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Review

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Proteomics of vitamin B_{12} processing

Abstract

The causes of cobalamin (B_{12}, Cbl) deficiency are multifactorial. Whether nutritional due to poor dietary intake, or functional due to impairments in absorption or intracellular processing and trafficking events, the major symptoms of Cbl deficiency include megaloblastic anemia, neurological deterioration and in extreme cases, failure to thrive and death. The common biomarkers of Cbl deficiency (hyperhomocysteinemia and methylmalonic acidemia) are extremely valuable diagnostic indicators of the condition, but little is known about the changes that occur at the protein level. A mechanistic explanation bridging the physiological changes associated with functional B_{12} deficiency with its intracellular processers and carriers is lacking. In this article, we will cover the effects of B_{12} deficiency in a *cblC*-disrupted background (also referred to as MMACHC) as a model of functional Cbl deficiency. As will be shown, major protein changes involve the cytoskeleton, the neurological system as well as signaling and detoxification pathways. Supplementation of cultured MMACHC-mutant cells with hydroxocobalamin (HOCbl) failed to restore these variants to the normal phenotype, suggesting that a defective Cbl processing pathway produces irreversible changes at the protein level.

Keywords: *cblC*; cobalamin deficiency; cobalamin processing; homocysteine; methylmalonic acid; MMACHC (methylmalonic aciduria type C with homocystinuria).

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Introduction

Vitamin B_{12} (cobalamin, Cbl), an ancient vitamin and 'Nature's most beautiful cofactor' [1, 2] is required by all cells in the body. Humans rely on dietary supplies of the vitamin since a biosynthesis pathway is lacking in higher organisms. Only a relatively few archaea and bacteria express the 30 or so enzymes required to synthesize the complex cobalt-containing macrocycle and its attached dimethylbenzimidazole moiety (Figure 1) [4]. In mammals, Cbl serves as a cofactor for methionine synthase (MS) and methylmalonyl-CoA mutase (MUT). Besides these canonical functions, new roles have been described for Cbl including intracellular signaling [5], apoptosis [6–8], oxidative stress [9] and cytokine and growth factor-mediated regulation [10, 11]. The exact mechanisms underlying these non-canonical actions remain largely unexplored. Dietary vitamin B_{12} enters the gastrointestinal tract first by complexation with the B_{12}-binder haptocorrin (HC), which is present in saliva. Once it reaches the stomach, the vitamin is relayed to a second B_{12}-binder, intrinsic factor (IF). Absorption of B_{12} occurs in ileal enterocytes of the lower intestine, where the vitamin dissociates from IF and binds to apo-transcobalamin (TC), the cellular transporter of vitamin B_{12} [12]. The transcobalamin receptor (TCblR) captures holo-TC (TC•XCbl) from circulation and internalizes the complex by absorptive endocytosis [12]. In the acidic milieu of the endosomal compartment, holo-TC dissociates from its receptor and TCblR recycles back to the cell surface [13]. Fusion of late endosomes carrying the holo-TC with lysosomes results in the proteolytic degradation of transcobalamin. Cobalamin is thereby released within the lysosome and exported to the cytosol via the *cblF* gene product LMBD1 [14–21]. It was recently reported that the *cblJ* gene product, ABCD4, may work in conjunction with LMBD1 to mediate transport out of the lysosome to the cytosol [22]. Cobalamin then undergoes processing and trafficking to cytosolic methionine synthase, or to mitochondrial methylmalonyl-CoA mutase.

Cellular processing of B_{12}: MMACHC (CblC)

Much of our knowledge of Cbl processing (defined as removal of the upper axial ligand with either concerted or subsequent reduction of the cobalt center) arose from

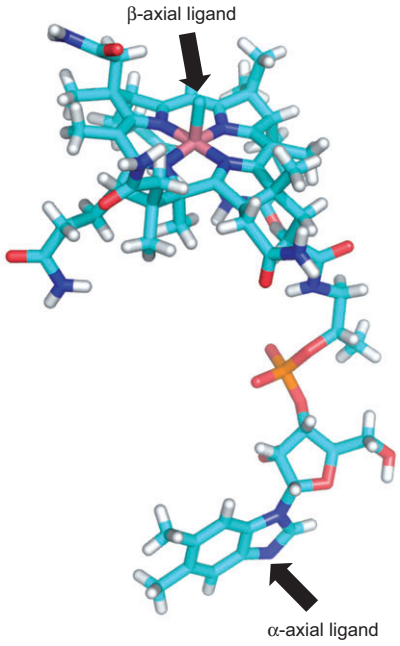

Figure 1 Structure of cobalamin.
The base-off conformation (free α-axial ligand) of methylcobalamin (MeCbl) is shown. The upper, β-axial position can be occupied by different ligands, including cyanide (CNCbl, vitamin B_{12}), 5′-adenosyl (AdoCbl) and hydroxo (HOCbl). The structure of MeCbl was taken from the MMACHC•MeCbl complex (PDB 3SC0 [3]). The Figure was generated with PyMol software (DeLano Scientific LLC).

