THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 18, pp. 12901–12909, May 3, 2013

Mammalian Target of Rapamycin Complex 1 (mTORC1)-mediated Phosphorylation Stabilizes ISCU Protein

*IMPLICATIONS FOR IRON METABOLISM**

Received for publication, October 1, 2012, and in revised form, March 4, 2013 Published, JBC Papers in Press, March 18, 2013, DOI 10.1074/jbc.M112.424499

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Background: ISCU facilitates assembly of iron-sulfur clusters (ISCs), the essential metabolic cofactors.
Results: mTORC1, a nutrient-sensing kinase, phosphorylates and stabilizes ISCU protein.
Conclusion: Through mTORC1, metabolic status affects ISC biogenesis.
Significance: This report is the first to show that ISC assembly is a part of mTORC1-modulated anabolism, and via mTORC1, coordinates with other biosynthetic processes to ensure cell growth and survival.

The scaffold protein ISCU facilitates the assembly of iron-sulfur clusters (ISCs), which are essential cofactors for many vital metabolic processes. The mTOR pathways are central to nutrient and energy-sensing networks. Here, we demonstrate that mTORC1 associates with ISCU and phosphorylates ISCU at serine 14. This phosphorylation stabilized ISCU protein. Insufficiency of ISCU triggered by mTORC1 inhibition prevented ISC assembly. Sustained ISCU protein levels enhanced by mTORC1 sensitized TSC2-null cells to iron deprivation due to constitutive ISC biogenesis-triggered iron demand, which outstrips supply. We conclude that the mTORC1 pathway serves to modulate iron metabolism and homeostasis, and we speculate that iron deprivation may be an adjunct in the treatment of cancers characterized by constitutive mTORC1 activation.

The scaffold protein ISCU, encoded by the *ISCU* gene, facilitates ISC[3] assembly (1). It is synthesized as a precursor in the cytosol and migrates to the mitochondria where it becomes the mature form after a two-step mitochondria target sequence (MTS) cleavage (2). The ISCs act as electron donors or receivers in many metabolic processes, including the electron transport chain and the TCA cycle. In humans, mutations of *ISCU* decrease its expression and ultimately the activities of muscle aconitase and succinate dehydrogenase, for which ISCs serve as essential cofactors (3). In mice, deletion of *ISCU* is embryonic lethal (4). It has been reported that *ISCU* gene expression is regulated by hypoxia as well as iron depletion (5–7). Despite what we know about ISCU protein, how *ISCU* gene expression and function are regulated remains enigmatic, in particular, the role of cellular metabolic status on its regulation is unclear.

The mammalian target of rapamycin, mTOR, a serine-threonine kinase, plays a central role in nutrient-sensing networks ensuring cell survival and growth. It can form two distinct complexes with other protein partners, mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2) (8). The latter is rapamycin-insensitive, modulates the activity of serum- and glucocorticoid-induced kinase, and contributes to the activation of the Akt pathway (8). The former is sensitive to rapamycin and responds to amino acids, growth factors, or ADP/ATP levels via the tuberous sclerosis complex (TSC1/TSC2). Upon nutrient starvation, TSC1/TSC2 inhibits mTORC1 activity, which activates autophagy that is adversely regulated by mTORC1, to produce energy and nutrients for survival. However, with nutrient abundance, the TSC1/TSC2 complex loses this inhibitory effect; therefore, mTORC1 stimulates cell growth and proliferation, particularly increasing biosynthesis, including protein translation by phosphorylating S6 kinase and eukaryotic translation initiation factor 4E binding proteins (4E-BP1 and 4E-BP2) (8) as well as lipogenesis via sterol regulatory element-binding proteins (9). Therefore, mTORC1 is crucial in balancing catabolism and anabolism, yet the known targets for mTORC1 are few.

Here, we establish a novel role of mTORC1 by demonstrating that mTORC1 phosphorylates ISCU at serine 14 (Ser-14), thus stabilizing ISCU protein in the cytosol and ultimately increasing its abundance in the mitochondria and function in ISC assembly. Furthermore, we demonstrate that unrestrained mTORC1-mediated stabilization of ISCU protein sensitizes cells to iron deprivation, due to constitutive ISC biogenesis-triggered iron demand that outstrips supply.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines used were human embryonic kidney cell line 293T, human cervical cancer cell line HeLa, normal mouse lung cell line MLg (10), mouse preadipocyte 3T3-L1, as

* This work was supported by National Institutes of Health Grant R01-HL058752 (to P. A. D.).
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[3] The abbreviations used are: ISC, iron-sulfur cluster; mTORC1, mammalian target of rapamycin complex 1; MTS, mitochondria target sequence; DFO, deferoxamine; MEF, mouse embryonic fibroblast.

