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# Tissue-specific and Nutrient Regulation of the Branched-chain α-Keto Acid Dehydrogenase Phosphatase, Protein Phosphatase 2Cm (PP2Cm)*[S]

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**Background:** PP2Cm regulates BCAA homeostasis via BCKD activity, but the underlying molecular mechanism is unknown.
**Results:** PP2Cm transcription and interaction with BCKD were studied *in vivo* and *in vitro*.
**Conclusion:** PP2Cm expression and function are nutrient-dependent.
**Significance:** BCAA homeostasis is contributed by dynamic regulation of PP2Cm.

**Branched-chain amino acid (BCAA) homeostasis is maintained through highly regulated catabolic activities where the rate-limiting step is catalyzed by branched-chain α-keto dehydrogenase (BCKD). Our previous study has identified a mitochondria-targeted protein phosphatase, PP2Cm, as the BCKD phosphatase and thus serves as a key regulator for BCAA catabolism. In this report, we performed comprehensive molecular and biochemical studies of PP2Cm regulation using both *in vivo* and *in vitro* systems. We show that PP2Cm expression is highly enriched in brain, heart, liver, kidney, and diaphragm, but low in skeletal muscle. The PP2Cm expression is regulated at the transcriptional level in response to nutrient status. Furthermore, we have established that PP2Cm interacts with the BCKD E2 subunit and competes with the BCKD kinase in a substrate-dependent and mutually exclusive manner. These data suggest that BCAA homeostasis is at least in part contributed by nutrient-dependent PP2Cm expression and interaction with the BCKD complex. Finally, a number of human PP2Cm single nucleotide polymorphic changes as identified in the public data base can produce either inactive or constitutive active mutant phosphatases, suggesting that putative PP2Cm mutations may contribute to BCAA catabolic defects in human.**

Leucine, isoleucine, and valine are collectively referred as branched-chain amino acids (BCAAs)[4] because they share a structural feature with a branched-side chain and common initiation steps of catabolism. BCAAs have a critical function not only as common amino acid ingredients for protein synthesis but also as potent nutrient signals to regulate mammalian target of rapamycin signaling for cell growth (1), autophagy (2), glucose utilization (3), lipid metabolism (4), neurotransmitter synthesis (5), as well as skeletal muscle growth/atrophy (6), among many others. BCAAs are essential amino acids to all animals, and their homeostasis is largely controlled by catabolic activities. Genetic defects in BCAA catabolic pathways can lead to several rare metabolic disorders, including maple syrup urine disease in which excess BCAA cannot be degraded due to mutations in the BCAA catabolic system (7). Classic maple syrup urine disease due to complete loss of BCAA catabolic activities can be lethal if untreated. On the other hand, a low BCAA concentration is also deleterious either as the result of malnutrition or unrestrained catabolism (8, 9). More recently, several studies have linked abnormal BCAA levels with common metabolic, cardiovascular, and neurologic diseases in both rodents and human (10–14). These observations implicate BCAA catabolic defects as contributing to both rare and common human diseases.

The first step of BCAA catabolism is a reversible transamination reaction catalyzed by the branched-chain aminotransferase to generate the corresponding branched-chain α-keto acids (BCKAs). The second and the rate-limiting step is an irreversible oxidative decarboxylation catalyzed by the branched-chain α-keto acid dehydrogenase (BCKD) complex. Following decarboxylation, each individual BCKA is catabolized through three separate pathways that eventually lead to the citric acid

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[4] The abbreviations used are: BCAA, branched-chain amino acid; BCKA, branched-chain α-keto acid; BCKD, branched-chain α-keto acid dehydrogenase; BCKDK, BCKD kinase; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; dpc, days postcoitus; LBD, lipoic acid-bearing domain; PP2Cm, protein phosphatase 2Cm; SNP, single-nucleotide polymorphism.

cycle. In animals, the BCAA catabolism is accomplished through a coordinated effort among multiple organs. The majority of the initial transamination occurs in skeletal muscle whereas BCKD capacity mostly resides in liver with brain playing a minor role in the BCAA catabolism (15). Contribution of heart muscle has not been well established.

As the flux-generating enzyme, BCKD activity is regulated by both covalent and allosteric mechanisms (8). The BCKD holoenzyme contains three subunits: a heterodimeric branched-chain $\alpha$-keto acid decarboxylase (E1$\alpha$ and E1$\beta$), a homo-24-meric dihydrolipoyl transacylase (E2), and a homodimeric dihydrolipoyl dehydrogenase (E3). Covalent phosphorylation of E1$\alpha$ subunits inhibits BCKD activity, and the BCKD kinase (BCKDK) that phosphorylates E1$\alpha$ has been identified (16). The BCKDK plays a crucial role in determining the BCKD activity, and its expression and activity are regulated by long and short term mechanisms (17). Long term control of BCKDK includes regulation of gene expression by nutritional status and hormonal signaling. Low protein diet increased BCKDK mRNA level in rat liver (18) whereas BCAA withdrawal in the medium increased the BCKDK protein expression in cultured hepatocytes (19). It has been shown that insulin, glucocorticoid, thyroid hormone, and sex hormones regulate the activity and expression of BCKDK as well (17, 20). In addition, the BCKDK activity is also subject to allosteric regulation by BCKAs, thiamine diphosphate, and other factors (7, 21). BCKAs inhibit the BCKDK activity and attenuates its interaction with the BCKD complex. It is plausible that these regulations serve as key to maintain BCAA homeostasis under different physiological and growth conditions (22, 23).

Although the role of a protein phosphatase that dephosphorylates and thus activates BCKD has been proposed for decades, the molecular identity of the BCKD phosphatase has only been revealed recently (24–26). The BCKD phosphatase is a mitochondrial targeted member of the protein phosphatase 2C family, PP2Cm (27). PP2Cm has high specificity for phosphor-E1$\alpha$. Genetic ablation of the PP2Cm gene in mice leads to hyperphosphorylation of the E1$\alpha$ subunit and BCAA catabolic defect associated with a mild form of maple syrup urine disease (25), thus implicating a critical role for PP2Cm as a key regulator of BCAA catabolism. However, the physiological and the molecular bases of PP2Cm function in BCAA catabolic regulation remain poorly understood. In the current study, we conducted a comprehensive physiological, molecular, and biochemical investigation focusing on the regulation of PP2Cm expression and function related to nutrient status. Using both *in vivo* and *in vitro* tools, we established that PP2Cm was expressed in discrete cell types of several vital organs, and its transcriptional activity was sensitive to nutrient levels. We further demonstrated that PP2Cm interacted with specific domains of the BCKD E2 subunit, and this interaction was regulated also by the level of BCKA substrate through competition with BCKDK. Finally, we characterized a number putative polymorphic changes in the PP2Cm coding sequence identified in human population. Therefore, our study provided the first comprehensive molecular and biochemical characterization of a newly discovered BCAA catabolic regulator, PP2Cm, in its nutrient-dependent regulation at transcriptional and biochemical levels. Our results indicate that the BCAA/BCKA-mediated regulation of PP2Cm expression and interaction with BCKD complex is another potentially important feedback mechanism to maintain BCAA homeostasis at both local and global levels.