ex vivo studies with fibroblasts from patients carrying inborn errors of Cbl metabolism. Mutations in the genes that encode the enzymes or proteins involved in Cbl processing, trafficking and biosynthesis are defined by Cbl complementation groups (*cblA-cblG* and *mut*) [23, 24]. A study by Chu et al. suggested that dietary methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) must undergo processing of their upper axial ligand prior to their incorporation into MS and MUT, respectively [25]. The first case report of functional Cbl deficiency caused by an inborn error of metabolism was provided by Harvey Mudd et al., more than 40 years ago [26]. The patient under study belonged to the *cblC* complementation group and presented with combined homocystinuria and methylmalonic aciduria [26]. Cultured patient fibroblasts displayed slightly reduced uptake of Cbl with respect to normal skin fibroblasts, efflux of Cbl at long incubation times, and impaired biosynthesis of both MeCbl and AdoCbl [26]. This was the first evidence that the gene responsible for the *cblC* complementation group was required for a step prior to both cofactors biosynthesis. A number of patients presenting with both early and late onset of the cblC disease were reported thereafter, which amounts to more than 360 cases to date [27].

In vitro assays with cell extracts showed that cblC fibroblasts possessed reduced Cbl β-transferase activity and/or Cbl reductase activity [28–32]. These studies suggested that the methylmalonic aciduria combined with homocystinuria type C (MMACHC) protein was involved in processing of the upper axial ligand of Cbls and/or reduction of the cobalt center. The work by Pezacka and Jacobsen also revealed a requirement for the most abundant intracellular thiol, glutathione, in a step preceding cofactor biosynthesis [28–32]. It has been postulated that MMACHC protein, the product of the *cblC* gene, is the immediate downstream acceptor of the Cbl cargo exiting the lysosome and the protein responsible for processing of the upper axial ligand of incoming dietary Cbls [33]. It was not until 2006 that the gene responsible for the cblC phenotype was identified and characterized [34]. According to the primary structure, the MMACHC protein is not a member of any previously identified gene family [34]. Although it is well-conserved among mammals, its C-terminal end does not seem to be conserved in eukaryotes outside mammalia, and no homologous proteins are found in prokaryotes [34]. The *MMACHC* gene is expressed in most tissues. High mRNA levels were detected in fetal liver with lower levels being detected in spleen, lymph node, thymus and bone marrow, and no message was detected in peripheral blood leukocytes [34]. Work by Koutmos et al. showed that a truncated form of cblC lacking the last 38 amino acid residues is predominantly expressed in most tissues [3]. Importantly, a comprehensive examination of the mitochondrion proteome identified MMACHC as one of its resident proteins [35]. How MMACHC is transported into the mitochondrion and its role in this compartment remains to be elucidated.

Biophysical and structural characterization of the B_{12}-processing enzyme MMACHC and its interactions with MMADHC (CblD)

The mystery of how decyanation of cyanocobalamin (CNCbl) occurs was recently solved by the in vitro studies of Kim et al. [36]. The reductive decyanation of CNCbl is catalyzed by the MMACHC protein in the presence of a flavoprotein reductase and NADPH [36]. The authors reported that *cblC* bound both MeCbl and AdoCbl inducing their base-off conformation [36]; however, it did not catalyze the

dealkylation of MeCbl and AdoCbl, the two major dietary forms of Cbl. This intriguing finding was re-examined via ex vivo studies [37], and a new function was uncovered for the *cblC* protein: MMACHC is also a Cbl dealkylase [37]. Mechanistically, dealkylation of AdoCbl and MeCbl is distinct from the decyanation pathway. MMACHC catalyzes the dealkylation of alkylcobalamins by a reaction involving the nucleophilic attack of the Co−C bond by the thiolate anion of glutathione [38]. Demethylation of MeCbl was much faster than the removal of the 5′-adenosyl group from AdoCbl (11.7±0.2 and 0.150±0.006/h, respectively) [38]. In addition, MMACHC was capable of dealkylating a series of MeCbl analogues namely, ethylcobalamin, propylcobalamin, butylcobalamin, pentylcobalamin and hexylcobalamin [37]. MMACHC catalyzed the removal of the alkyl group at the upper axial position of all of the MeCbl analogs, however, the rate of dealkylation decreased with increasing alkyl chain length [38]. Whether the latter is a result of conformational alterations in the MMACHC protein induced by the more bulky alkyl moieties or due to an unfavorable incorporation of the longer alkyl carbocations into glutathione remains to be elucidated.

