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Mutations in *ABCD4* cause a new inborn error of vitamin B_{12} metabolism

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Inherited disorders of vitamin B_{12} (cobalamin) have provided important clues to how this vitamin, which is essential for hematological and neurological function, is transported and metabolized. We describe a new disease that results in failure to release vitamin B_{12} from lysosomes, which mimics the cblF defect caused by *LMBRD1* mutations. Using microcell-mediated chromosome transfer and exome sequencing, we identified causal mutations in *ABCD4*, a gene that codes for an ABC transporter, which was previously thought to have peroxisomal localization and function. Our results show that ABCD4 colocalizes with the lysosomal proteins LAMP1 and LMBD1, the latter of which is deficient in the cblF defect. Furthermore, we show that mutations altering the putative ATPase domain of ABCD4 affect its function, suggesting that the ATPase activity of ABCD4 may be involved in intracellular processing of vitamin B_{12}.

ABCD4 is an ATP-binding cassette (ABC) transporter that has been classified as a member of the D subfamily of peroxisomal ABC half transporters with an unknown function[1]. We provide evidence that this protein is involved in a new inherited defect affecting vitamin B_{12} (cobalamin) metabolism. In humans, cobalamin (Cbl) is converted into two active cofactors: methylcobalamin (MeCbl), required by the cytosolic enzyme methionine synthase (MTR) that catalyzes methylation of homocysteine to methionine, and adenosylcobalamin (AdoCbl), required by the mitochondrial enzyme methylmalonyl-CoA mutase (MUT) that converts methylmalonyl-CoA to succinyl-CoA. Much of the knowledge about processing of Cbl—from uptake of its circulating form by cells to its conversion into cofactors—was obtained from studies of individuals affected with rare inherited defects. Eight defects in the intracellular processing of Cbl are known, classified as eight complementation groups from cblA to cblG and mut, that lead to either isolated methylmalonic aciduria or isolated homocystinuria or both[2]. Here, we show that a new genetic defect in this pathway is caused by mutations in *ABCD4*. We also provide evidence that the active ABCD4 protein colocalizes with the lysosomal proteins LAMP1 and LMBD1 (encoded by *LMBRD1*), the latter of which is deficient in the cblF complementation group[3].

Detailed investigations in cultured fibroblasts from two unrelated individuals presenting with methylmalonic aciduria and hyperhomocysteinemia led to the simultaneous identification in North America and in Europe of a new genetic defect in intracellular Cbl processing. Subject 1 (from North America) presented at birth with an abnormality in newborn screening, hypotonia, lethargy, poor feeding and bone marrow suppression. Subject 2 (from Europe) presented in the newborn period with poor feeding, macrocytic anemia and heart defects (for clinical details, see the **Supplementary Note**). Studies of cultured fibroblasts from both subjects showed elevated total Cbl uptake but virtually absent synthesis of MeCbl and AdoCbl, together with deficient but hydroxocobalamin-responsive function of MUT (estimated indirectly by incorporation of label from [^{14}C]propionate into cellular proteins) and MTR (estimated indirectly by incorporation of label from [^{14}C]methyltetrahydrofolate (methylTHF) into proteins or from [^{14}C]formate into methionine) (**Supplementary Table 1**). In addition, there was accumulation of free Cbl in the cells (not bound to MUT or MTR as in control cells) (**Fig. 1**). This cellular phenotype mimicked that of the cblF disorder[4] (**Fig. 1** and **Supplementary Table 1**). No *LMBRD1* mutations were detected in either subject. Somatic cell complementation analysis showed that these subjects share the same genetic defect (**Fig. 2**), hereby named the cblJ complementation group.