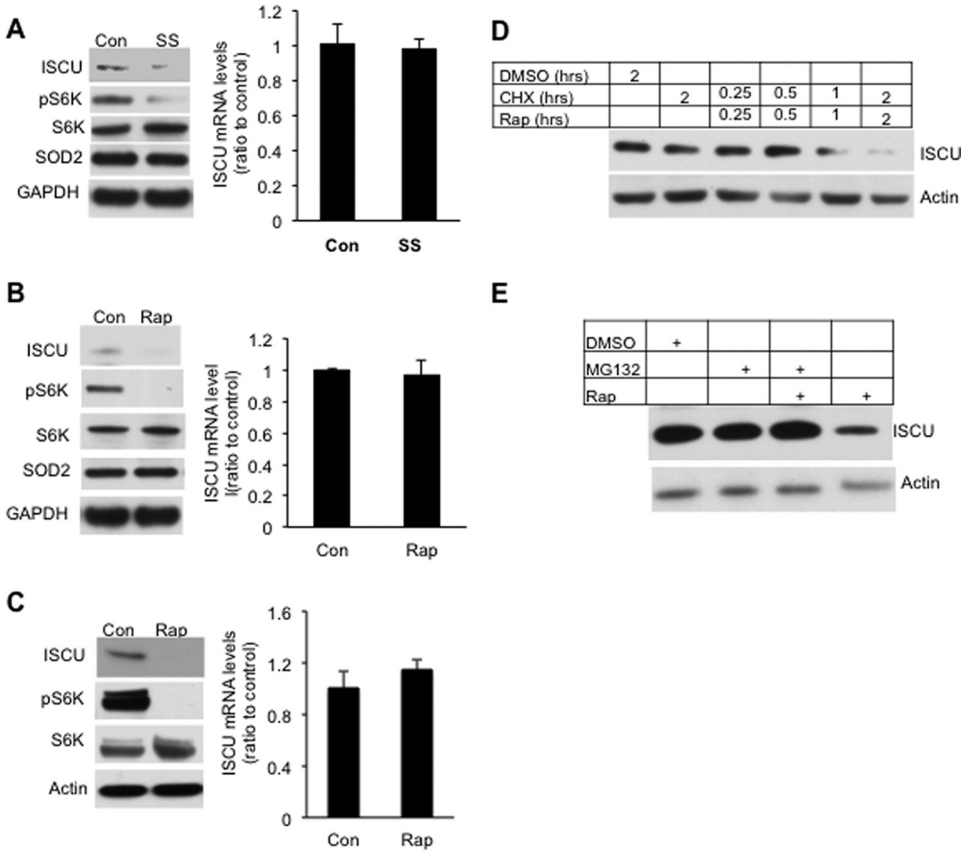

FIGURE 1. **mTORC1 inhibition destabilizes ISCU protein.** *A*, 3T3-L1 cells control (*Con*) or serum-starved overnight (*SS*) were analyzed by Western blot and Taqman RT-PCR assays. *B*, 3T3-L1 cells were incubated with 200 nM rapamycin (*Rapa*) or dimethyl sulfoxide (*DMSO*; *Con*) in complete medium for 4 h and further analyzed by Western blot and Taqman RT-PCR assays. *C*, MLg cells, incubated with 200 nM rapamycin in complete medium for 7 h, were analyzed by Western blot and Taqman RT-PCR assays. *D*, MLg cells were treated with 20 μM rapamycin and/or 25 μM cycloheximide (*CHX*) for 2 h. The resultant cell pellets were subjected to Western blot analysis. *E*, MLg cells, pretreated with or without 10 μM MG132 for 0.5 h, were further co-incubated with 200 nM rapamycin or dimethyl sulfoxide for 4 h. The resultant cell pellets were analyzed by Western blot.

well as $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs (11, 12). All cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), under 5% CO_2 and at 37 °C. For drug treatment, rapamycin, MG132 and cycloheximide were purchased from LC Laboratories. Transfection reagents Lipofectamine 2000 (Invitrogen) were applied per the manufacturer's instructions. For evaluating viable cells, crystal violet staining was performed following the standard protocols; total and live cells were counted with Mini automated cell counter (ORFLO).

Antibodies—Anti-ISCU, anti-actin (Santa Cruz Biotechnology), anti-FLAG, anti-FLAG M2 affinity gel (Sigma), anti-phosphoserine (Enzo Life Sciences, Inc.), anti-S6 kinase, anti-pS6 kinase, anti-mTOR (Cell Signaling Technology, Inc.), anti-Raptor, anti-tubulin, and anti-GAPDH (Millipore) antibodies were used.

Protein Function Assays—Mitochondrial and cytosolic fractions were isolated with a commercially available isolation kit (Pierce). Immunoprecipitation and *in vitro* kinase assays were as described previously (13, 14). Aconitase activity was measured using an Aconitase assay kit (BioVision Corp.).

Quantitative Real-time PCR (Taqman RT-PCR)—Using the Taqman Gene expression assay (Applied Biosystems), ISCU mRNA levels were analyzed and further normalized to GAPDH mRNA levels.

Plasmid Construction—To express ISCU, human ISCU cDNA was cloned in p3XFLAG-CMV-14 (Sigma) and further used for generating S14A, S20A, S29A, or cMTS-ISCU-FLAG mutant by the site-directed mutagenesis method. For expressing 4E-BP1, human 4E-BP1 cDNA was cloned in p3XFLAG-CMV-14. shRNA lentiviral clones for ISCU or Raptor were purchased and applied per instructions from Thermo Fisher Scientific.

Statistical Analysis—All data represent the mean ± S.D. of three experiments. Data were analyzed by standard Student's *t* test. Statistical significance was considered at $p < 0.05$.

RESULTS

mTORC1 Inhibition Destabilizes ISCU Protein—To determine whether *ISCU* expression was modulated by nutrient limitation, mouse preadipocyte 3T3-L1 cells were serum-starved. In human, another isoform, ISCU1, is also derived from the *ISCU* gene (7). So far, only ISCU (aka ISCU2 in some publications) has been identified in mice. Thus, as shown in Fig. 1*A*, the protein bands, immunoreactive with anti-ISCU antibody, represented murine ISCU (4). As demonstrated in Fig. 1*A*, ISCU protein levels decreased by serum starvation, whereas

