, we propose that Stk40 may 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 Stk40 depletion-caused adipogenesis

To explore the mechanism through which Stk40 KO/KD promoted the 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 revert 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 MAPK/Erk1/2 and PI3K/Akt signaling were activated by MDI induction, but consistently attenuated in KO MEFs compared to WT MEFs (See Fig. S1B in supplementary material), 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 adipogenic signals of MAPK/Erk1/2 and PI3K/Akt.

Between C/EBPβ and C/EBPδ, C/EBPβ is more potent for the induction of lineage commitment and differentiation, while 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 via specific KD of C/EBPβ. Silencing of C/EBPβ efficiently abrogated the enhanced adipocyte differentiation in Stk40 KO MEFs (Fig. 3C, D), suggesting an essential role of C/EBPβ in Stk40 KO-caused 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 and see Fig. S2A in supplementary material).
However, C/EBPβ KD abrogated the increased adipocyte differentiation in Stk40 KD C3H10T1/2 cells efficiently (Fig. 3F-H). Interestingly, we noticed that elevated levels of Stk40 transcripts and proteins in C/EBPβ KD cells, hints 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/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 at early hours after MDI induction, opposite to their protein levels (Fig. 4A, 4B). Similarly, the mRNA level of C/EBPβ was also lower in Stk40 KD BM MSCs than in control cells (See Fig. S2B in supplementary material). Consistently, the mRNA levels of C/EBPβ and C/EBPδ were lower in Stk40 KD C3H10T1/2 cells than in control cells as well (See Fig. 2C in supplementary material). 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 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 and see Fig. S2D in supplementary material). 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 levels of the C/EBPs were 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 of a protein synthesis inhibitor, Cyclohexamide.
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/KD cells might result from the enhanced protein synthesis, rather than changes in their transcription or protein degradation.

An increased eIF4E mediated translation of C/EBPβ may account for the enhanced adipogenesis in Stk40-deficient cells

Protein synthesis is subjected to multifaceted controls. As microRNAs were reported to regulate the 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 (See Table S1 in supplementary material). We then turned our attention to RNA binding proteins, including calreticulin and protein disulfide isomerase family A, member 3, which were previously reported to regulate C/EBPα/β 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 (See Fig. S3A in supplementary material).

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 5’-cap structure, while 4E-BP1 dissociates from eIF4E upon hyper-phosphorylation, in turn facilitating the translation. The major signaling 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-/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/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 (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 to mention that overexpression of Stk40 in Stk40 KO/KD cells reverted 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 independent 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 activation, while phosphorylation of 4E-BP1 in Stk40 KO MEFs retained increased with all rapamycin dosages tested (See Fig. S3B in supplementary material). These results favor a notion that Stk40 may repress phosphorylation of 4E-BP1 independent 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 for adipogenesis. To surmount this problem, we looked at global gene expression profiling between Stk40 KO and WT fetal livers at E18.5. Two thousand and one hundred forty 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 starts the hepatic metabolism at this stage. The Gene Ontology (GO) analyses of differentially expressed genes (DEGs) in livers enriched genes associated with oxidation/reduction, immune/inflammatory response and a large proportion of metabolic process of steroid, glucose, hexose, monosaccharide and cholesterol (See Fig. S4 in supplementary material). 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) shared by the liver and lung, 81 genes up-regulated and 56 genes down-regulated in both organs (Fig. 6A). These genes might reflect the general physiological impact of Stk40 on cellular functions independent of its specific role in particular organs. GO analyses of the common DEGs revealed that a large proportion of genes was involved in white/brown fat cell differentiation, lipid transport and lipid localization (Fig. 6B, indicated by asterisk *).

The expression of several important genes participating in adipogenesis, like aP2, adipisin, adiponectin and PPARγ2, which was significantly higher in Stk40 KO livers than in WT/Het livers was further validated by RT-qPCR (Fig. 6C). These data indicate that Stk40 may 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/eIF4E-dependent translation might contribute to the altered expression of genes associated with adipogenesis and metabolism in Stk40 KO fetal organs. Indeed, similar to 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/Het 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 Stk40 is a novel repressor of adipogenesis, acting through 4E-BP1/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 the conclusion: 1) KO or KD of Stk40 leads to increased adipogenesis in mouse MEFs, fetal liver stromal cells, and MSCs; 2) Protein levels of C/EBPβ and C/EBPδ substantially increase in Stk40-KO MEFs or Stk40-KD MSCs. Knockdown of C/EBPβ abolished the enhanced adipogenic potential of Stk40-KO MEFs and Stk40-KD MSCs; 3) Levels of C/EBPβ and C/EBPδ increase by enhanced cap-dependent translation, rather than by transcriptional or degradation regulation. In Stk40-KO MEFs and Stk40-KD MSCs, phosphorylation of 4E-BP1 increases, releasing more eIF4E for cap-dependent translation initiation; 4) Knockdown of eIF4E in MSCs decreases C/EBP protein translation; 5) Forced expression of Stk40 abrogates the increased phosphorylation of 4E-BP1, decreases the translation of C/EBPβ and C/EBPδ, and blocks the adipogenesis; 6) Stk40 KO fetal livers display an increased adipogenic program and aberrant lipid/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 potential of adipogenic lineage commitment, but not in specified 3T3-L1 preadipocytes. This phenomenon argues for its primary role 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 low level of Stk40 or a lack of special functional context for Stk40 in 3T3-L1 cells. Thus, Stk40 may function predominantly in the adipogenic commitment of mesenchymal cells.