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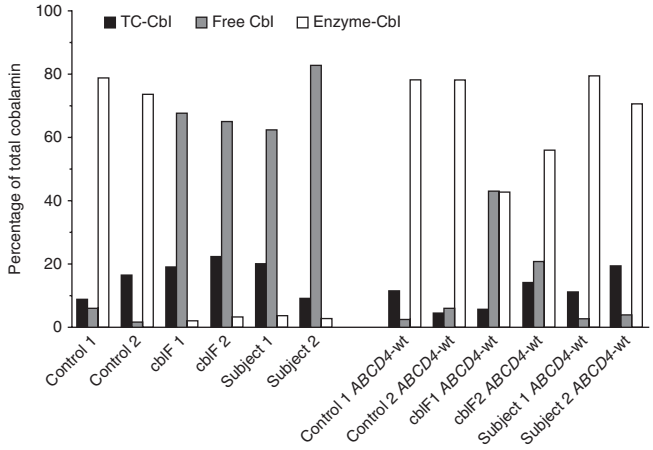

Figure 1 Distribution of free and protein-bound cobalamin in cell lines from controls and affected individuals. Fibroblasts from two controls, two subjects with the cblF defect and subjects 1 and 2 were not transfected (left) or transfected with wild-type *ABCD4* (*ABCD4*-wt) (right). Cells were incubated in medium containing [^{57}Co]cyanocobalamin (CNCbl). Cell homogenates were subjected to fast protein liquid chromatography, and fractions containing labeled Cbl co-eluting with the Cbl-dependent enzymes (enzyme-Cbl), with the Cbl transporter transcobalamin (TC-Cbl) and without bound proteins (free Cbl) were separated and counted for radioactivity. Results are single determinations expressed as the percentage of total radioactivity in all fractions in each sample. For the Cbl binding pattern after transfection with wild-type *LMBRD1* cDNA, see **Supplementary Figure 1**.

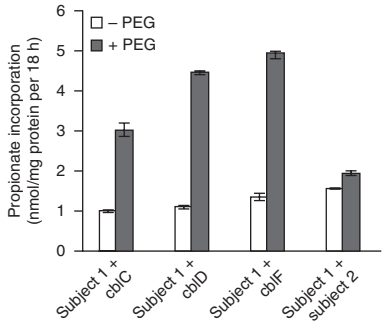

Figure 2 Somatic complementation analysis confirms a new cobalamin complementation group cblJ. Fibroblasts from subject 1 were mixed with cells belonging to the three known Cbl complementation groups that are also associated with combined deficiency in AdoCbl and MeCbl synthesis (cblC, cblD and cblF) and with cells from subject 2. We then fused cells with 40% polyethylene glycol (+ PEG) and compared incorporation of [^{14}C]propionate into cellular proteins with that in mixed but non-fused cells (– PEG). Bars represent the mean, and error bars show the range of triplicate determinations in a representative experiment.

To identify the gene responsible for this defect, the North American team performed exome sequencing of genomic DNA from subject 1 and identified three candidate genes, each carrying two potentially damaging variants (**Supplementary Table 2**). The European team localized the gene responsible for the cblJ defect to chromosome 14 using microcell-mediated chromosome transfer with cells from subject 2 (**Supplementary Table 3**). Exome sequencing of genomic DNA from subject 2 and filtering for variations present on chromosome 14 using stringent criteria for analysis (**Supplementary Table 4**) led to the identification of nine potentially damaging variants, two of which were located in the same gene, namely *ABCD4* (**Supplementary Table 5**). Segregation analysis of the candidate variants in the families of both subjects identified *ABCD4* as the disease-causing gene and confirmed that two mutations were located on different alleles in each subject. The two *ABCD4* variants identified in each subject and their parents were confirmed by Sanger sequencing. Subject 1 carried a missense mutation, c.956A>G (p.Tyr319Cys) (NM_005050.3), predicted to be probably damaging with a score of 0.984 using the PolyPhen-2 program[5], and a dinucleotide insertion, c.1746_1747insCT (p.Glu583Leufs*9), resulting in a frameshift and the introduction of a premature stop codon leading to removal of 14 amino acids from the C terminus. Subject 2 carried two mutations that are predicted to disrupt consensus splice sites: c.542+1G>T, located at the last nucleotide of exon 5, and c.1456G>T (p.Gly486Cys), located at the last nucleotide of exon 14. RT-PCR amplification of RNA from subject 2 showed skipping of exon 5 and of exons 13 and 14, resulting in in-frame deletions of 39 (p.Asp143_Ser181del) and 43 (p.Gly443_Ser485del) amino acids, respectively (data not shown). All four variants were absent from the 1000 Genomes Project data set.