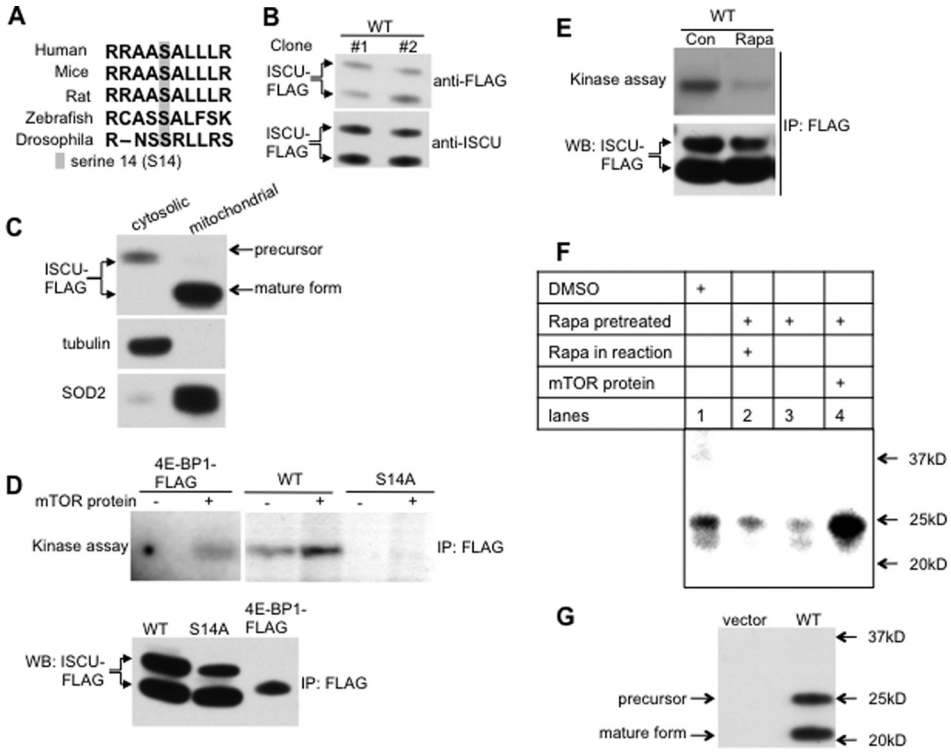

FIGURE 2. **mTORC1 phosphorylates ISCU protein at serine 14.** *A*, sequence alignment of the putative mTOR phosphorylation site in ISCU was derived by the Cluster W method. *B*, 293T cells were transfected with plasmids expressing ISCU-FLAG WT. In 30 h post-transfection, the cell pellets were analyzed by Western blot with respective antibodies as indicated. *C*, as stated in *B*, 293T cells expressing ISCU-FLAG WT were subjected to the isolation of mitochondrial and cytosolic fractions and further analyzed by Western blot. SOD2 and tubulin served as the markers for mitochondrial and cytosolic fractions, respectively. *D*, 293T cells were transfected with plasmids expressing ISCU-FLAG WT, S14A mutant, or the positive control 4E-BP1-FLAG. After 20 h post-transfection, immunoprecipitation (*IP*) with anti-FLAG M2 beads was performed and followed by an *in vitro* kinase assay with or without recombinant mTOR protein (*upper panel*). To monitor the amount of proteins used, the immunopurified proteins were also probed with anti-FLAG antibody by Western blot (*WB*) (*lower panel*). *E*, as in D, 293T cells expressing WT were pretreated with 200 nM rapamycin (*Rapa*) for 1 h in complete medium followed by immunoprecipitation and kinase assays with additional 200 nM rapamycin (*upper panel*). Immunopurified proteins were also analyzed by Western blot (*lower panel*). *F* and *G*, as described in *D* and *E*, 293T cells, expressing ISCU-FLAG WT was analyzed in an *in vitro* kinase assay (*F*), whereas as a side-by-side comparison, a proportion of the immunoprecipitated protein complex used for *lane 1* in *F* was analyzed by Western blot with anti-FLAG antibodies (*G*). 293T cells transfected with vectors served as a control (*G*). *DMSO*, dimethyl sulfoxide.

mRNA levels remained unchanged, suggesting ISCU protein was post-transcriptionally regulated. Because the mTORC1 pathway typically affects gene expression at the post-transcriptional level and is modulated by nutrient availability as well as growth factors, we tested whether mTORC1 affected ISCU levels. Indeed, incubation with rapamycin, an allosteric mTORC1 inhibitor, decreased ISCU protein content but not mRNA levels in 3T3-L1 (Fig. 1*B*). In contrast, the amount of another mitochondrial protein SOD2 remained unchanged, indicating specificity. Phosphorylated ribosomal protein S6 kinase was also reduced, confirming inhibition of mTORC1 activity (Fig. 1). Similar effects of rapamycin on ISCU protein were also observed in MLg mouse lung fibroblasts cells (Fig. 1*C*), thereby excluding a 3T3-L1 cell-specific cause. To test whether this effect occurred at the translational or post-translational level, MLg cells were treated with rapamycin and/or protein translation inhibitor cycloheximide. Shown in Fig. 1*D*, despite inhibition of *de novo* protein synthesis by cycloheximide, rapamycin incubation steadily decreased ISCU protein levels, implying that a post-translational process was involved. Furthermore, co-incubation with MG132, a proteasome inhibitor, blocked the rapamycin-mediated decrease in ISCU protein (Fig. 1*E*), thus suggesting that mTORC1 inhibition destabilizes ISCU protein.

mTOR Phosphorylates ISCU Protein at Serine 14—mTORC1 primarily activates protein translation (8) and also phosphorylates proteins, subsequently altering protein stability (13, 14). Thus, we tested whether mTOR phosphorylated ISCU protein. Ser-14 of ISCU is well conserved among different species (Fig. 2*A*), and its phosphorylation was confirmed by two phosphoproteomic studies (15, 16). Typical mTOR phosphorylation sites are bulky hydrophobic motifs or proline-directed sites. However, atypical mTOR targeting sites are also found in insulin receptor substrate 1 (17), proline-rich Akt/PKB substrate 40 kDa, and repressor of RNA polymerase III transcription MAF1 homolog (18, 19). In these examples, mTORC1-targeting sites are either flanked by small hydrophobic or non-hydrophobic residues. Therefore, we targeted Ser-14 located in the MTS of ISCU and surrounded by hydrophobic amino acids as a potential mTOR phosphorylation site (Fig. 2*A*) and mutated Ser-14 to an alanine (S14A) in human ISCU cDNA fused with FLAG epitope (ISCU-FLAG, WT). Consistent with what has been reported previously (2), the expression of ISCU-FLAG WT resulted in two immunoreactive bands with anti-ISCU or anti-FLAG antibodies (Fig. 2*B*), in which the slower migrating band represented the precursor form in the cytosol, whereas the faster migrating mature form was in the mitochondria (Fig. 2*C*). Furthermore, after immunopurification with anti-FLAG M2