## EXPERIMENTAL PROCEDURES

*Animals*—Male wild-type C57BL/6 mice and PP2Cm knockout mice on the same background were considered as adult at the age of 8–10 weeks. Animals were euthanized for collection of tissues or other experiments. Frozen tissues were ground with a pestle and mortar in a liquid nitrogen bath. The resulting powders were dissolved in lysis buffer for Western blot analyses or TRIzol reagent (Invitrogen) for RNA. For starvation experiments, the mice were deprived of food but were given free access to drinking water. Animal handling was approved by the Committee for Humane Treatment of Animals at Shanghai Jiao Tong University School of Medicine.

*Histochemical Assay of Mouse Embryo and Tissues for LacZ Activity*—Transgenic embryos were collected from pregnant females at 10.5 and 14.5 days postcoitus (dpc), considering 12 noon of the day on which a copulatory plug was found as 0.5 dpc. The neonatal mice (0 d) were dissected immediately after being born. The 1-week, 4-week, and adult mice tissues were collected after dissecting. For the 10.5-dpc embryos, the whole bodies were fixed in fresh PBS with 5 mM EGTA, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 0.02% Nonidet P-40, and 0.1% paraformaldehyde 90 min at 4 °C; but the 14.5-dpc embryos, 0-d, 1-week, 4-week, and adult tissues were frozen in Optimal Cutting Temperature compound (Tissue Tek), tandemly sectioned by a cryostat, and then fixed. The samples were incubated with 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM $MgCl_2$, 5 mM $K_3Fe_4(CN)_6$, 5 mM $K_4Fe_3(CN)_6$, 20 mM Tris-Cl, and 1 mg/ml X-Gal for 16 h in the dark at 37 °C.

*Real-time RT-PCR Analysis*—Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse transcribed into the first-strand cDNA using the Superscript First-strand synthesis kit (Invitrogen). Then, cDNA transcripts were quantified by the Step-One Plus Real-time PCR System (ABI) using SYBR Green (ABI). Each reaction was performed in triplicate, and values were averaged to calculate the relative expression level. Primers sequences will be presented upon request.

*Luciferase Expression Constructs, Cell Culture, Transient Transfection, and Luciferase Assay*—Utilizing the NCBI GenBank, we identified the genomic sequence of mouse PP2Cm (*ppm1k*). We therefore amplified the proximal 5′ region (−2764 to +20 bp relative to transcript start site) and the 3′-untranslated region of mouse PP2Cm (4216 bp) by PCR. Promoter PCR product was cloned into a firefly luciferase reporter pGL3-Basic vector (Promega) to drive luciferase expression (PP2Cm-luc). The 3′-UTR PCR product was cloned into psi-CHECK2 vector (Promega) in which *Renilla* luciferase is used as a primary reporter (PP2Cm-3′UTR-luc). SHS-Y5Y and HepG2 cell lines were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. Transient transfections were performed with the use of Lipofectamine 2000 reagent (Invitrogen). Customized BCAA (leucine, isoleucine, and

valine)-free DMEM was produced by Invitrogen. Briefly, HepG2 and SHS-Y5Y were seeded into 12-well plates at a density of $2 \times 10^5$ cells/well the day before transfection. For each well of cells 0.2-$\mu$g promoter constructs were co-transfected with 0.02-$\mu$g pSV40-*Renilla* vectors, and 0.2-$\mu$g 3′-UTR constructs were transfected in each well. The transfected cells were collected after transfection for 48 or 24 h, respectively, in promoter and 3′-UTR assay. Luciferase activities were measured with the Dual-Luciferase Reporter Assay system (Promega). To normalize for transfection efficiency, the promoter activity was expressed as the ratio of firefly activity to *Renilla* activity, but the 3′-UTR activity was *Renilla* to firefly. For each construct, more than three independent experiments were performed in triplicate.

*Immunoblotting, Immunoprecipitation, and Glycerol Gradient Sedimentation Assay*—Cells were washed twice with ice-cold PBS and harvested in Buffer A (50 mM HEPES, pH7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 20 mM NaF, 1 mM PMSF, 1 $\mu$g/ml aprotinin, leupeptin, and pepstatin). Samples were separated on 4–12% Bis-Tris gels (Invitrogen) and transferred onto a nitrocellulose blot (Amersham Biosciences). The blot was probed with the indicated primary antibodies. Protein signals were detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL) Western blotting detection regents (Pierce). Rabbit polyclonal antisera against the E1 and E2 subunits of BCKD complex were a kind gift from Dr. Yoshiharu Shimomura (Nagoya Institute of Technology). For Fig. 7, human PP2Cm antibody was purchased from Novus Biologicals (H00152926-B01P). For anti-FLAG immunoprecipitation assay, total cell lysates were incubated with anti-FLAG M2 beads (Sigma) for 2 h at 4 °C and washed with Buffer A six times, followed by elution with FLAG peptide (200 $\mu$g/ml). For sedimentation on glycerol gradient, total cell lysate (2 mg) or anti-FLAG immunoprecipitates were loaded onto a 10-ml 10–40% glycerol gradient in Buffer A. After centrifugation at 156,000 × *g* for 14.5 h, 330-$\mu$l fractions were collected from the top to the bottom of the gradient and subjected to SDS-PAGE analysis.

*Generation of Recombinant Proteins and Pulldown Assay*—GST-tagged and His-tagged recombinant proteins were expressed in *Escherichia coli* BL21(DE3) and purified with glutathione-Sepharose 4B beads (Amersham Biosciences) and TALON resins (Clontech), respectively. For generation of full-length PP2Cm from His-tagged protein, Factor Xa was used to cleave the fusion protein by targeting the predesigned digestion site. 1 $\mu$g of GST-E2 was incubated with full-length PP2Cm and other His-tagged subunits in 500 $\mu$l of Buffer B (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 1 mM PMSF, 1 $\mu$g/ml aprotinin, leupeptin, and pepstatin) at 4 °C overnight. The GST-tagged E2 and its interacting proteins were captured using glutathione-Sepharose 4B beads. Alternatively, full-length PP2Cm was incubated with individual subunits with and without BCKA in 500 $\mu$l of buffer B, and PP2Cm interaction with individual subunits was captured by His-tagged protein purification using TALON resins. After being washed with Buffer B six times, beads were boiled in SDS loading buffer followed by SDS-PAGE analysis.