To prove that *ABCD4* mutations cause the cblJ phenotype, we tested the ability of *ABCD4* to correct the cblJ defect. Expression of wild-type *ABCD4* cDNA in fibroblast cell lines from affected individuals led to rescue of the biochemical phenotype with normalization of intracellular enzyme-bound Cbl levels (**Fig. 1**), significantly increased synthesis of both Cbl cofactors (**Fig. 3**; for *P* values, see **Supplementary Table 6**) and increased methylTHF incorporation (MTR activity) and propionate incorporation (MUT activity) in both cell lines (data not shown). We observed no rescue when these cell lines were transfected with *LMBRD1* (**Fig. 3** and **Supplementary Fig. 1**). Furthermore, expression of *ABCD4* alleles with c.956A>G or c.1456G>T mutation did not significantly increase the synthesis of AdoCbl and MeCbl compared to the wild type, proving their

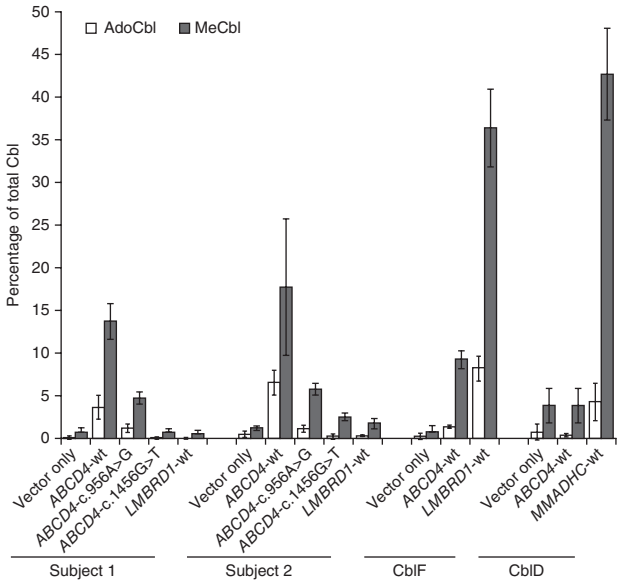

Figure 3 Expression of wild-type and mutant *ABCD4* alleles. We transiently transfected immortalized fibroblasts from subjects 1 and 2 (cblJ), a subject with cblF and a subject with the cblD combined defect with different constructs in the pTracer vector by electroporation. We measured rescue of function by assay of AdoCbl and MeCbl synthesis. Wild-type (wt) constructs were employed for *ABCD4*, *LMBRD1* (associated with the cblF defect) and *MMADHC* (associated with the cblD defect). Transfections with empty vector (vector only) were used as negative controls. Bars represent the mean, and error bars show the s.d. of results from four or five replicate experiments with single determinations.