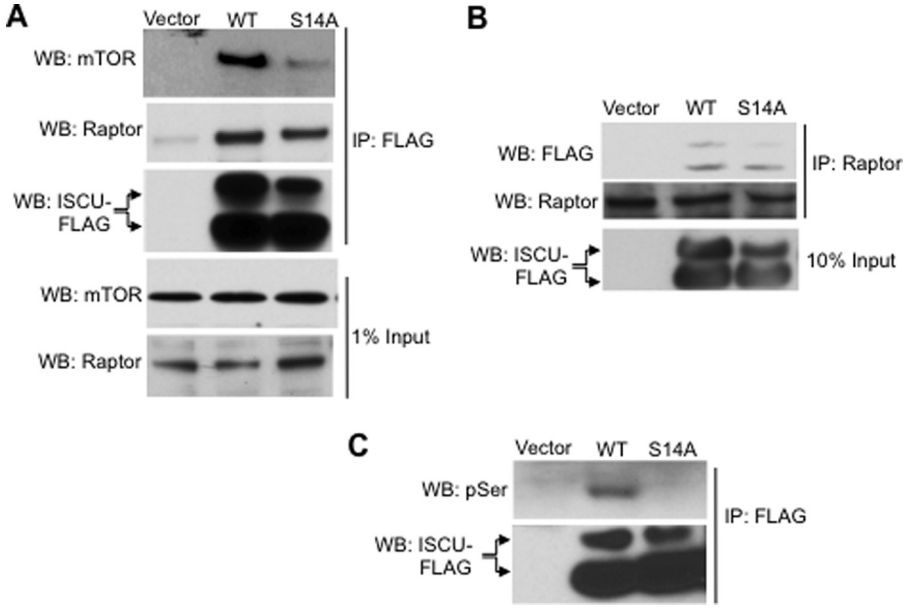

FIGURE 3. **mTORC1 associates with ISCU protein.** *A*, immunoprecipitation (*IP*) with anti-FLAG M2 beads was performed with 293T cells transfected with control vectors or plasmids expressing WT or S14A mutant. Immunopurified protein complexes were probed with anti-mTOR, anti-Raptor, or as an immunoprecipitation quality control, anti-FLAG antibodies. *B*, as described in *A*, the same cells were used for immunoprecipitation with anti-Raptor antibody followed by Western blot (*WB*) analysis. *C*, as stated in *A*, immunopurified ISCU-FLAG WT or S14A mutant were analyzed by Western blot with anti-phosphorylated serine (pSer) or anti-FLAG antibodies. The relative amount of WT, S14A, mTOR, or Raptor protein in the immunoprecipitation reactions was examined by Western blot with the *input*.

beads, the potential phosphorylation of these proteins was evaluated by an *in vitro* kinase assay. Shown in Fig. 2*D*, phosphorylated bands appeared in immunopurified ISCU-FLAG WT and positive control 4E-BP1-FLAG with addition of recombinant mTOR protein. Strikingly, ISCU-FLAG WT was even phosphorylated without mTOR protein, although to a lesser extent (Fig. 2*D*). In contrast, the S14A mutant did not yield any phosphorylated bands, even in the presence of mTOR protein (Fig. 2*D*).

Because ISCU protein has not been reported to have any kinase activity and immunopurified ISCU-FLAG WT was phosphorylated in the absence of recombinant mTOR protein (Fig. 2*D*), we suspected that a kinase associated with immunopurified ISCU-FLAG and also phosphorylated it. To test whether this kinase was mTOR, 293T cells expressing ISCU-FLAG WT were pre-incubated with rapamycin and rapamycin was also added to the subsequent immunopurification and to the kinase activity assays. Because rapamycin acts as an inhibitor via interaction with FK506 binding protein 12, which further binds mTOR and inhibits the kinase activity in mTORC1 (20), rapamycin presumably would lessen the phosphorylation signal if mTORC1 was involved. Shown in the *upper panel* of Fig. 2*E*, rapamycin attenuated phosphorylation of WT, whereas it did not affect total protein levels (Fig. 2*E*, *lower panel*), suggesting that the endogenous kinase phosphorylating ISCU-FLAG WT was mTOR.

ISCU is a nuclear gene-encoded mitochondrial protein. It is seen as a newly translated and full-length precursor form in the cytosol as well as a mitochondrial form with the MTS cleaved off (Fig. 2*B*). Considering that mTORC1 functions as a kinase in the cytosol and Ser-14 only exists in the MTS of ISCU, we wanted to further confirm that the phosphorylation of ISCU indeed occurred in the cytosolic precursor form. Thus, an *in vitro* kinase assay and Western blot were performed in parallel. As we expected, the phosphorylated band, whose intensity was enhanced by the additional mTOR protein and weakened by rapamycin incubation (Fig. 2*F*), migrated at the same speed as the precursor form in the Western blot (Fig. 2*G*). These data confirmed that mTORC1 phosphorylates Ser-14 of ISCU precursor that exists in cytosol.

ISCU Protein Associates with mTORC1—As shown in Fig. 2, mTORC1 associated with ISCU-FLAG, which causes the phosphorylation of immunopurified ISCU-FLAG WT. To further explore this possibility, immunoprecipitation assays with 293T cells expressing ISCU-FLAG WT and S14A was performed. Shown in Fig. 3*A*, endogenous mTOR and the protein Raptor, a specific subunit of mTORC1, were coimmunoprecipitated with WT and S14A by anti-FLAG M2 beads although mTORC1 had a higher affinity to WT. This was substantiated by a reciprocal immunoprecipitation with anti-Raptor antibodies, showing that WT and S14A mutant were coimmunoprecipitated with Raptor (Fig. 3*B*). Notably, in Fig. 3*B* generated with whole cell lysates, the mature forms of both ISCU WT and the S14A mutant were coimmunoprecipitated with Raptor, implying that the domain responsible for interacting with mTORC1 was located downstream of the MTS. In addition, an antibody targeting phosphorylated serines (pSer) revealed that an immunoreactive band migrating at the same speed as the precursor of ISCU-FLAG existed only in immunopurified WT but not in the S14A mutant (Fig. 3*C*). Thus, these data demonstrate that, although endogenous mTORC1 associates with both the WT and the S14A mutant, it only phosphorylates WT.

Phosphorylation of ISCU at Ser-14 Stabilizes ISCU Protein in Cytosol—Because Ser-14 is located in the MTS of ISCU, which is cleaved off after the ISCU precursor form migrates to the mitochondria, we anticipated that mTORC1 phosphorylation