*Molecular Cloning and Mutagenesis of Human PP2Cm*—Human PP2Cm cDNA was cloned into pShuttle-CMV expression vector and sequenced. Mutants were generated by a QuikChange Site-directed Mutagenesis kit (Stratagene).

*Statistics*—Statistical analyses were performed with Student's *t* test when two groups were compared. A $p$ value $< 0.05$ was considered statistically significant.

## RESULTS

*Tissue-specific Expression of PP2Cm in Adult Mouse*—BCAA catabolism is ubiquitous but more concentrated in selected tissues. In rats, skeletal muscle and liver are two major organs that coordinate to complete the disposal process (15). We first examined the tissue specificity of PP2Cm expression by taking advantage of PP2Cm knock-out mouse where a LacZ coding sequence is inserted in the PP2Cm second exon (25) (supplemental Fig. 1). Real-time PCR was performed to assess the level of LacZ and PP2Cm mRNA in various tissues from the heterozygous mice (Fig. 1, *A* and *B*). The highest transcript levels of both LacZ and PP2Cm were detected in the brain whereas significant levels were also found in heart, liver, and diaphragm. Unexpectedly, the skeletal muscle had relatively low levels expression for both genes, and a number of other tissues including lung, pancreas, stomach, and spleen had very low expression (Fig. 1, *A* and *B*). These results confirmed the original report from our previous study on tissue-specific distribution of the PP2Cm mRNA (25) and demonstrated that the LacZ expression faithfully reflected the endogenous PP2Cm expression pattern. A similar pattern of tissue distribution for PP2Cm was also observed at protein level by immunoblotting (Fig. 1*C*). Using histochemical staining for $\beta$-galactosidase on tissue sections prepared from adult PP2Cm knock-out mice, we further identified high levels of PP2Cm expression in neuronal cells, myocytes in heart and diaphragm, hepatocytes in liver, and smooth muscle cells of pulmonary brachia. However, only very low LacZ staining was detected in skeletal muscle, and no detectable signal was observed in spleen and pancreas (Fig. 1*D*). These data indicated that brain, heart, liver, and diaphragm may have relatively high level of PP2Cm-dependent BCAA catabolism.

*PP2Cm Expression during Development*—PP2Cm expression is critical for normal fish development (27) although PP2Cm-deficient mice can develop to term (25). To evaluate the potential role of BCAA catabolic regulation during early development, we examined the LacZ expression at different stages of development in the PP2Cm knock-out mouse. Whole mount staining of mouse embryos showed clear LacZ expression in heart and brain as early as 10.5 dpc (Fig. 2*A*). In 14.5-dpc embryo, LacZ expression was clearly detected in brain, heart, and liver, with particularly concentrated expression in choroid plexus of the brain (Fig. 2*B*). In neonatal to young mice at 0, 7, and 28 day of age, LacZ staining was sustained in heart, liver, and brain (Fig. 2*C*), but it remained absent in skeletal muscle, lung, spleen, and pancreas (Fig. 2*C* and supplemental Fig. 2). These data suggested that PP2Cm-dependent BCAA catabolism is also present in brain, liver, and heart of embryo and young mice. The concentrated expression of PP2Cm in devel-