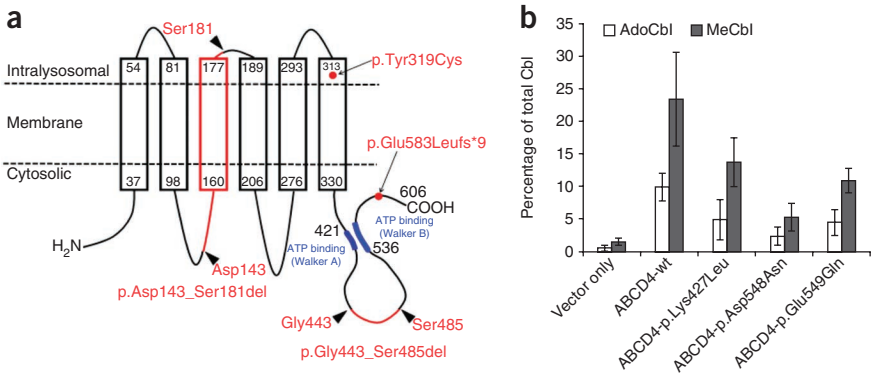

Figure 4 ABCD4 is a membrane protein with ATPase function. (**a**) Schematic of the predicted topology of ABCD4 with transmembrane helices. The putative ATP-binding site (Walker A and B consensus sequences) in the NBD is highlighted in blue. The position of the p.Tyr319Cys and p.Glu583Leufs*9 alterations (detected in subject 1) are represented with a red dot, and the polypeptide fragments deleted in the p.Asp143_Ser181del and p.Gly443_Ser485del protein products (detected in subject 2) are highlighted in red. Transmembrane positions were predicted by the PHDhtm program of the PredictProtein suite[11]. The dashed horizontal lines represent the boundaries of the membrane. (**b**) ABCD4 function requires ATPase activity. Mutant alleles encoding changes to highly conserved amino-acid residues known to be involved in ATPase activity, for example, p.Lys427Leu in the Walker A site, p.Asp548Asn in the Walker B site and p.Glu549Gln in the putative catalytic domain of ABCD4, were transfected transiently into immortalized fibroblasts from subject 1 by electroporation and were assayed for rescue of AdoCbl and MeCbl synthesis. Concurrent transfections with an empty vector (vector only) and wild-type ABCD4 were used as negative and positive controls, respectively. Bars represent the mean, and error bars show the s.d. of results from eight replicate experiments with single determinations.

functional consequences (**Fig. 3**). Notably, expression of *ABCD4* in cblF cells led to partial rescue of function (**Figs. 1** and **3**). This effect was not seen in cblD cells (**Fig. 3**), a cell line that has a combined defect in AdoCbl and MeCbl synthesis.

ABCD4, also known as P70R and PMP69, was originally described as a member of the D subfamily of ABC half transporters[1,6]. The other members of this subfamily (ALDP, ALDR and PMP70) combine as homo- or heterodimers to form transporters of very-long-chain fatty acids across the peroxisomal membrane[7,8]. ABCD4 has 25–27% amino-acid identity with the other three proteins and lacks their N-terminal domain[9]. The protein consists of a transmembrane domain and an ABC transporter or nucleotide-binding domain (NBD), characterized by the presence of highly conserved motifs involved in the binding of ATP and Mg^{2+}, including the ABC signature and the Walker A and B motifs[10] (**Fig. 4a**). Among the four mutations detected in our affected subjects, two (encoding p.Asp143_Ser181del and p.Tyr319Cys) occur in the predicted transmembrane domain, whereas the other two (encoding p.Gly443_Ser485del and p.Glu583Leufs*9) are located in the predicted NBD[11]. Expression of an *ABCD4* construct encoding a mutant Walker B motif (p.Asp548Asn) in cells from subject 1 led to very low levels of Cbl cofactor synthesis (**Fig. 4b**; $P < 0.00001$ for AdoCbl and 0.0001 for MeCbl synthesis compared with wild-type *ABCD4* construct), indicating that this Walker B motif aspartate is necessary for ABCD4 function. Moreover, expression of constructs with selective changes affecting the Walker A motif (p.Lys427Leu) and the putative catalytic site (p.Glu549Gln) also led to reduced synthesis of both Cbl cofactors (**Fig. 4b**; p.Lys427Leu: $P = 0.003$ for AdoCbl and 0.007 for MeCbl synthesis; p.Glu549Gln: $P = 0.001$ for AdoCbl and 0.0021 for MeCbl synthesis). Notably, the level of expression, determined by protein blot analysis, of red fluorescent protein (RFP)-tagged ABCD4 protein was similar for wild-type and mutant constructs, ruling out a destabilizing effect of the alterations introduced (**Supplementary Fig. 2**). Taken together, our results suggest that the ATPase activity of ABCD4 might be involved in the intracellular processing of Cbl.

Although initially reported to be peroxisomal[1], ABCD4 was suggested by a more recent study to be localized to the endoplasmic reticulum[9]. Moreover, several studies aimed at characterizing all peroxisomal proteins did not detect ABCD4 under conditions where the three other ABCD proteins were detected[12–14]. In addition, *in vitro* studies reported that PEX19p, a peroxisomal biogenesis protein, binds ABCD1, ABCD2 and ABCD3 but not ABCD4 (ref. 15). Finally, we showed that Cbl cofactor synthesis in three different cell