Stk40 can activate the MAPK/Erk1/2 pathway (Li et al., 2010; Yu et al., 2013), which is known essential for early pro-adipogenic 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/KD cells could be attributed to the attenuated activation of MAPK/Erk1/2 signaling. In spite of lower transcriptional levels of C/EBPβ and C/EBPδ, their translation was increased in Stk40 KO/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 dependent on the eIF4E activity, either in an isoform selective fashion via 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’ to the synthesis of proteins and 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 down-stream targets of mTOR. Usually, mTOR activation leads to the phosphorylation of S6K1 and 4E-BP1 to increase 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), while knockout of 4E-BP1 results in a reduction of the adipose tissue due to brown-like transition of white adipocytes and increased energy consumption (Tsukiyama-Kohara et al., 2001). Moreover, 4E-BP1/4E-BP2 double KO mice suffered 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 signalings (von Manteuffel et al., 1996; Le Bacquer et al., 2007; Laplante and Sabatini, 2009). Our data indicate that Stk40 may repress
4E-BP1 phosphorylation through inhibiting a specific kinase or activating a phosphatase of 4E-BP1 independent 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 novel 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 were provided (See Table S2 in supplementary material). 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 one 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 (Bone marrow MSC line was a gift from Yufang Shi, Institute of Health Sciences, Shanghai).

To induce MEFs to differentiate into adipocytes, two days after confluence, the cells were treated with a culture medium containing 0.5 mM IBMX (3-isobutyl-1-methylxanthine, Sigma), 1 μM dexamethasone (Sigma), 10 mg/L insulin for 96 hours, 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 hours and then switching to the maintaining medium with 1 mg/L insulin. C3H10T1/2 cells and fetal liver stromal cells were induced in 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 three days.

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

**Virus package and transduction**

For retro/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: Control, 5’-GTGCGCTGCTGTGCAAC-3’; Stk40, 5’-GGACCCATCGGATAACTAT-3’. The small RNA interference sequences for lentiviral vector pLKO.1: C/EBPβ, 5’-ACAAGCTGAGCGAGTACA-3’; eIF4E-1, 5’-GGTGTTCACTTCTGTGCAAAT-3’; 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 7.5, 150 mM NaCl) with protease inhibitor phenylmethanesulfonyl fluoride and phosphatase inhibitors, sodium fluoride and sodium orthovanadate) were collected and quantified by 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 hours before harvest.

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

**RNA extraction and RT-qPCR**

Total RNA was extracted using the TRIzol reagent (Invitrogen) in accordance to the
manufacturer’s instructions. Reverse transcription was performed with Fastquant reverse kit (Tiangen). Quantitative real-time PCR (RT-qPCR) was carried out on ABI 7900 using the FastStart Universal SYBR Green Master (Roche). Primers used in this study are provided in Supplementary Table 2.

**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 da 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 $1 \times 10^5$ 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)(Stoneley et al., 2000). Cells were harvested and luciferase activities were measured 48 hours later. Cap-dependent translation levels in cells were calculated by normalizing Renilla luciferase levels to Firefly luciferase (human hepatitis C virus IRES-directed) levels.

**Statistical analysis**

All results were analyzed with SigmaPlot version 10.0. Data are presented as the mean ± s.d.. Two-tailed Student’s $t$ test was used to compare the differences between two groups with at least three independent experiments or samples. *, p<0.05; **, p<0.01; ***, p<0.001.
**Author contribution**

Conception, design and analysis: HY, KH and YJ. Experiment performance: HY, KH, LW, JH, JG, CZ, RL, JG. Manuscript writing: HY and YJ.

**Competing interests**

The authors declare that they have no conflict of interest.