An interesting feature of MMACHC is its stability. Froese et al. demonstrated that MMACHC is naturally thermolabile (T_m=39°C) and that some of the most frequent mutations that occur in humans exacerbate this property [39] as well as its ability to bind Cbls [40]. Studies with the bovine isoform of MMACHC revealed that the reduced form of glutathione stabilizes MMACHC, suggesting that intracellular redox control could play a role in the regulation of the protein's lifetime [41–44]. Koutmos [3] and Froese [45] have independently obtained high-resolution X-ray crystal structures of MMACHC. The work by Koutmos et al. revealed that MMACHC possesses an N-terminal flavodoxin nitroreductase domain, which can use FMN or FAD to catalyze the reductive decyanation of CNCbl [3]. MMACHC possesses a large cavity for binding B_{12} in its base-off configuration (Figure 2, panel A), a binding mode thought to facilitate the reductive removal of the cyanide group at the β-axial position. Unlike other Cbl-dependent enzymes, the base-off Cbl binding by MMACHC does not involve the coordination of a His residue from the protein backbone [3]. Binding of MeCbl to MMACHC induces measurable conformational changes in three different loop-structured domains around the B_{12} cavity (Figure 2, panel A, arrows). Froese et al. elucidated the first structure of MMACHC bound to AdoCbl [45]. The overall fold of MMACHC does not differ markedly from that reported of MMACHC complexed with MeCbl, but revealed a highly conserved dimerization cap for the β-axial 5′-adenosyl ligand, and an arginine-rich

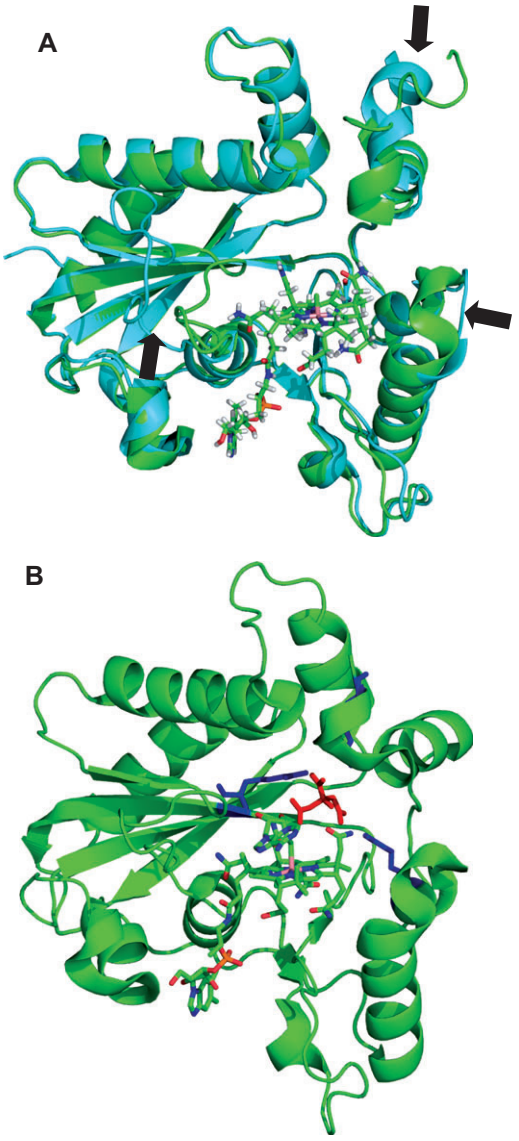

Figure 2 Structures of MMACHC.
(A) Apo-MMACHC (PDB 3SBZ, cyan) and the MMACHC•MeCbl complex (PDB 3SC0 [3], green). The arrows indicate the major regions that undergo conformational change upon binding of Cbl. (B) The MMACHC•AdoCbl complex (PDB 3SOM [45]) displays three conserved Arg residues within the Arg-rich pocket (Arg161, Arg206 and Arg230, blue) and a citrate molecule (red) at a site predicted to be occupied by GSH under physiological conditions. The Figures were generated with PyMol software (DeLano Scientific LLC).