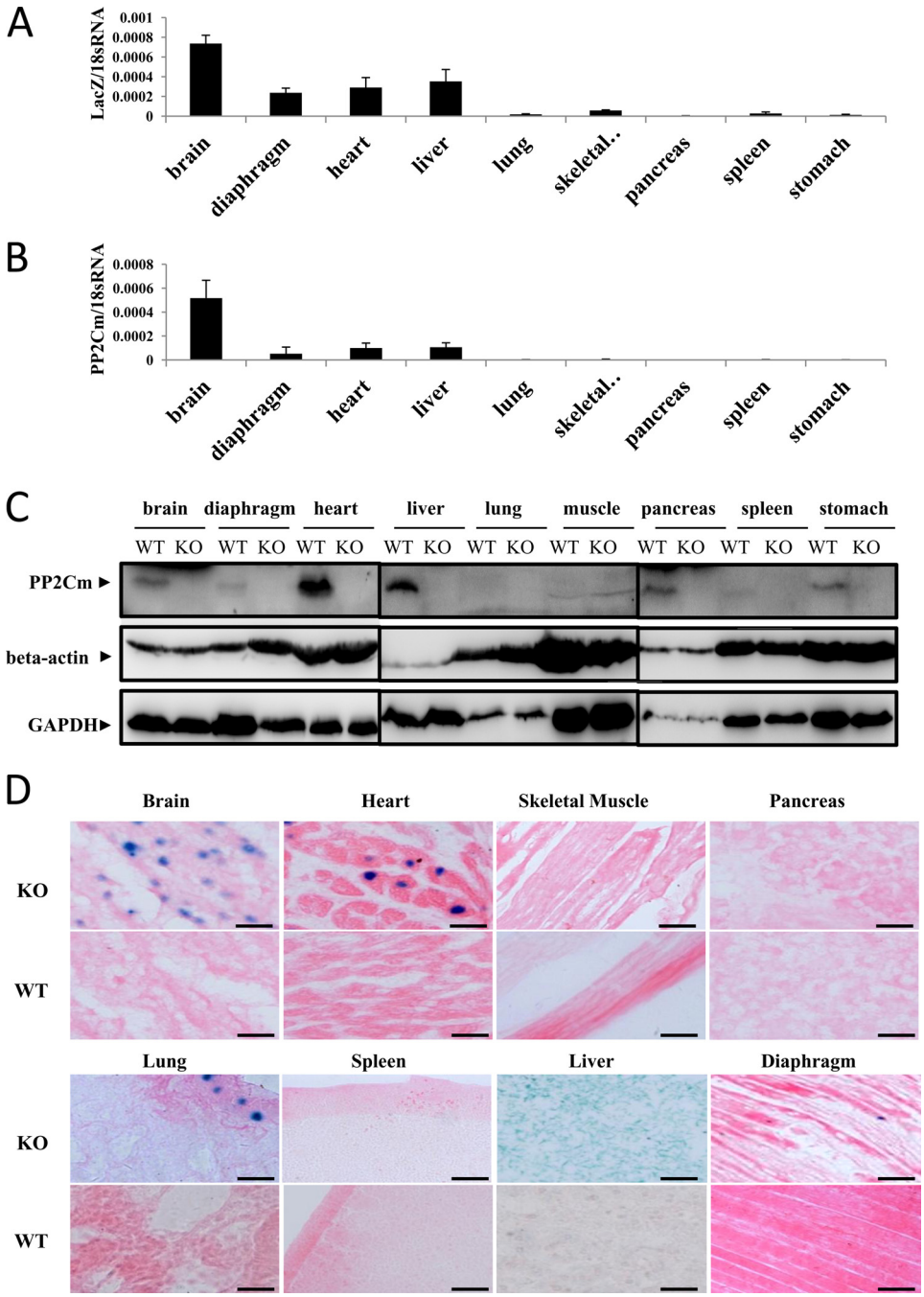


FIGURE 1. **Tissue-specific expression of PP2Cm.** *A* and *B*, real-time RT-PCR results of LacZ (*A*) and PP2Cm (*B*) using mRNA from different tissues of PP2Cm heterozygous knock-out (*ppm1k*$^{+/-}$) mouse. PP2Cm mRNA level was normalized to 18S RNA. The data represented the average values with S.D. (*error bars*) of three male mice. *C*, PP2Cm protein expression in tissues of adult wild-type (*WT*) and PP2Cm knock-out (*KO*) mice. The same amount of total protein was loaded from each tissue. β-Actin and GAPDH were used as loading reference for immunoblotting. *D*, LacZ staining of different tissues from adult WT or PP2Cm KO mouse. Images were originally taken at magnification of ×400. *Scale bars*, 25 μm.

oping choroid plexus suggests an unrecognized role of BCAA in the homeostasis of cerebrospinal fluid production.

*BCAA Controls PP2Cm Gene Transcription*—Nutritional status controls BCKD subunits and BCKDK level in rat (17, 18, 28). In adult mice, 48-h food deprivation caused down-regulation of PP2Cm mRNA to varying degrees in brain, heart, and liver (Fig. 3*A*). To show a direct impact of BCAA on PP2Cm expression, we examined the mRNA level of PP2Cm in cultured cells. 24-h BCAA depletion significantly down-regulated PP2Cm expression in HepG2 cells whereas replenishment of BCAA brought the PP2Cm mRNA level back (Fig. 3*B*). To reveal whether BCAA controls PP2Cm transcriptional or post-transcriptional regulation, we examined activity of the PP2Cm promoter and 3′-UTR in cell culture using luciferase reporter constructs as described under "Experimental Procedures." In both HepG2 liver cells (Fig. 3*C*) and SHS-Y5Y neuronal cells (supplemental Fig. 3*A*), the PP2Cm promoter activity was decreased by ~50% when BCAAs were removed from medium whereas adding back BCAAs restored the PP2Cm promoter activities. In contrast, no changes were observed in either cell

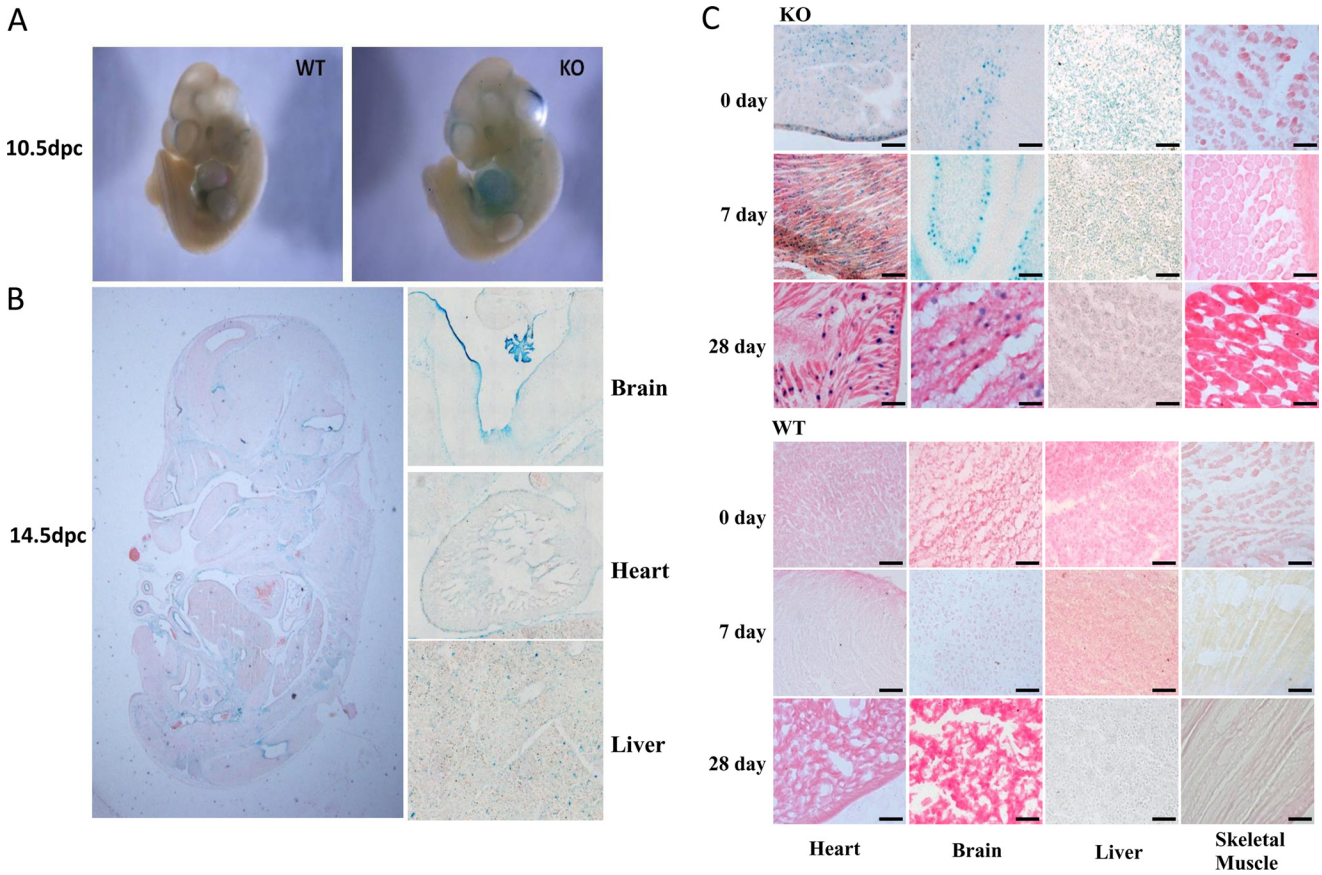


FIGURE 2. **PP2Cm expression during development.** *A*, whole mount LacZ staining of mouse early embryos (10.5 dpc) of wild-type (*WT*, *left*) and PP2Cm knock-out (*KO*, *right*) mouse. *B*, tissue sections from mouse embryos (14.5 dpc) of PP2Cm KO mouse stained for LacZ expression detected by β-galactosidase histochemistry. *Left*, whole embryo. *Right*, different tissues. *C*, tissue sections from PP2Cm KO and WT mouse with different ages stained for LacZ expression detected by β-galactosidase histochemistry. Images were originally taken at magnification of ×200. *Scale bars*, 25 μm.