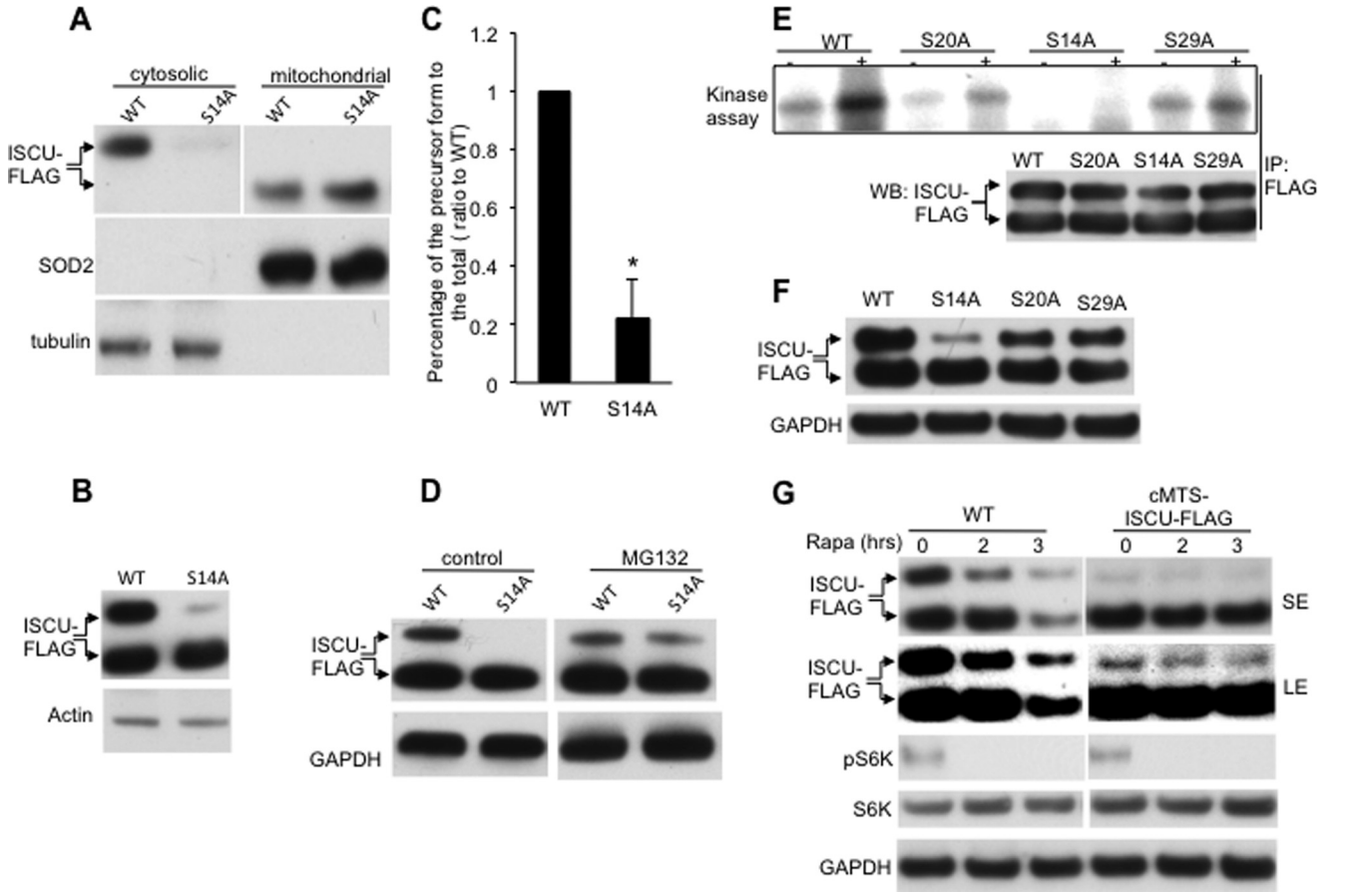

FIGURE 4. **mTORC1 phosphorylation of Ser-14 enhances the protein stability of ISCU.** *A*, after 30 h post-transfection, 293T cells expressing WT or mutant S14A were subjected to the cellular fractions isolation followed by Western blot analysis. SOD2 and tubulin served as quality controls for mitochondrial and cytosolic fractions, respectively. *B*, the whole cell lysates isolated from 293T cells expressing WT or mutant S14A were analyzed by Western blot. *C*, signals of immunoreactive bands for precursor (slow-migrating) and mature (fast-migrating) form in *B* were quantified with NIH ImageJ software, *, $p < 0.05$ *versus* WT. The total levels of ISCU represent the combined signals of precursor and mature form. *D*, in 20 h post-transfection, 293T cells expressing WT or S14A mutant were further treated with 5 μM MG132 in complete medium for 24 h and followed by Western blot (*WB*) analysis. *E*, in 16 h post-transfection, 293T cells expressing ISCU-FLAG WT, S20A, S14A, or S29A were subjected to immunoprecipitations (*IP*) with anti-FLAG M2 beads. Immunopurified proteins were analyzed by *in vitro* kinase (*upper panel*) and Western blot (*lower panel*) assays. *F*, after 30 h post-transfection, 293T cells expressing ISCU-FLAG WT, S20A, S14A, or S29A were analyzed by Western blot. *G*, in 20 h post-transfection, HeLa cells expressing WT or cMTS-ISCU-FLAG were serum-starved for 3 h followed by 200 nM rapamycin incubation for 2 or 3 h. Cell pellets were analyzed by Western blot. *SE*, short exposure; *LE*, long exposure; *Rapa*, rapamycin.

of ISCU at Ser-14 played a role in stabilizing ISCU protein in the cytosol and ultimately regulated the levels of the mature form. First, we verified that the fast migrating band of S14A (the mature form) was also enriched in the mitochondrial fraction, whereas the slower migrating band (the precursor) appeared only in the cytosolic fraction (Fig. 4*A*). Furthermore, as we predicted, the precursor form of S14A mutant was reduced to 20% of that in WT (Fig. 4, *B* and *C*), even to a greater extent with a longer interval after transfection (Data not shown). However, with MG132 incubation, S14A was able to maintain the precursor form at a level similar to WT (Fig. 4*D*), implying that the mutation of Ser-14 triggered ISCU protein degradation.

As shown in Fig. 4*B*, the Ser-14 mutation decreased ISCU precursor levels yet maintained a comparable amount of mature form as in WT. In recent studies, other potential phosphorylation sites have been suggested to Ser-20 and Ser-29 residues in the MTS of ISCU (21). Thus, we wondered whether Ser-20 and Ser-29 were also mTORC1 phosphorylation sites and whether dephosphrylation of Ser-20 and/or Ser-29 was required for further decreases in the ISCU-FLAG mature form. Shown in Fig. 4*E*, individual mutation of Ser-20 and Ser-29 did not abolish the phosphorylation of ISCU-FLAG with or without mTOR protein but slightly weakened it, suggesting that the Ser-20 and Ser-29 might be accessory but not essential mTOR phosphorylation sites on ISCU. Furthermore, in contrast to the significant decrease in S14A precursor after a longer post-transfection interval, S20A and S29A mutants maintained both of the precursors and mature forms at a steady level (Fig. 4*F*), implying Ser-20 and Ser-29 might not play as important a role in stabilizing ISCU protein as Ser-14 does. This, however, cannot exclude the possibility that, in addition to Ser-14, dephosphorylation at these sites might further lead to a decline in ISCU-FLAG mature form. To this end, we replaced the native MTS of ISCU-FLAG with one derived from subunit VIII of human cytochrome *c* oxidase (referred to as cMTS-ISCU-FLAG) (22). However, similar to S14A mutant, cMTS-ISCU-FLAG maintained the mature form at a steady level as WT (Fig. 4*G*, 0-h rapamycin treatment). Furthermore, upon rapamycin incubation, the precursor and the mature form in the WT were decreased, whereas neither of these was affected in the cMTS-ISCU-FLAG mutant (Fig. 4*G*), proving that phosphorylation of Ser-14 was essential for mTORC1-mediated preservation of