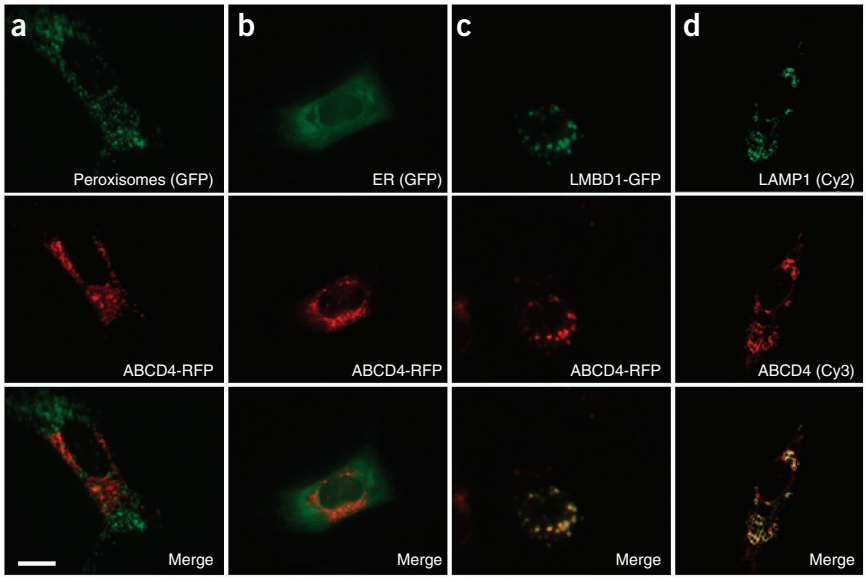

Figure 5 Subcellular localization of ABCD4 detected by fluorescence and confocal microscopy. Green fluorescence is indicative of GFP and Cy2, and red fluorescence is indicative of RFP and Cy3. (**a**) Peroxisomes stained with Peroxisome CellLight-GFP do not colocalize with ABCD4-RFP transiently expressed in immortalized fibroblasts. (**b**) Endoplasmic reticulum (ER) stained with ER CellLight-GFP does not colocalize with ABCD4-RFP. (**c**) Lysosomal LMBD1-GFP colocalizes with ABCD4-RFP. (**d**) Lysosomal LAMP1 stained with antibody to LAMP1 and Cy2-conjugated secondary antibody colocalizes with ABCD4 stained with antibody to ABCD4 and Cy3-conjugated secondary antibody, as shown by confocal microscopy. Scale bar, 20 μm.

lines with severe defects in peroxisome biogenesis was completely normal (**Supplementary Table 7**), arguing against an involvement of peroxisomes in Cbl metabolism.

To investigate the subcellular localization of ABCD4, we cotransfected fibroblasts with a vector coding for ABCD4 fused to RFP and vectors coding for different organelle-targeting sequences fused to green fluorescent protein (GFP). Fluorescence microscopy showed no colocalization of ABCD4 with peroxisomes or endoplasmic reticulum (**Fig. 5**). In contrast, ABCD4 did colocalize with the two lysosomal proteins, LMBD1 and LAMP1, as shown in images of fibroblasts co-expressing ABCD4-RFP and LMBD1-GFP or of fibroblasts expressing ABCD4 that were stained with antibodies to ABCD4 and LAMP1 (**Fig. 5**). Lysosomal localization of ABCD4 is further supported by the finding that expression of the tagged ABCD4-RFP protein still rescued the cblJ phenotype, ruling out mislocalization (**Supplementary Fig. 2**).

Taken together, our biochemical findings and subcellular localization studies elaborate on the function of ABCD4 and suggest that the cblJ defect affects the lysosomal release of Cbl into the cytoplasm with a marked similarity to cblF[4]. On the basis of the accumulation of free Cbl in cblJ cells, which mimics the cblF biochemical phenotype, we suggest that it is likely that ABCD4, possibly in close collaboration with LMBD1, is involved in the lysosomal release of Cbl into the cytoplasm. This notion is supported by the partial rescue of function in cblF cells by ABCD4 overexpression.