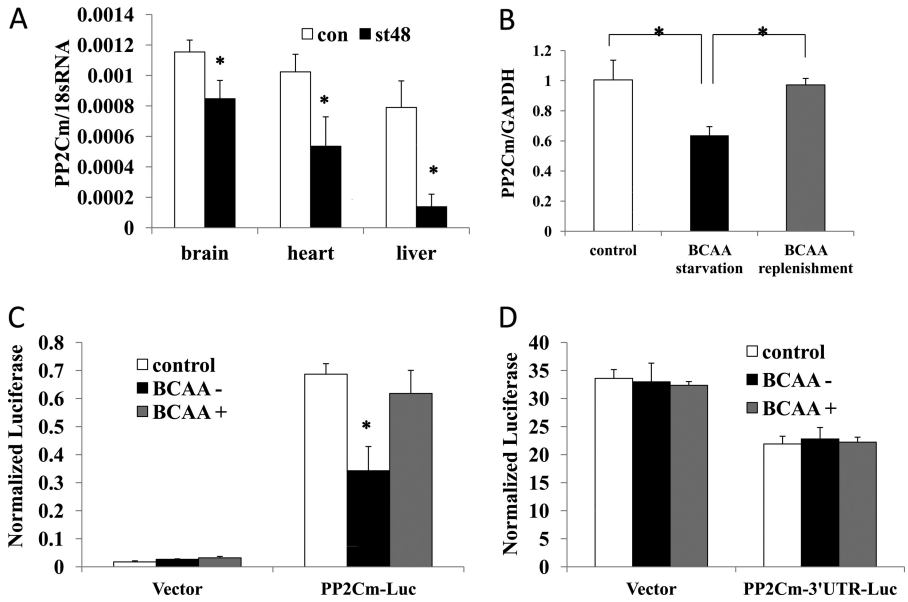


FIGURE 3. **Nutritional regulation of PP2Cm transcription by BCAA.** *A*, real-time RT-PCR results of PP2Cm gene using mRNA from different tissues of wild-type mice with (*st48*) or without starvation (*con*) for 48 h. PP2Cm mRNA level was normalized to 18S RNA. The data represent the average values with S.D. (*error bars*) of three male mice. *, $p < 0.05$ compared with control. *B*, real-time RT-PCR results of PP2Cm gene using mRNA from HepG2 cells without treatment (control), with 24-h BCAA starvation, or with 12-h BCAA starvation then 12-h BCAA replenishment. PP2Cm mRNA level was normalized to GAPDH. The data represent the average values with S.D. of three groups. *, $p < 0.05$. *C* and *D*, luciferase assay result of PP2Cm promoter-luciferase (*C*) and PP2Cm 3′-UTR-luciferase (*D*) and corresponding vectors expression in HepG2 cells. The cells were not treated (control), cultured in BCAA-free medium (DMEM without leucine, isoleucine, and valine) for 24 h (*BCAA −*), or replenished with BCAA (complete DMEM containing 0.8 mM leucine, isoleucine, and valine) for 12 h after 24 h BCAA starvation (*BCAA +*). The data represent the average values with S.D. of triplicate samples from one experiment representative of three independent experiments. *, $p <$ 0.05 compared with control or BCAA replenishment.

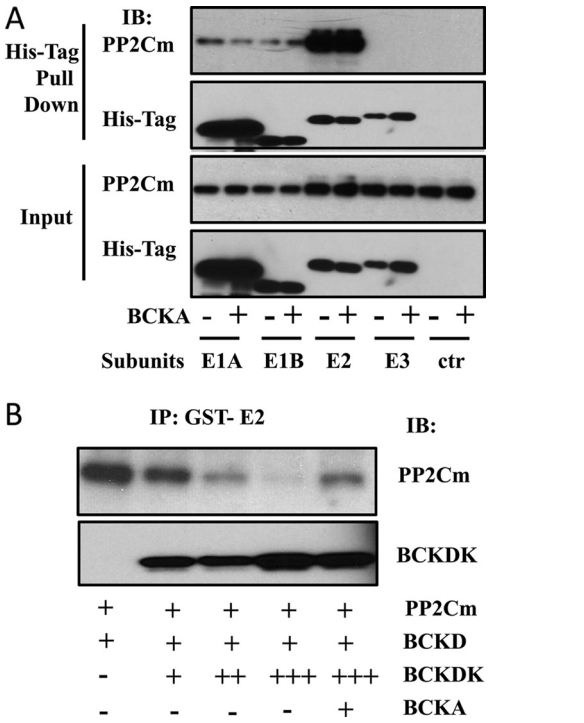


FIGURE 4. **BCKA regulates competition between PP2Cm and BCKDK *in vitro*.** *A*, His-tagged E1$\alpha$, E1$\beta$, E2, and E3 subunits of BCKD and His-tagged PP2Cm recombinant proteins were expressed in *E. coli* and purified. His tag was then cleaved by Factor Xa using the predesigned cleavage site during cloning to get full-length PP2Cm. Each individual subunit then incubated with PP2Cm, His tag pull-down was performed, and immunoprecipitate (*IP*) was analyzed by immunoblotting (*IB*) with antibodies labeled. *ctr*, no His-tagged subunits were incubated with PP2Cm. 1% input was loaded for control. *B*, PP2Cm protein was incubated with BCKD subunits (His-tagged E1$\alpha$, E1$\beta$, E3, and GST-tagged E2) for 4 h. Then, a different amount of His-tagged BCKDK was added to the incubation with or without three BCKAs (500 $\mu$M each) overnight. Glutathione-Sepharose beads were used for immunoprecipitation, and immunoprecipitate was analyzed by immunoblot with antibodies labeled. All recombinant proteins were N-terminally tagged.

line with a PP2Cm 3′-UTR reporter construct under the BCAA starvation or replenishment protocol (Fig. 3*D* and supplemental Fig. 3*B*). All of these data indicate that, similar to its counterpart BCKDK, the PP2Cm expression is also regulated at the transcriptional level by the nutrient level of BCAA. This result suggests a coordinated response between BCKDK (increased by starvation) and PP2Cm (reduced by starvation) to achieve optimal BCKD regulation according to the local level of BCAA.