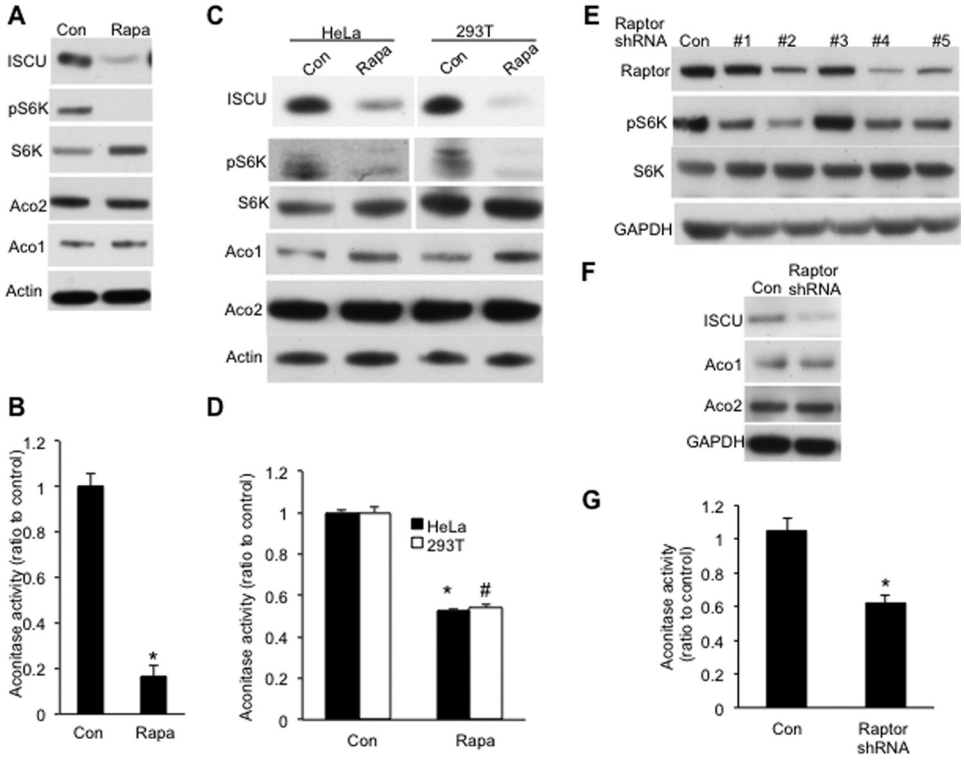

FIGURE 5. **mTORC1 inhibition-mediated degradation of ISCU decreases ISC assembly.** *A* and *B*, MLg cells were incubated with rapamycin for 7 h in complete medium followed by Western blot (*A*) and aconitase activity (*B*) assays. *, $p < 0.05$ *versus* control (*Con*). *C* and *D*, 293T or HeLa cells were incubated with 200 nM rapamycin (*Rapa*) for 2 h following serum starvation overnight. The resultant cell pellets were analyzed by Western blot (*C*) and aconitase activity (*D*) assay. *, $p < 0.05$ *versus* HeLa control; #, $p < 0.05$ *versus* 293T control. *E*, Mlg cells were infected with lentiviruses control or Raptor shRNA and further selected with puromycin. Puromycin-resistant cells were analyzed by Western blot. *F* and *G*, cell line 4 stated in *E*, were further analyzed with Western blot (*F*) and aconitase activity (*G*) analyses. *, $p < 0.05$ *versus* control. *S6K*, S6 kinase; *Rapa*, rapamycin.

ISCU protein. Moreover, the WT precursor was diminished by rapamycin incubation in a time-dependent manner, and the mature form was also decreased after a longer period of incubation (3 h), implying that the decline of the mature form was secondary to the decrease in the precursor. Together, these data demonstrate that mTORC1 phosphorylation of Ser-14 is required for ISCU protein stabilization in the cytosol, whereas dephosphorylation of Ser-14, due to the inhibition of mTORC1, leads to degradation of the precursor form and ultimately to a decrease in the mitochondrial mature form.

mTORC1 Inhibition-triggered ISCU Degradation Inhibits ISC Assembly—ISCU is crucial to ISC assembly. Therefore, we tested whether ISCU degradation caused by mTORC1 inhibition affected ISC assembly. As shown in Fig. 5, *A–D*, with the reduction of ISCU protein in MLg, 293T, and HeLa cells, all aconitase activity, for which ISCs are indispensable, was inhibited by rapamycin, whereas the levels of aconitase enzyme Aco2 (mitochondrial form) or Aco1 (cytosolic form) was unaffected, suggesting that the decrease in aconitase activity was due to insufficient ISC biogenesis and that ISCU degradation triggered by mTORC1 inhibition suppressed ISC assembly. To further confirm this, we inhibited the Raptor protein expression using five different raptor shRNA clones. As shown in Fig. 5*E*, the Raptor protein levels were decreased by clones 2, 4, and 5. Because Raptor is a distinct and essential subunit of mTORC1, the decrease in Raptor protein further inhibited S6 kinase phosphorylation. Thus, we determined ISCU protein levels and ISC assembly activity in cell line 4 where Raptor protein levels were decreased most significantly by clone 4 compared with others. As shown in Fig. 5*F*, ISCU protein levels were decreased in cell line 4 compared with the control cell line. Consistent with this, aconitase activity was also reduced, whereas the amount of aconitase protein remained unaffected, suggesting a decrease in ISC biogenesis along with ISCU protein reduction in Raptor-inhibited cells. Together, all these data demonstrated that mTORC1 inhibition, due to rapamycin treatment or Raptor deficiency, triggers ISCU degradation and inhibits ISC assembly.

The Constitutive mTORC1 Activation-induced ISCU Stabilization Sensitizes Cells to Iron Deprivation—Due to an inhibitory effect of the tumor suppressor TSC2 on mTORC1, mTORC1 activity in TSC2 null mouse embryonic fibroblasts ($TSC2^{-/-}$ MEFs) was constitutively active even under the nutrient and growth factor starvation. Thus, we explored the levels and function of ISCU in these MEFs and their impacts on overall iron homeostasis. As shown in Fig. 6*A*, in $TSC2^{-/-}$ MEFs ISCU levels and aconitase activity but not protein content was increased compared with those in TSC2 wild-type ($TSC2^{+/+}$) MEFs (Fig. 6*A*). In contrast, the mRNA levels of ISCU remained similar (data not shown). Moreover, incubation of $TSC2^{-/-}$ MEFs with rapamycin reduced ISCU protein (Fig. 6*B*), although it spared mRNA levels (data not shown). Along with this, aconitase activity was also reduced (Fig. 6*C*). These data suggested increased ISCU protein levels and ISC biogenesis in $TSC2^{-/-}$ MEFs, which can be diminished by rapamycin treatment.