glutathione-binding pocket up above the β-axial ligand position [45]. Importantly, the arginine-rich pocket comprises residues Arg161, Arg206 and Arg230 (Figure 2, panel B, blue) all of which are sites for point mutations that occur in humans, leading to *cblC* disease. A citrate molecule from the solvent was identified in the region predicted to be occupied by glutathione during catalysis (Figure 2, panel B, red). Froese et al. further showed that

recombinant mutant Arg206Gln was insoluble suggesting a structural role for this residue, and that mutants Arg161Gln and Arg230Gln abolished GSH binding and dealkylase activity, which is a strong indication that these amino acid residues are critical for GSH binding [45]. The authors noted that FMN, and to a lesser extent Cbl, induces the dimerization of MMACHC, a previously unrecognized feature of the protein [45].

The exact sequence of events that occur upon processing of the upper axial ligand of Cbl by MMACHC prior to its delivery to the two Cbl-dependent enzymes is unknown. Methylmalonic aciduria combined with homocystinuria type D (MMADHC), the product of the *cblD* gene has been proposed to have an adapter function to target newly processed Cbl into MS or MUT [3, 24–45]. The *cblD* gene has been recently mapped to chromosome 2q23.2 and has been designated MMADHC [46]. The predicted gene product has sequence similarity with a bacterial ATP-binding cassette transporter, possesses a putative Cbl binding motif and a putative mitochondrial targeting sequence [46]. The remarkable heterogeneity [47] of the *cblD* disorder in humans can be partially rationalized in terms of mutation sites. Mutations affecting the putative Cbl binding site would lead to a disrupted delivery of newly processed Cbls to both MS and MUT, resulting in combined homocystinuria and methylmalonic aciduria (a phenotype common to the *cblC* disorder), whereas mutations in the mitochondrial targeting sequence would result in disrupted delivery of Cbl to MUT, and therefore, would lead to isolated methylmalonic aciduria. Work by Stucki et al. indicated that MMADHC is a single protein with two different domains that interact with either cytosolic or mitochondrial targets [48]. Plesa et al. were the first to demonstrate that MMACHC and MMADHC interact [49]. Surface plasmon resonance studies showed that the two proteins interact at a 1:1 stoichiometry with an affinity in the mid-nanomolar range [49]. Phage display assays identified five putative MMACHC-binding sites. Two of these belong within the ABC-transporter homology domain, and the other three potential binding sites comprise residues in the C-terminal portion of MMADHC [49]. Further, phage display studies performed by Deme et al. identified several putative MMACHC binding sites in MMADHC, as well as self-binding regions in MMACHC [50]. In agreement with previous findings by Plesa et al., the authors proposed that the MMADHC functionality involves its C-terminal residues, which interact with MMACHC to orchestrate the fate of newly processed Cbl. Interestingly, none of these studies found evidence for Cbl binding to MMADHC [49, 50], despite the presence of a partially conserved B_{12}-binding motif in its primary sequence.

Metabolic profile of cells with a defective MMACHC protein

An assessment of the metabolic and morphological characteristics of three *cblC* cell lines [WG1801 (c.217C>T/c.217C>T), WG2176 (c.1–234A>G/c.609G>A) and WG3354 (c.435_436delAT/c.435_436delAT)] was first conducted to determine whether this genetic system would be a suitable model of cobalamin deficiency.

The Cbl processing activity of these *cblC* cell lines has been characterized ex vivo [37]. All *cblC* cell lines released increased levels of homocysteine and methylmalonic acid compared to the normal cell line [51]. Supplementation with hydroxocobalamin (HOCbl) reduced the levels of methylmalonic acid exported by the *cblC* cell lines, but failed to substantially diminish the levels of homocysteine [51]. All *cblC* cell lines displayed reduced uptake and impaired processing of HOCbl compared to the normal cell line [51]. This is in line with the finding that all *cblC* mutant cell lines take up or retain lower amounts of Cbl compared to the normal fibroblasts [26], a phenomenon well-mirrored in total levels of intracellular folates [51]. Therefore, it appears that the positive response of the *cblC* cell lines to HOCbl supplementation (lowering of methylmalonic acid) may not be directly associated with the activity of mitochondrial MUT. An altered cellular morphology was observed when *cblC* cells were seeded at very low densities, but this vanished once the cultures became confluent. Whether this is an unwanted effect of elevated homocysteine and methylmalonic acid, or the presence of a defective MMACHC protein is uncertain. Altogether, the selected *cblC* cell lines displayed properties consistent with a model of functional deficiency.