*PP2Cm Competes with BCKDK in Binding with the BCKD Complex in Vitro*—It has been shown that BCKA triggers dissociation of the BCKDK from the BCKD complex (23). As shown before (25) and in Fig. 4*A*, PP2Cm showed a specific interaction with the E2 subunit, and its interaction with the BCKD E1$\alpha$ and E1$\beta$ subunits was relatively weaker. No interaction between PP2Cm and the E3 subunit was observed. However, the specific interactions between PP2Cm and E1/E2 subunits were not affected by the presence or absence of BCKA based on our *in vitro* binding assay (Fig. 4*A*). However, the presence of BCKDK significantly reduced the interaction between PP2Cm and the BCKD complex (Fig. 4*B*), and adding BCKA in the binding mixture attenuated this competition. A previous report has established that BCKA can diminish BCKDK interaction with the BCKD complex. Therefore, the BCKDK and the phosphatase PP2Cm compete to bind to the BCKD complex, and the balance of this competitive binding is modulated by the presence of BCKA substrate.

*BCKA Regulates PP2Cm Interaction with BCKD Holoenzyme in Cells*—To further characterize the BCKD complex interaction with PP2Cm in intact cells under different levels of BCKA, FLAG-tagged PP2Cm was expressed in HEK293 cells followed by culturing in medium with or without BCKA supplement. Based on immunoblot analysis, BCKA supplement led to a significant loss of the BCKDK protein as well as reduction in the E1$\alpha$ and the E1$\beta$ protein levels (Fig. 5*A*). Using immunoprecipitation with anti-FLAG antibodies, PP2Cm-interacting proteins were separated on a glycerol gradient and analyzed by immunoblotting for BCKD complex and BCKDK. Under normal BCAA/BCKA culture condition (Fig. 5*B*), the vast majority of the PP2Cm protein was detected in a low molecular mass fraction co-migrating with the E2 subunit only (fractions 2–4). In contrast, the majority of the E2 subunit was found in the high molecular mass fractions (20–26), co-migrating with the BCKDK and E1 subunits. This is consistent with previous findings that at basal state BCKD holoenzyme contains the BCKDK and has higher E1$\alpha$ phosphorylation status. Following BCKA treatment, there was a major shift of the E2 subunit to the high molecular mass fractions where the BCKDK was no longer detectable. In contrast, the level of PP2Cm in the high molecular mass fractions was significantly enhanced. These data suggest that PP2Cm binds to the BCKD holoenzyme complex in the presence of high levels of BCKA substrate. All of these findings support a dynamic change in the BCKD complex in response to high level of BCKA by replacing the inhibitory BCKDK with the activating PP2Cm phosphatase.

*Motifs Responsible for PP2Cm and E2 Interaction*—To delineate the BCKD E2 binding domains interacting with PP2Cm, we generated a series of PP2Cm N-terminal truncation mutants to perform co-immunoprecipitation assays. We found that the region between the residues 46 and 61 was critical to the association of PP2Cm with the complex (Fig. 6*A*). This region has no homologous counterparts among the other PP2C family members and may contribute to the specificity between PP2Cm and the BCKD. This conclusion was further supported by the observation that PP2C$\alpha$, a prototypic PP2C family member, was not able to interact with the BCKD complex based on our *in vitro* binding assay (Fig. 6*A*). To determine which domain of E2 interacts with PP2Cm, serial E2 truncation mutants were generated. Based on binding assays, PP2Cm did not interact with the lipoic acid-bearing domain (LBD) or LBD plus C-terminal hinge domain of the E2 alone. Instead, an intact E2 di-domain including both the LBD and the subunit binding domain was required to form a stable complex with PP2Cm (Fig. 6*B*). These data indicate that PP2Cm and BCKDK have potentially overlapping binding motifs within the LBD plus C-terminal hinge domain of the E2 (29). This may explain their mutually exclusive interaction with the BCKD complex.

*Single-nucleotide Polymorphisms (SNPs) in Human PP2Cm Gene Affected Enzymatic Action in Response to BCKAs*—A significant number of SNPs in the human PP2Cm coding sequence are listed in the NCBI dbSNP data base. By computational prediction, these SNPs can cause either synonymous,

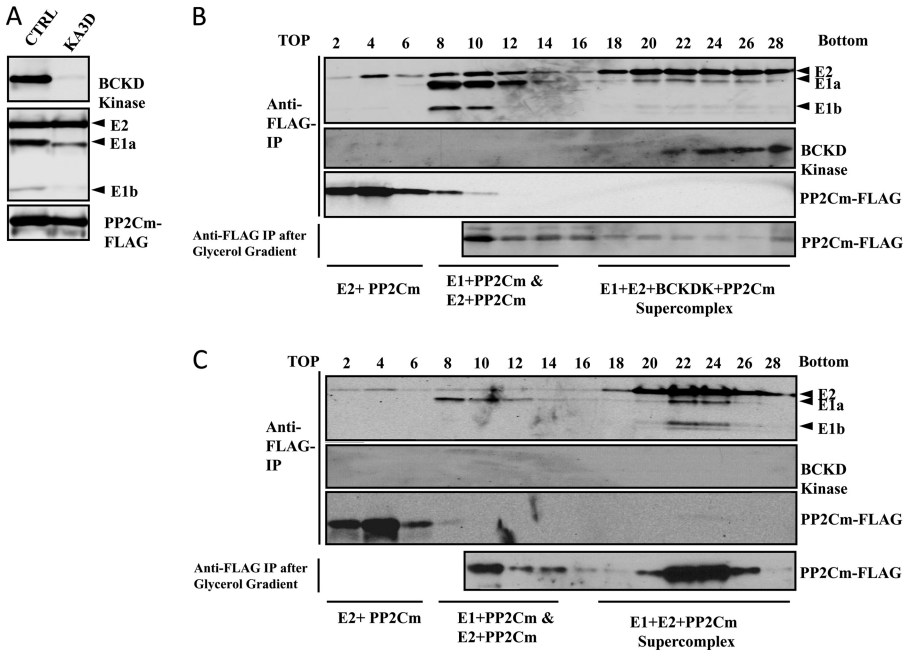


FIGURE 5. **BCKA enhanced interaction between PP2Cm and BCKD complex in cells.** *A*, HEK293T cells were transfected with the plasmids expressing FLAG-tagged PP2Cm. 48 h later, cells were treated with or without BCKAs (5 mM each) for 3 days (KA3D or CTRL, respectively). Total cell lysates were immunoprecipitated with anti-FLAG M2 beads, followed by immunoblotting with antisera against E1, E2, BCKDK, or FLAG. *B* and *C*, using the same cell lysates as treated with (*C*) or without BCKAs (*B*) for 3 days, the anti-FLAG immunoprecipitated (*IP*) proteins were separated on a glycerol gradient, and fractions were collected and enriched via second round of anti-FLAG immunoprecipitation in each fraction. BCKD components were examined by immunoblotting in each section.