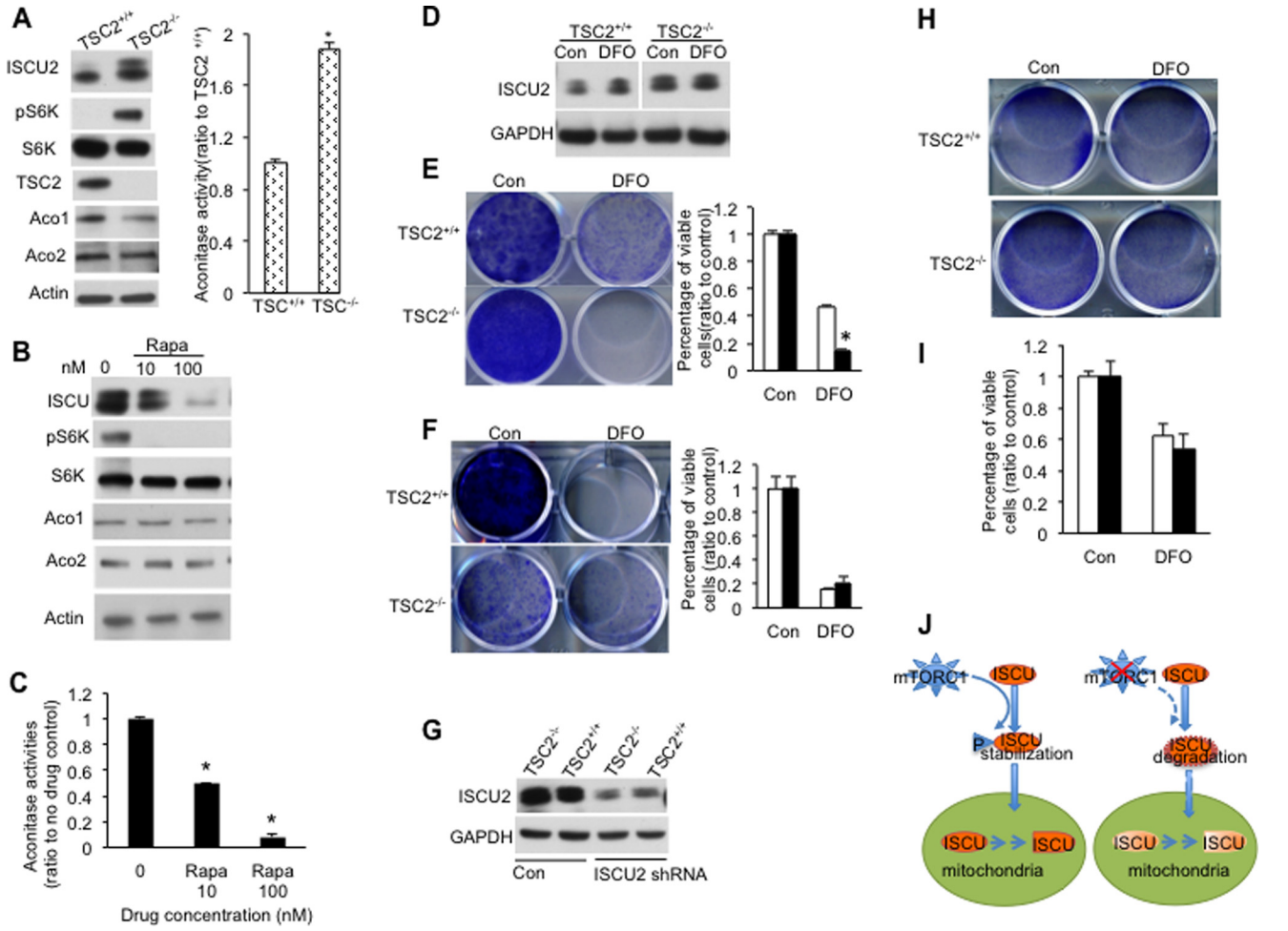

FIGURE 6. **Constitutively active mTORC1-mediated stabilization of ISCU sensitizes cells to iron deprivation.** *A*, $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were examined with Western blot and aconitase activity analyses, *, $p < 0.05$ *versus* $TSC2^{+/+}$ MEFs. *B* and *C*, $TSC2^{-/-}$MEFs were incubated with different concentrations of rapamycin in complete medium for 24 h followed by Western blot (*B*) and aconitase activity (*C*) assays. *D*, $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were incubated with 15 μM DFO for 24 h and further examined by Western blot. *E*, after overnight culture, 1000 $TSC2^{+/+}$ or $TSC2^{-/-}$ MEFs per well in 12-well plate were incubated with 15 μM DFO for 24 h. Thereafter, cells were recovered for 5 days followed by crystal violet staining (*left panel*) or cell counting (*right panel*). *F*, 5000 MEFs per well were pretreated with 200 nM rapamycin for 6 h and further combined with DFO for 21 h followed by assays as described in *E*. *G*, cells were infected with lentivirus control (*Con*) or ISCU shRNA and further selected with puromycin. Puromycin-resistant cells were analyzed by Western blot. *H* and *I*, as in G, puromycin-resistant cells were treated with 15 μM DFO for 24 h and further stained with crystal violet (*H*) and cell counting (*I*). *Empty bar*, $TSC2^{+/+}$; *solid bar*, $TSC2^{-/-}$. In *C*, the *single asterisk* indicates $p < 0.05$ *versus* 0 nM rapamycin (*Rapa*) treatment. In *E*, the *single asterisk* indicates $p < 0.05$ *versus* $TSC2^{+/+}$ MEFs with the respective treatment as to $TSC2^{-/-}$ MEFs. *J*, graphic abstract. ISCU associates with and is phosphorylated by mTOR in the cytosol. This phosphorylation stabilizes ISCU protein and thus increase ISCU levels in mitochondria, whereas the dephosphorylation destabilizes ISCU protein in cytosol and decreases its mitochondrial levels.