Proteomics of defective B_{12} processing in *cblC* cell lines

The recent years have witnessed much progress in the elucidation of the genes, the proteins and the diverse phenotypes associated with B_{12} processing and trafficking disorders [6, 9, 27, 34, 36, 38–40, 45, 49–71]. However, considerably less is known about the changes that accompany Cbl deficiency at the protein level. The proteome of fibroblasts of three genetically unrelated, severely ill *cblC* patients were examined in order to identify changes associated with functional Cbl deficiency [51]. The effect of supplementation with HOCbl, a form of Cbl used in the treatment of *cblC* patients, was also evaluated [51]. Proteomic studies

performed with diseased individuals are often challenged by the availability of their cells or tissues, and the examination of the cblC fibroblast proteome by two-dimensional difference gel electrophoresis (2-D DIGE) was no exception. Skin fibroblasts were the only sample available from the patients, WG1801, WG2176 and WG3354, all of whom had deceased by the time the study was conducted. Likewise, fibroblasts from healthy family members without *cblC* disease were not available. The problem of small sample size (three *cblC* fibroblasts samples and three from normal individuals), was partially solved by identifying protein expression differences that were common to all three *cblC* fibroblasts sources and by excluding the changes that were particular to an individual sample. Although these hurdles prevent the direct extrapolation of protein changes identified in fibroblasts to other cell types unless appropriate validation is conducted, the study provides a global view of the changes associated with the *cblC* disease by utilizing cells that are genetically stable, easy to grow and for which genetically unrelated controls are readily available (fibroblasts from normal patients).

Table 1 shows the changes in protein expression in *cblC* vs. normal fibroblasts. Major changes occurred in proteins related to cytoskeleton, the nervous system, signaling and cellular detoxification [51]. Table 2 presents the changes in protein expression in *cblC* vs. normal fibroblasts supplemented with 723 nM (1 μg/mL) HOCbl. Supplementation of the cell cultures with HOCbl led to a global downregulation of the *cblC* fibroblast proteome. The target proteins, namely MMACHC, MS and MUT could not be detected in the study, possibly due to their naturally low levels of expression and the limit of detection of the 2-D DIGE technique (~0.25 ng protein) [72]. An independent study by Sourmala et al. demonstrated that MS activity of 5 cblC fibroblast patient cell lines was four to five-fold lower than normal fibroblasts, and that MUT activity was two to five times lower in cblC compared to normal fibroblasts [73]. No differences in the expression of HDL binding protein, collagen VI α1 precursor, eukaryotic elongation factor 2 (eEF2), glutathione-S-transferase omega 1 (GSTO1), GST M3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were observed between the normal and *cblC* fibroblasts upon supplementation with HOCbl, which suggests that supplying the vitamin corrected for the alterations caused by its deficiency, an effect that must be independent of the cblC processing route.

Protein	Gene ID	Fold change	Mascot score	t-test (p-Value)
Proteins whose expression was upregulated in cblC fibroblasts				
HDL binding protein	42716280	2.87	807	0.029
Eukaryotic translation elongation factor 2	4503483	2.5	1234	0.042
Collagen type VI, α2, isoform 2C2	115527062	2.1	295	0.028
Collagen type VI, α1 precursor	87196339	2.1	662	0.028
Ribosomal protein S14	5032051	2.45	260	0.033
H2B histone family member A	4504257	2.45	90	0.033
Proteins whose expression was downregulated in cblC fibroblasts				
Serine (or Cys) proteinase inhibitor (protease inhibitor 6 or serpin B6)	41152086	−2.09	2322	0.032
Caldesmon-1, isoform 2, Ct truncated	4826657	−2.09	956	0.032
β-actin	4501885	−2.11	1116	0.024
Tubulin α, ubiquitous	57013276	−2.17	907	0.028
Chloride intracellular channel 4	7330335	−2.04	1507	0.018
Collagen type VI, α2, isoform 2C2	115527062	2.1	295	0.028
Collagen type VI, α1 precursor	87196339	2.1	662	0.028
Ubiquitin carboxyl esterase L1	21361091	−2.19	1261	0.021
Plastin 3	7549809	−2.9	1471	0.023
Vimentin	62414289	−2.44	3652	0.032
Glutathione transferase omega 1	4758484	−2.1	172	0.0096
Glutathione transferase	4504183	−2.84	821	0.016
Glutathione transferase M3	2306552	−2.19	281	0.021
PDI associated 3 precursor	21361657	−2.9	529	0.023
SH3 domain binding Glu-rich protein like 3	13775198	−2.23	312	0.042
S100 Ca-binding protein A6	7657532	−2.23	121	0.042
GAPDH	7669492	−2.11	761	0.024

Table 1 Protein changes in *cblC* vs. normal fibroblasts grown in the absence of exogenous hydroxocobalamin (n=3, α=0.05). Adapted from Hannibal et al. [51].