The exact contributions of LMBD1 and ABCD4 to the intracellular transport of Cbl remain unclear. In bacteria, the ABC transporter BtuCD specifically transports Cbl across the inner membrane[16]. ABCC1, another ABC transporter, is responsible for the efflux of free Cbl out of cells[17]. These previous studies support the idea that ABCD4 is the actual lysosomal Cbl transporter, with LMBD1 having a regulatory or accessory role. However, the bacterial BtuCD-F system imports Cbl into the cytosol, whereas eukaryotic ABC transporters generally mediate efflux from the cytosol[18]. Also, it is known that, in humans, the sulfonylurea receptor is constituted by the pore-forming Kir6.2 protein, whose opening is regulated by the ATPase activity of the ABC transporter SUR1 (ref. 19). Thus, it is tempting to hypothesize a similar model whereby LMBD1 would constitute the Cbl transport channel that is regulated by the ATPase activity of ABCD4.

We conclude that this newly discovered disorder, named cblJ, is an autosomal recessive disorder caused by mutations in *ABCD4*. Our results show that ABCD4, an ABC transporter, is an essential component of intracellular Cbl metabolism and suggest that it interacts with LMBD1 in the lysosomal release of Cbl.

URLs. 1000 Genomes Project, http://www.1000genomes.org/home; PredictProtein, http://www.predictprotein.org/; method for transfection of retroviral vector into Phoenix helper-free retrovirus producer cell line, http://www.stanford.edu/group/nolan/retroviral_systems/phx.html.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Genetic data relevant to this study are available upon detailed request to the corresponding authors. Non-relevant exome data are not publicly available to protect the identity of single individuals with rare diseases.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.R.B., F.R., B.F. and D.S.R. supervised the project. M.R.B., F.R., B.F., D.S.R., J.C.K., T.S. and D.C. designed the study, analyzed the data and wrote the manuscript. T.S., M.d.M. and D.C. performed microcell-mediated chromosome transfer. M.S., D.C. and M.F. designed primers and built constructs. M.d.M., I.B., J.C.K., I.R.M., D.W. and D.C. performed sequencing analysis. J.M., P.N. and H.T. performed whole-exome sequencing. S.F. and E.A.S. performed immortalization and stable transduction of fibroblasts. T.S., P.B., J.C.K. and D.W. performed somatic cell complementation, enzymatic assays, Cbl coenzyme synthesis and expression studies. H.R., I.B. and D.C. analyzed the subcellular localization of ABCD4. D.C. and P.B. performed protein blots. W.H. and D.C. analyzed the structure of ABCD4. N.L., M.P. and E.M. provided clinical information about the affected individuals. All authors discussed the results and reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Two individuals with a previously unknown defect in Cbl metabolism and their families were investigated. The studies were approved by the institutional ethics committees of Münster University and the McGill University Health Centre. Subjects were included after informed consent was obtained.

Fibroblast cultures. Skin fibroblasts, obtained for diagnostic purposes, were used for biochemical evaluation and were routinely grown in standard culture medium supplemented with 10% FCS, with or without antibiotics[20,21]. For expression studies, the North American group immortalized fibroblasts by transduction with a retroviral vector expressing the E7 gene from human papilloma virus type-16 and human telomerase[22], and the European group used the pRNS14 plasmid[23] delivered by electroporation[2]. Immortalization did not markedly affect cellular phenotype.

Exome sequencing and mutation analysis. A total of 3 µg of DNA from subject 1 was used for exome capture with the Agilent SureSelect All Exon 38Mb kit, and massively parallel sequencing was performed on 76-bp reads using the Illumina Genome Analyzer IIx, as previously described[24], to generate a mean 30× coverage of the targeted regions. Variants were compared against a pool of in-house exomes, and those previously seen in two or more individuals were discarded. Novel variants were defined as those absent from dbSNP that had an allele frequency of less than 0.005 in 1000 Genomes Project data. This allowed us to consider rare variants observed in the 1000 Genomes Project at very low frequencies. Potentially damaging variants included nonsynonymous substitutions caused by missense and nonsense SNPs, splice-site SNPs and frameshift changes due to insertions and/or deletions (indels)[24]. Candidate genes were defined as those containing either a homozygous or two potentially compound heterozygous variants in the same gene, satisfying the above criteria. This filtering strategy led to the identification of three candidate genes. DNA of index subject 2 was enriched for protein-coding genes with the Agilent SureSelect Human All Exon 38Mb kit and was run on two lanes of the Illumina Genome Analyzer IIx sequencer using the paired-end protocol and a read length of 100 bp at each end. Alignment and variant calling were performed, using MAQ for SNP detection (version 0.7.1)[25] and BWA-short (version 0.5.7)[26] in combination with SAMtools (version 0.1.7)[27] for indel detection. Scripts developed in house were applied to detect protein changes, affected splice sites and overlap with known variants (**Supplementary Table 3**). All mutations in candidate genes in the affected subjects and family members were confirmed by Sanger sequencing.