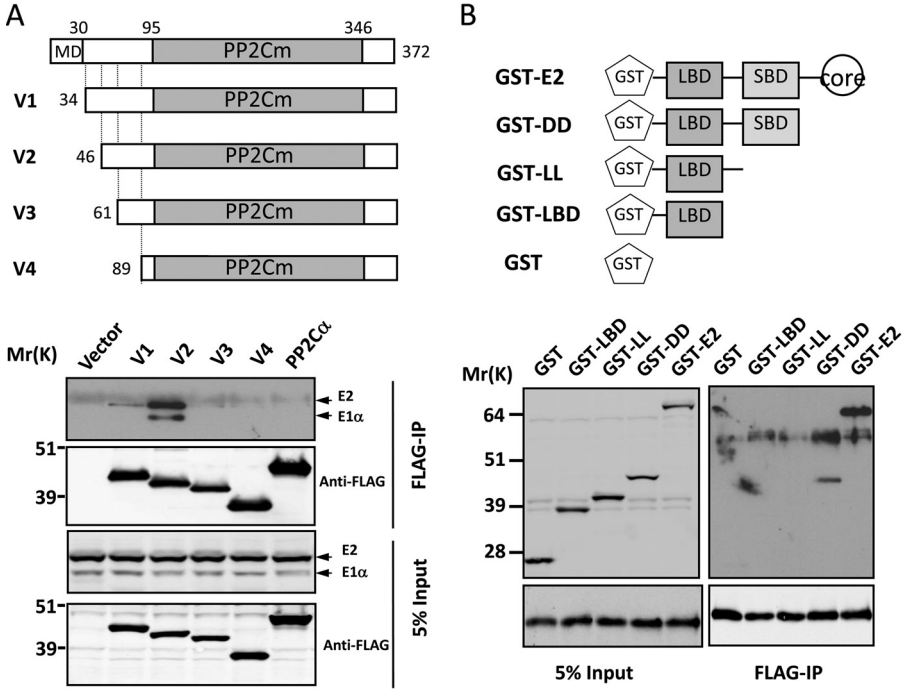


FIGURE 6. **Mapping of interaction domains between PP2Cm and BCKD E2.** *A*, full-length or truncated PP2Cm-FLAG expressing plasmid was transfected into HEK293T cells. Anti-FLAG immunoprecipitation (*IP*) was carried out, and the immunoprecipitate was analyzed by immunoblotting with the antibodies labeled. *MD*, predicted mitochondrial targeting signal (amino acids 1–30). *B*, PP2Cm-FLAG-expressing plasmid was co-transfected with either GST-tagged wild-type E2 or truncation mutants (shown on the *left*) into HEK293T cells. Anti-FLAG immunoprecipitation was carried out, and the immunoprecipitate was analyzed by immunoblotting with the GST-antibody.

missense, or frameshift mutations. To evaluate whether these SNPs affect PP2Cm enzymatic action in response to BCKAs, we cloned the wild-type human PP2Cm and generated five mutants with SNPs that cause missense or frameshift mutations through directed mutagenesis (Fig. 7*A*). By expressing these proteins in HEK293 cells where the endogenous PP2Cm level is very low, we can determine their function by examining BCKD phosphorylation with or without BCKA treatment.

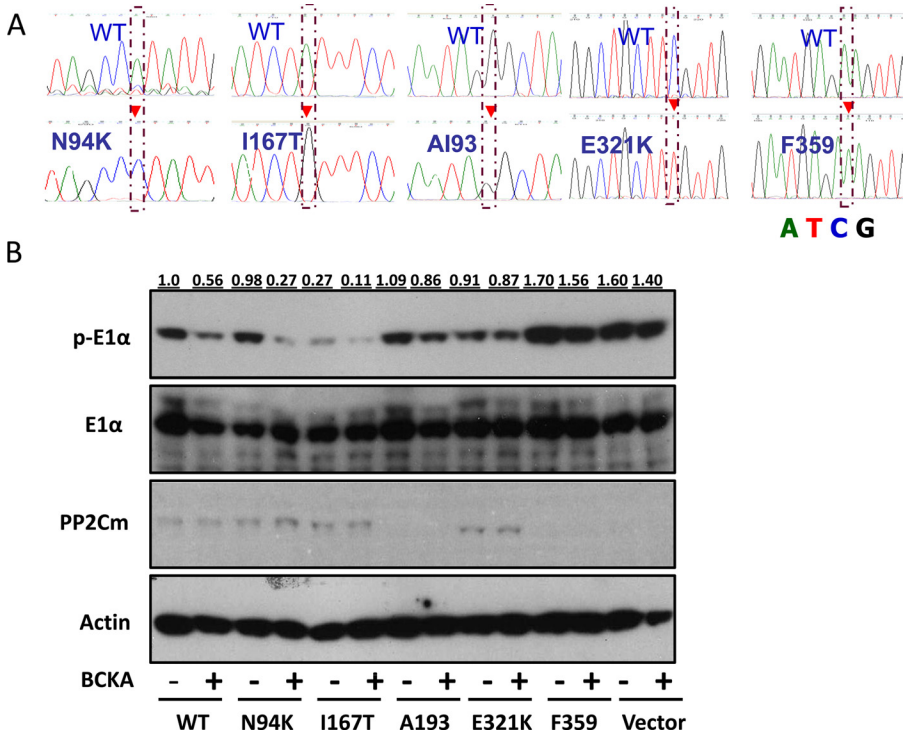


FIGURE 7. **Human PP2Cm SNPs affect phosphatase activity.** *A*, sequencing results of wild-type human PP2Cm and variants with SNPs generated through mutagenesis. *B*, Western blotting results of BCKD E1$\alpha$ phosphorylation. PP2Cm variants were expressed in HEK293 cells and treated with BCKAs (500 $\mu$M each) for 30 min. Cell lysates were analyzed by immunoblotting with labeled antibodies. The relative densitometric values of the pE1$\alpha$ bands are shown on *top* of *panels* in *B*.

Expression of the wild-type and N94K PP2Cm significantly decreased the E1$\alpha$-Ser$^{293}$ phosphorylation compared with the vector control (CMV promoter only) in response to BCKA treatment (Fig. 7*B*), suggesting that the N94K mutation has no impact on PP2Cm activity. In contrast, the I167T mutant PP2Cm significantly dephosphorylated BCKD in the absence of BCKA, suggesting a constitutively elevated activity. However, the Ala$^{193}$ and E321K mutants demonstrated some basal activity against BCKD but impaired responsiveness to BCKAs. Finally, the Phe$^{359}$ frameshift mutant showed no phosphatase activity at basal or after BCKA treatment. The Ala$^{193}$ and Phe$^{359}$ PP2Cm mutants were not detected by immunoblot using human PP2Cm antibody. Therefore, it remains unclear whether these mutant proteins are phosphatase dead due to loss-of-function mutations or unstable due to premature truncation. These analyses suggest that in human populations, individuals carrying different PP2Cm SNPs may have different capability of BCAA catabolism.