Protein	Gene ID	Fold change	Mascot score	t-test (p-Value)
Hsp 90 protein 1, β	20149594	−2.03	1758	0.042
Hsp 90 α (cytosolic), class A, member 1, isoform 2	40254816	−2.03	1393	0.042
Transgelin 2	4507357	−2.03	1364	0.04
Annexin V	4502107	−2.08	1285	0.031
Phosphoglycerate dehydrogenase	23308577	−2.13	2316	0.033
Inosine monophosphate dehydrogenase 2	66933016	−2.13	778	0.042
Hsp70 protein 8, isoform 1	5729877	−2.15	2218	0.042
Annexin VI isoform 1	71773329	−2.15	1678	0.042
ATPase, H+ transporting, lysosomal	19913424	−2.15	701	0.042
Voltage dependent anion channel 1	4507879	−2.17	657	0.034
Voltage dependent anion channel 2	42476281	−2.17	405	0.034
S100 Ca-binding protein A6	7657532	−2.19	141	0.031
Peroxirredoxin 2, isoform a	32189392	−2.23	899	0.031
Septin 11	8922712	−2.25	1237	0.031
Cofilin 1	5031635	−2.26	473	0.031
Hsp70, protein 5,	16507237	−2.28	3310	0.031
PDI associated 4	4758304	−2.28	435	0.031
Peroxiredoxin 1	4505591	−2.29	269	0.031
Lamin A/C, isoform 2	5031875	−2.3	2672	0.049
Dihydropyrimidase -like 2	4503377	−2.3	1622	0.049
Chaperonine containing TCP1, sub3	63162572	−2.3	829	0.049
Peroxiredoxin 6	4758638	−2.34	600	0.042
PDI associated 3 precursor	21361657	−2.38	1140	0.042
Tubulin α6	14389309	−2.38	1014	0.042
Chloride intracellular Channel 4	7330335	−2.38	621	0.034
DJ-1	31543380	−2.52	152	0.031
Glutathione transferase	4504183	−2.54	926	0.031
Tryptophanyl-tRNA synthetase isoform a	47419914	−2.63	2129	0.033
Collagen type VI, isoform 2C2	17402875	−2.72	953	0.039
Vinculin isoform VCL	4507877	−2.72	660	0.039
Ubiquitin and ribosomal protein S27a precursor	4506713	−2.79	382	0.031
Annexin V, A2, isoform I	50845388	−2.9	355	0.039
Triosephosphate isomerase 1	4507645	−2.93	954	0.039
SH3 domain binding Glu-rich protein like 3	13775198	−3.1	565	0.032
Ubiquitin carboxyl esterase L1	21361091	−3.16	949	0.037
Vimentin	62414289	−5.03	3908	0.031

Table 2 Protein changes in *cblC* vs. normal fibroblasts supplemented with 723 nM hydroxocobalamin (n=3, α=0.05). The proteins are listed in descending order of fold change in expression. Adapted from Hannibal et al. [51], with permission.

Cytoskeleton: assembly and remodeling

Substantial changes in protein expression levels were identified for cytoskeletal proteins with structural and regulatory roles. These included collagen VI, α1 and 2C2 isoforms, vimentin (VIM), tubulin-α (TUBA1B), β-actin (ACTB), vinculin isoform, plastin 3 (PLS3), lamin A/C isoform 2, chaperonin TCP1, caldesmon 1 (CALD1), cofilin 1 and transgelin 2. Changes in cytoskeletal proteins have been also reported for a patient cell line belonging to the *cblD* complementation group [74] and also, for human fibroblasts [75] and colonocytes [76] under conditions of folate deficiency. Collagen VI, isoform 2C2 was upregulated in *cblC* fibroblasts. Supplementation of the *cblC* cultures with HOCbl downregulated its expression. Mutations in the genes that code for collagen VI subunits result in Ullrich syndrome [77] and Bethlem myopathy [78], an autosomal dominant disorder. An upregulation of collagen VI α2 was also noted in a patient with *cblD* disease [74]. The patient described in the study presented with isolated methylmalonic aciduria. Another study performed in human smooth muscle cells demonstrated that high levels of homocysteine cause an upregulation in the production of collagen, which could be related to