Microcell-mediated chromosome transfer. Mouse-human monochromosomal hybrid cell lines (donor cells), each carrying a single human chromosome tagged with a hygromycin resistance gene[28], were used to serially transfect immortalized fibroblasts from subject 2 (recipient cells) through microcell-mediated chromosome transfer, as described previously[2,29] (see also **Supplementary Table 3**). Cells containing each transfected human chromosome were selected on the basis of growth in medium containing hygromycin (0.1 mg/ml). Colonies were subcultured, and rescue of function was assayed by measuring Cbl coenzyme synthesis.

Functional assays. The functional integrity of methylmalonyl-CoA mutase (MUT) was evaluated by measuring the degree of formation of AdoCbl from [^{57}Co]CNCbl and incorporation of label from [^{14}C]propionate into macromolecules, and the integrity of methionine synthase (MTR) was evaluated by measuring the degree of formation of MeCbl from [^{57}Co]CNCbl and incorporation of label from [^{14}C]methylTHF into macromolecules or from [^{14}C]formate into methionine.

Total uptake of Cbl and synthesis of Cbl cofactors. Fibroblasts were incubated for 3 or 4 d in medium containing 25 pg/ml [^{57}Co]CNCbl (MP Biomedicals). Medium contained 10% human serum that was preincubated for 30 min at 37 °C with [^{57}Co]CNCbl to allow its binding to transcobalamin[30,31]. Cells were harvested, and total uptake (pg/mg protein) was estimated in a small portion of the cells by quantifying radioactivity in a gamma counter and measuring protein by the Lowry assay. The rest of the cells were disrupted by freezing and thawing, Cbl was extracted in hot ethanol (80 °C), and Cbl derivatives were separated by high performance liquid chromatography using a LiChrosorb RP-C8 column (Phenomenex or Supelco). Radioactivity in fractions co-eluting with hydroxcobalamin (OHCbl), CNCbl and the cofactors AdoCbl and MeCbl were expressed as the percentage of radioactivity in all fractions. Small amounts (0–8%) of unidentified [^{57}Co]labeled compounds were also detected (data not shown).

Propionate and methylTHF incorporation assays. Fibroblasts were incubated for 16 or 18 h in medium containing [^{14}C]propionate[20,21] or [^{14}C]methylTHF[21]. To study *in vitro* Cbl responsiveness, medium of parallel cultures was supplemented with 0.7 or 1.5 µM OHCbl, respectively. After incubation, cells were harvested, cellular macromolecules were precipitated with 5% trichloroacetic acid and dissolved in NaOH, and incorporated radioactivity was quantified in a liquid scintillation spectrometer. Protein concentrations were determined by the Lowry assay.

Methionine synthesis. Formation of [^{14}C]methionine from [^{14}C]formate was measured as described previously[32]. Cells were incubated for 16 h in medium containing 0.3 mM [^{14}C]formate and 0.1 mM homocysteine. Cells were then harvested, and cellular macromolecules were precipitated with 5% trichloroacetic acid, dissolved in NaOH and oxidized for 16 h with performic acid. Proteins were hydrolyzed by 16 h of treatment with 6 N HCl at 105 °C, and oxidized methionine was separated by high-voltage paper electrophoresis and quantified by counting strips of the chromatogram in a scintillation spectrometer. Protein concentrations were determined by the Lowry assay. *In vitro* Cbl responsiveness was estimated in parallel cultures grown for 3 d in medium supplemented with 0.7 µM OHCbl before addition of the radioactive medium.