## DISCUSSION

Essential amino acids are critical to normal cell growth and physiological function, and their homeostasis is largely maintained by highly regulated catabolic activities. For BCAAs, the key to their catabolic regulation is the phosphorylation status of the BCKD subunit E1$\alpha$. In this study, we performed the first comprehensive molecular and biochemical analysis of the BCKD phosphatase, PP2Cm, focusing on its expression and function under different nutrient environment. Our data indicate that, in adult mice as well as during embryonic development, the PP2Cm expression is highly enriched in several vital organs but low in skeletal muscle. The PP2Cm transcription is repressed by starvation or low BCAA condition, opposite from the changes observed for the BCKDK (19, 20). Whereas PP2Cm binds to BCKD E2 and E1 subunits in a BCKA-independent manner, its binding motifs overlap with the BCKDK binding domain on E2 (29, 30). This competition in BCKD binding is dynamically regulated by the BCKA levels. Consequently, low BCKA favors kinase association with BCKD, and high BCKA favors phosphatase association with BCKD. Therefore, our study has established a nutrient-sensitive regulation of BCAA catabolism as the result of coordinated regulation of both BCKDK and phosphatase at transcriptional and posttranslational levels (Fig. 8).

The tissue expression profile of PP2Cm may reflect the relative levels of the BCAA catabolic activities in different tissues. A low level of PP2Cm expression is associated with a low BCKD activity in skeletal muscle whereas liver is the primary organ for BCKA degradation (15). In brain, a high level of PP2Cm expression is more restricted in the cortex and the choroid plexus. It is known that BCAA is involved in the biosynthesis of glutamate and GABA, both critical neurotransmitters (15), and choroid plexus is responsible for cerebrospinal fluid production and metabolic waste removal. It is plausible that a high level PP2Cm expression in these subregions of the brain may reflect the necessary role of BCAA catabolism in normal biosynthesis and transport of neurotransmitters. This information provided additional insight to the pathogenesis of seizure and other neurotoxic effects in maple syrup urine disease patients. It is intriguing to observe that the PP2Cm level is high in both car-

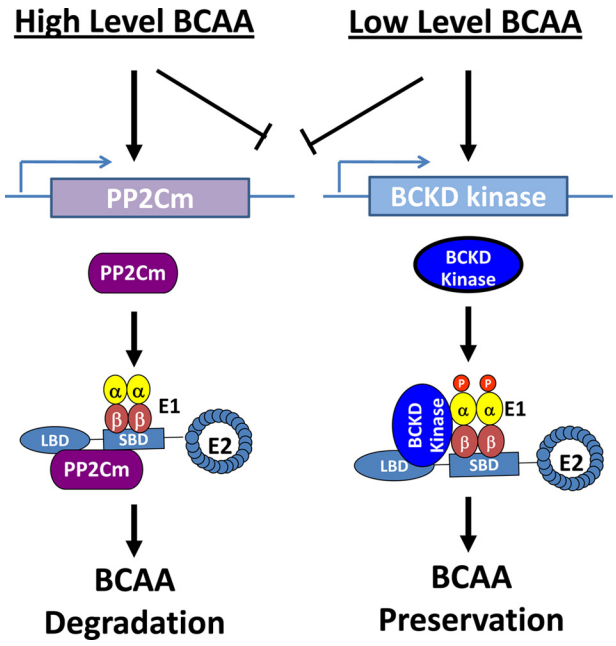


FIGURE 8. **Schematic representation of BCAA homeostasis maintenance through coordinated regulation of BCKDK and phosphatase at transcriptional and posttranslational levels.** A high level of BCAA promotes PP2Cm transcription while suppressing BCKDK expression. In the meantime, PP2Cm and BCKDK compete with each other to interact with BCKD complex. A high level of BCKA favors PP2Cm binding, whereas a low level of BCKA favors kinase binding. The coordinated response between BCKDK and PP2Cm achieves optimal BCKD activity to maintain BCAA homeostasis.

diac and diaphragm muscle but low in skeletal muscle. The low BCKD phosphatase and high BCKDK activities in skeletal muscle (31) are consistent with almost 100% suppression of the BCKD activity in the tissue (9, 15). This finding suggests that skeletal muscle may serve as a storage pool for the BCAAs *in vivo* where protein synthesis capacity is high whereas the BCAA catabolic activities are low. However, BCAA has not been implicated as an important source of energy for the heart. The functional role of high levels of BCAA catabolic activity in cardiac and diaphragm muscle remains to be further investigated.

Our study showed that PP2Cm competes with BCKDK for binding with BCKD E2 subunit in a keto acid-dependent manner. Within the BCKD holoenzyme, E1 and E3 subunits bind to E2 through the subunit binding domain (30). BCKDK interacts with E2 via the LBD-linker domain (29). More structural analysis would be needed to define the binding motifs between PP2Cm and the E2 and the molecular basis for the sensitivity to BCKA. It is conceivable that BCKA binds with BCKDK, leading to conformational change and dissociation from the E2, opening E2 motifs for PP2Cm binding. Nevertheless, our study provided the first direct biochemical evidence that dynamic interaction between BCKD and its kinase *versus* phosphatase is part of the feedback regulation at posttranslational level to maintain BCAA homeostasis.

The abnormal plasma BCAA level has been identified in patients under different pathological conditions including neurodegenerative, cardiovascular, and metabolic diseases (10–14). However, it remains unclear whether the disruption of BCAA homeostasis is a consequence of diseases or whether it contributes to pathological processes. In a previous report, we demonstrated that the PP2Cm level was dramatically decreased in stressed murine heart (27). Meanwhile, it has been reported that the plasma BCAA level is increased in rodents with failing heart (14). Therefore, it is conceivable that pathological stresses down-regulate PP2Cm, leading to impaired BCAA catabolism. However, whether BCAA and BCKA accumulation in turn affect disease progression still remains unknown, although BCKA induces oxidative stress in cells (25). Meanwhile, it remains unclear how PP2Cm gene expression is regulated under pathological conditions. In our previous and present studies, PP2Cm expression can be regulated by stress as well as nutrients. It will be interesting to discover how nutrient and stress signaling modulate PP2Cm gene transcription.

The intricate regulation of PP2Cm expression and function by the BCAA/BCKA nutrient status supports the notion that the homeostasis of BCAAs must be tightly regulated. The fact that defects of BCKD activity due to mutations are the major cause of maple syrup urine disease further highlight the importance of BCAA catabolic regulation in normal development. In this report, we find that a number of SNPs in the coding sequence of human PP2Cm significantly alter the phosphatase activity or BCKA responsiveness. Therefore, the carriers of these polymorphic alleles may have impaired PP2Cm activity and BCAA catabolism. Further study in human would be needed to validate this hypothesis.

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