the pathogenesis of homocystinuria [79]. Patients with untreated homocystinuria have widespread premature atherosclerosis with intimal thickening and collagen-rich fibrous plaques [80]. An altered collagen expression may contribute to the pathology of cblC given the deregulation in the fibroblasts from patients (elevated Hcy and increased collagen expression). VIM was also downregulated in *cblC* fibroblasts. VIM is a cytoskeletal protein whose major role is stabilizing the architecture of the cytoplasm. An in vitro study conducted by Mor-Vaknin et al. revealed that monocyte-derived macrophages secrete VIM into the extracellular space [81]. Secretion of VIM was stimulated by tumor necrosis factor-α (TNF-α) and inhibited by IL10, suggesting that the protein is also involved in the immune response [81]. It was found that *cblC* fibroblasts expressed lower levels of VIM compared to normal cells, and, interestingly, downregulation was further exacerbated by supplementation with HOCbl [51]. The abnormal expression of VIM in *cblC* fibroblasts could be responsible for their altered cellular morphology. Less is known about the roles of other cytoskeleton-related proteins identified in the study. However, it appears that their functions are broadly related to the regulation of smooth muscle, non-muscle contraction (CALD1, a calmodulin and actin-binding protein) [82] and axonogenesis (PLS3) [83] and its related neuromuscular disease, spinal muscular athrophy [84]. Neurological and muscular disorders commonly seen in *cblC* patients are often alleviated by the administration of HOCbl. PLS3 levels corrected to the normal pattern of expression upon supplementation with HOCbl, which could partly account for the improvement observed in some *cblC* patients.

Nervous system and signaling

Ubiquitin carboxy-terminal hydrolase L1 (UCHL1) is another of the downregulated proteins in *cblC* fibroblasts. UCHL1, also known as PGP9.5, is an essential component of the ubiquitin-proteasome system (UPS), a major pathway for protein degradation [85]. UCHL1 is one of the most abundant proteins in the brain and is predominantly localized in neurons [86] and cells of the diffuse neuroendocrine system [87]. Downregulation and extensive oxidative modification of UCHL1 occurs in brain tissue of patients with Alzheimer's as well as Parkinson's diseases [88–90]. Downregulation of UCHL1 in *cblC* fibroblasts was not restored to normal levels upon supplementation with HOCbl. Therefore, downregulation of UCHL1 could contribute to the neurocognitive manifestations of the *cblC* disorder and the poor improvement observed after treatment with HOCbl [91]. Three other proteins were downregulated in *cblC* fibroblasts grown in the presence of exogenous HOCbl: DJ-1 (Parkinson's disease protein 7), dihydropyrimidase-like 2 (DPYLS2), and annexin V A2 isoform I. DJ-1 belongs to a family of peptidases that act as a positive regulator of androgen receptor-dependent transcription. DJ-1 may also function as a redox-sensitive chaperone and it is thought to protect neurons from oxidative damage [92]. Defects in this gene are the cause of early-onset Parkinson's disease 7 [92, 93]. DPYLS2 shares homology with dihydropyrimidase and it is expressed actively in the fetal and neonatal brains of mammals and chickens. Little is known about this family of proteins, however, they are thought to be intracellular transducers in the development of the nervous system [94].

Gene regulation and protein synthesis

Fibroblasts with a defective *cblC* gene grown in the absence of exogenous HOCbl displayed an upregulation in the expression levels of eEF2, ribosomal protein S14 (RPS14) and H2B member A, and a downregulation in the levels of the calcium binding protein S100A6 [51]. Supplementation with HOCbl restored the expression of eEF2, RPS14 and H2B to normal levels in *cblC* fibroblasts, however, the expression pattern of S100A6 was unaffected. Two other proteins, septin 11 (SEPT11) and ubiquitin and ribosomal protein S27a precursor (URPS27a) were dowregulated in *cblC* fibroblast grown in the presence of HOCbl. Septins are a novel family of GTP-binding proteins that appear to play an important role in cytokinesis, membrane dynamics, vesicle trafficking, apoptosis, and cell polarity [95]. SEPT11 was also downregulated in fibroblasts from patients with the *cblD* disorder [74].