Because the constitutive activation of mTORC1 enhanced ISCU levels and thus ISC biogenesis in $TSC2^{-/-}$ MEFs, we wondered whether this would affect iron homeostasis. To test this, iron homeostasis was challenged with the incubation of an iron chelator, deferoxamine (DFO) in MEFs. Contrary to what was reported previously (6, 7), DFO incubation did not decrease ISCU levels (Fig. 6*D*). We suspect that this is likely due to the type of cells and low concentrations of DFO applied in this study. Moreover, as shown in Fig. 6*E*, fewer viable $TSC2^{-/-}$ MEFs survived DFO incubation compared with $TSC2^{+/+}$ MEFs. Yet, these effects were abolished by rapamycin pretreatment and further co-incubation with DFO, as demonstrated by a comparable number of viable cells remaining in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs (Fig. 6*F*). In addition, supporting a previous report (23), rapamycin treatment alone was even more toxic to $TSC2^{-/-}$ than $TSC2^{+/+}$ MEFs (data not shown), strengthening the antagonizing effect of rapamycin on DFO incubation in $TSC2^{-/-}$ MEFs. To further explore the role of ISCU protein in the iron starvation-triggered cell death, the endogenous ISCU knockdown was performed with lentiviral shRNA (Fig. 6*G*). As shown in Fig. 6, *H* and *I*, knockdown of ISCU partially prevented the iron starvation-triggered cell death in $TSC2^{-/-}$ MEFs, resulting in a similar number of surviving $TSC2^{-/-}$ MEFs as $TSC2^{+/+}$ MEFs. As a comparison, control shRNA did not modulate the iron deprivation-induced cell death in $TSC2^{-/-}$ or $TSC2^{+/+}$ MEFs (data not shown). Therefore, these data demonstrate that ISCU protein synthesis and subsequent ISC assembly, enhanced by unrestrained mTORC1 activation, affect iron homeostasis, and thus sensitize cells to iron deprivation.

DISCUSSION

In this study, we demonstrate that ISCU, a critical scaffold protein for ISC assembly, is an mTOR kinase target and that

phosphorylation of ISCU by mTORC1 prevents its degradation and increases ISC biogenesis. Given that the upstream stimuli of mTORC1 are nutrients, growth factors, and energy, our findings demonstrate that metabolic cues play an important role in ISCU gene expression and function and thus broaden our knowledge of iron homeostasis mediated by metabolism. Also, these studies expand the mTORC1 targets to ISC biogenesis/iron homeostasis and elucidate that unrestrained mTORC1-mediated ISC biogenesis can aggravate cell vulnerability to iron starvation.

ISC assembly protein complexes have been identified outside of mitochondria. However, ISC assembly primarily occurs in mitochondria, which is essential for ISC function in the cytosol and nucleus. Herein, ISCU is a nuclear gene-encoded mitochondrial protein. Thus, its protein stability and amount available to mitochondria can be regulated in the cytosol. In this study, the mutation of the potential phosphorylatable serines (cMTS-ISCU-FLAG) or Ser-14 alone (S14A) significantly reduced the content of the cytosolic precursor yet left mitochondrial mature form unchanged. Likely, this is due to the expression system used here, which often triggers exogenously expressed protein unfolding and greatly accelerates mitochondrial translocation (24). Thus, this competition favoring mitochondrial translocation over protein stability in the cytosol eventually leads to the accumulation of the mature form. Supporting this notion is the observation that, with the post-transfection interval prolonged, the amount of cytosolic ISCU-FLAG WT decreased, whereas the levels of mitochondrial form were not affected. However, in WT but not cMTS-ISCU-FLAG mutant, the precursor and mature form were both reduced by mTORC1 inhibition, implying that other potential mechanism involved. Furthermore, 14-3-3 proteins binding, well established as a regulator of protein stability and translocation, has been implied to the phosphorylated S14 and S29 in ISCU (25). However, similar levels of the precursor and mature forms existed in S20A and S29A mutants, and yet S20A still demonstrated binding of 14-3-3 proteins.[4] Therefore, the binding of 14-3-3 cannot exclude the other as-yet-unknown mechanisms involved. This needs to be further explored in future. Nonetheless, as others have shown that mTORC1 preserves the stability of transcription factors and further regulates their levels and activities in nucleus (18, 26), the protein stability of ISCU appears to be regulated by mTORC1 in the cytosol, prior to its mitochondrial translocation.

mTORC1 is structured to coordinate metabolic demand and supply. The inability to suppress mTORC1-mediated biosynthesis following nutrients starvation causes cell death due to demand outstripping supply. As such, glucose or amino acid starvation induces $TSC2^{-/-}$MEF death due to the protein translation and lipid synthesis outdoing substrate supply (27). In line with this, we showed that mTORC1 significantly activates ISC synthesis in $TSC2^{-/-}$ MEFs and that the starvation for iron, the particular substrate of ISC biogenesis, leads to notorious cell death. Thus, as protein and lipid syntheses, ISC biogenesis is also a part of mTORC1-mediated metabolic homeostasis, imbalance of which leads to the deleterious effect on cells. Conceivably, via mTORC1 responding to nutrient availability, ISC assembly, and other biosytheses, including protein and lipid productions, are accommodated to each other to ensure cell survival and growth. Furthermore, iron starvation has been applied in cancer treatment (28). Considering the high sensitivity of $TSC2^{-/-}$ MEFs to iron starvation and that mTORC1 is frequently activated in tumors (8), it would benefit patients to apply iron chelators to tumors where mTORC1 is activated. Hence, the current study provides a molecular mechanism to potentially guide iron deprivation treatment to cancer.

Some mTORC1 inhibitors are used clinically in a wide spectrum of diseases, including immune and non-immune-mediated disorders (29). One of the most challenging and frequent adverse effects of the mTORC1 inhibitors is anemia due to dysregulation of cellular iron metabolism. ISC assembly can impact erythrocyte-specific heme synthesis, the dysfunction of which always results in anemia (30). The study presented here shows that rapamycin decreases ISCU protein levels and its function in assembly of ISCs. Thus, it would be interesting to investigate the potential effect of rapamycin or its analogs on heme synthesis. This may help explain how anemia occurs with rapamycin treatment in clinical studies.

In conclusion, we identified that mTORC1 associates with and phosphorylates ISCU protein, consequently stabilizing ISCU and enhancing ISC assembly.

Acknowledgments—We thank Dr. David Kwiatkowski at the Brigham and Women's Hospital for sharing the $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs. We also thank members of the group of P. A. Dennery for the critical reading of this manuscript.

[4] P. La, unpublished observations.

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