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Fig. 1. *Stk40* knockout enhances adipocyte differentiation of mouse embryonic fibroblasts (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 + Dexamethasone; MDI, MD + insulin. The cells of each genotype were pooled from at least 10 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. Error bars represent s.d.; Student's *t*-test. **, *p*<0.01; ***, *p*<0.001.

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 after 8 days of differentiation. Error bars represent s.d.; Student's t-test. **, p<0.01; ***, p<0.001.
Fig. 2. *Stk40* knockdown enhances adipocyte differentiation in mouse bone marrow mesenchymal stem cells (BM MSC) and C3H10T1/2 cells

A, Knockdown of *Stk40* in BM MSCs. CtrlI, control shRNA; Stk40i, *Stk40* shRNA. α-tubulin was used as a loading control. Protein molecular weight markers are indicated at the right.

B, Oil Red O staining of BM MSCs after 9 days of differentiation. Scale bars, 50
C. mRNA levels of adipogenic markers were increased in \textit{Stk40} KD BM MSCs at indicated time points. Error bars represent s.d.; Student's $t$-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

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 \textit{Stk40} in control or \textit{Stk40} KD C3H10T1/2 cells treated with or without BMP4 treatment after 8 days of differentiation. Error bars represent s.d.; Student's $t$-test. ***, $p<0.001$.

F. mRNA levels of adipogenic markers were increased in \textit{Stk40} KD C3H10T1/2 cells after 8 days of differentiation. Error bars represent s.d.; Student's $t$-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

G. \textit{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 \textit{Stk40} and adipogenic markers in 3T3-L1 cells at indicated time points. Error bars represent s.d..
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 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 + Dexamathasone + Insulin. α-tubulin was used as a loading control. Protein molecular weight markers are indicated at 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
shRNA. 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 (C/EBPβi) abrogated increased adipocyte differentiation in Stk40 KO MEFs. -/-, Stk40 knockout; Ctrl, 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. Ctrl, control shRNA; Stk40i, Stk40 shRNA.

F, Knockdown of C/EBPβ in C3H10T1/2 cells recovered Stk40 protein. N.S, non-specific band recognized by Stk40 antibody as a sample loading control. Ctrl, 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. Ctrl, control shRNA; Stk40i, Stk40 shRNA; C/EBPβi, C/EBPβ shRNA. Error bars represent s.d.; Student's t-test. *, p<0.05; **, p<0.01; ***, p<0.001.

H, Oil Red O staining of C3H10T1/2 cells treated as in G after 8 days of differentiation. Ctrl, control shRNA; Stk40i, Stk40 shRNA; C/EBPβi, C/EBPβ shRNA.
Fig. 4. C/EBPβ and C/EBPδ are post-transcriptional 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 indicated time points after MDI induction. Error bars represent s.d.; Student's t-test. *, p<0.05; **, p<0.01; ***, p<0.001.

B, Protein levels of C/EBPβ and C/EBPδ increased in Stk40 KO MEFs at indicated time points after MDI induction. LAP*, full length active C/EBPβ, 36 kDa; LAP, active C/EBPδ, 34 kDa; +/+, Stk40 wild type; -/-, Stk40 knockout; MDI, IBMX + Dexmthasone + Insulin. α-tubulin was used as a loading control. Protein molecular weight markers are indicated at the right.

C, C/EBPβ and C/EBPδ were degraded through 26S proteasome pathway. MEFs were treated with 30 μM MG132 for 4 hours 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 in D. The gray density of blots was measured by software ImageJ. The levels of C/EBPβ and C/EBPδ at 0.5 hour after cyclohexamide treatment were set as 100. LAP*, full length active C/EBPβ, 36 kDa; LAP, active C/EBPβ, 34 kDa. Error bars represent s.d.
Fig. 5. Cap-dependent translation of C/EBPs is enhanced in Stk40 KO/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 C3H10T1/2 cells. 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
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/firefly luciferase system with the human hepatitis C virus IRES driving firefly 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/firefly luciferase plasmid were indicated in schema (upper). Error bars represent s.d.; Student's t-test. *, p<0.05; ***, p<0.001.

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.
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. Error bars represent s.d.; Student's t-test. ***, p<0.001.

D, The protein levels of 4E-BP1 and S6K1 in livers at E18.5. Adipocyte marker aP2 as well as phosphorylation of 4E-BP1 and S6K1 increased in Stk40 KO livers. α-tubulin was used as a loading control.
E, Relative protein levels of phospho-4E-BP1, phospho-S6K1 and aP2 increased in Stk40 KO livers. +/-, Stk40 wild type or heterozygous, n=15; -/-, Stk40 knockout, n=14. Error bars represent s.d.; Student’s t-test. *, p<0.05; ***, p<0.001.