Intracellular trafficking and protein folding

A number of proteins involved in protein folding and intracellular trafficking were downregulated in *cblC* fibroblasts compared to the normal cell line, a pattern that was not reversed by exogenous supplies of HOCbl. These include: protein disulfide isomerase (PDI) precursor 3 and PDI associated 4, heat shock protein 90 (Hsp90) 1 and Hsp90 α-class A member 1 isoform 2, heat shock protein 70 (Hsp70) proteins 5 and 8, annexin VI isoform 1, annexin V, annexin V A2, a lysosomal – H^+

transporting ATPase, and voltage dependent anion channels (VDACs) 1 and 2. Importantly, PDI, Hsp70 and Hsp90 play important roles in folding of newly synthesized proteins or stabilizing and refolding of denatured proteins after stress [96, 97]. Annexins are a family of Ca^{2+}-dependent and membrane-binding proteins, which are involved in membrane trafficking and various other processes including signaling, proliferation, differentiation, and inflammation [98–100]. Lysosomal H^+-transporting ATPase is a vacuolar enzyme that mediates acidification of eukaryotic intracellular organelles, a critical step for processes such as protein sorting, zymogen activation, receptor-mediated endocytosis and synaptic vesicle proton gradient generation [101]. The VDACs are the major channels by which small hydrophilic molecules cross the mitochondrial outer membrane. Evidence suggests that VDAC isoforms in mammals may act in the cross-talk between mitochondria and the cytoplasm by direct interaction with enzymes involved in energy metabolism and proteins involved in mitochondrial-induced apoptosis [102]. VDACs also interact with anti-apoptotic proteins from the Bcl-2 family, and this interaction inhibits the release of apoptogenic proteins from the mitochondrion [103]. The expression of both VDAC1 and VDAC2 was diminished in *cblC* fibroblasts compared to normal cells, a pattern that also prevailed when cells were grown in the presence of HOCbl.

General metabolism and cellular detoxification

A number of proteins involved in general metabolism and cellular detoxification were identified as downregulated in the *cblC* fibroblast proteome. These include: high density lipoprotein binding protein (HDLBP), GAPDH and glutathione-S-transferase (GST) (various isoforms) among others. Three isoforms of GST were downregulated in *cblC* fibroblasts grown without HOCbl supplementation: GST Ω1, GST, and GST M3. Of these, only GST remained downregulated under conditions of HOCbl supplementation, whereas the expression levels GST Ω1 and GST M3 did not differ significantly from that of normal cell lines. Glutathione transferases utilize glutathione to detoxify drugs, xenobiotics, and oxidants. A recent report showed strong associations between the age of onset of Alzheimer's and Parkinson's diseases and polymorphisms of GST Ω1 and 2 [104]. The mu (M) class of GST functions in the detoxification of carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress by conjugation with GSH [105]. Immunoblotting analysis revealed that GST M3 is the predominant isoform in the brain [106]. Activity assays confirmed that *cblC* fibroblasts have reduced total GST activity [51]. Reduced GST expression and activity may compromise the detoxification of metabolites, which in turn could aggravate the manifestation of the *cblC* disease.

Conclusions and future perspective

The elucidation of the biophysical and structural properties of MMACHC led to a greater understanding of its function including its interactions with downstream MMADHC. The instability of MMACHC suggests it is likely short-lived in vivo, or that its existence may require an unknown stabilizing factor.

The *cblC* fibroblast proteome exhibited expression patterns that are significantly different from that of the normal cultured skin fibroblasts. A defective or absent MMACHC protein caused profound changes of cellular metabolism and regulation, including cytoskeleton assembly, nervous system proteins, signaling, and cellular detoxification [51]. A number of the proteins identified in the study have been linked to skeletal and muscular diseases as well as neurological diseases, which concurs with the clinical manifestations of the *cblC* disorder. The identification of proteins whose expression was altered by the *cblC* mutation could be useful targets for further research, and perhaps, for designing alternative therapies to alleviate the symptoms of the *cblC* disease. For instance, administration of UCHL1, a protein that is mutated or downregulated in patients with Alzheimer's and Parkinson's disease, and also downregulated in *cblC* fibroblasts, was shown to alleviate the β-amyloid-induced synaptic dysfunction and memory loss associated with a mouse model of Alzheimer's disease [107]. Likewise, it is possible that therapies utilized to treat patients with skeletal and muscular diseases would be effective in ameliorating such affections in *cblC* patients; we now know that at least some of the proteins involved in the progression of these diseases have a common set of contributors, the cytoskeletal proteins. Although these notions remain largely speculative until further research is conducted, these results provide a platform for investigating the protein-phenotype relationships underlying the most common inborn error of vitamin B_{12} metabolism.

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Conflict of interest statement

Author's conflict of interest disclosure: The authors state that there are no conflicts of interest regarding the publication of this article.

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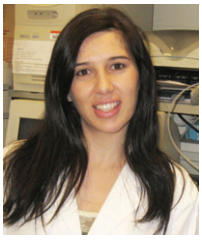

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