Somatic cell complementation analysis. For complementation analysis, equal numbers of fibroblasts from affected individuals and reference fibroblasts belonging to known complementation groups were mixed and fused by exposure to 40% PEG (J.T. Baker), and functional assays were performed as described[21]. Parallel cultures of mixed but not fused cells were used to measure basal activity. A clear increase in activity after fusion to at least twice the basal activity indicated complementation.

Distribution of free and protein-bound Cbl in fibroblasts. Fibroblasts were incubated for 4 d in medium containing 25 pg/ml [^{57}Co]CNCbl. Cells were harvested, resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and homogenized by sonication using a Soniprep 150 (MSE Scientific Instruments). Disrupted cell membranes were removed by ultracentrifugation at 171,500 *g* at 5 °C for 30 min, and free Cbl, transcobalamin-bound Cbl, and MUT- and MTR-bound Cbl were separated by fast protein liquid chromatography using a Superose 12 column (GE Healthcare Life Sciences). Radioactivity of collected fractions was quantified by gamma counting, and the distribution of Cbl was expressed as the percentage of radioactivity in all fractions.

Stable transduction of fibroblasts with wild-type *ABCD4* and *LMBRD1*. Wild-type human *ABCD4* cDNA in pENTR221 vector (DQ892847.2) was purchased from DNAFORM, cloned into the Gateway-modified retroviral expression vector pBabe[33] using LR Clonase II Enzyme Mix (Invitrogen) and transiently transfected into the Phoenix Amphotrophic packaging cell line using the HEPES-buffered saline (HBS)-$Ca_3(PO_4)_2$ method (see URLs). After a 48-h incubation period, virus-containing medium was collected, supplemented with 4 µg/ml polybrene and used to infect immortalized fibroblast cell lines. Fibroblasts were grown for 2 weeks in medium containing 1 µg/ml puromycin for selection[34]. The protocol for stable transduction of fibroblasts with wild-type *LMBRD1* cDNA was identical, with the following difference. Wild-type human *LMBRD1* cDNA in pBlueScriptR vector (BC047073.1) was purchased from Open Biosystems and cloned into empty pENTR221 vector using BP Clonase II Enzyme Mix. This clone was then used for LR recombination reaction and subsequent transfection.

Transient expression of wild-type and mutant *ABCD4* cDNA in fibroblasts. Constructs containing wild-type and mutant *ABCD4* cDNA sequences were cloned into the pTracer-CMV2 expression vector (Invitrogen), as

described previously[2,35]. For RFP-tagged constructs, the *ABCD4* coding sequence was subcloned into a modified version of pRFP-N1 (Clontech) with sequence encoding a valine in place of the RFP initiation methionine. Constructs were transfected into immortalized fibroblasts by electroporation, and their effects on cellular function were tested by measuring MeCbl and AdoCbl synthesis[2,35].

Statistical analysis. The statistical significance of data on rescue of function was tested by unpaired *t* test (two tailed) with Welch's correction for unequal variances and GraphPad Prism software (version 4). *P* values less than 0.05 were considered to indicate statistical significance.

Immunofluorescence analysis. For fluorescence microscopy, fibroblasts were transfected with cDNA constructs of human *ABCD4* or *LMBRD1* that were subcloned into a modified version of pRFP-N1 or pGFP-N1 (Clontech) with a sequence encoding valine in place of the initiation methionine. For peroxisome and endoplasmic reticulum staining, cells were incubated with the corresponding CellLight reagents (Invitrogen) according to the manufacturer's instructions. Cells were examined under a Leica DM IL fluorescence microscope with a Leica DFC420C digital camera. For confocal microscopy, fibroblasts were transfected with cDNA constructs of human *ABCD4* that were subcloned into pTracer. Cells were then grown on glass coverslips, washed with PBS, fixed with 4% paraformaldehyde (Sigma) for 10 min, permeabilized in blocking buffer (PBS with 0.05% Tween-20 and 1% BSA) overnight at 4 °C and incubated for at least 1 h with a mouse antibody to LAMP1 (CD107a, BD Biosciences) and with a rabbit antibody to ABCD4 (HPA003396, Sigma-Aldrich) in blocking buffer. After washing with PBS, cells were incubated for 1 h with a Cy2-conjugated antibody against mouse and a Cy3-conjugated antibody against rabbit in the blocking buffer and were rinsed with PBS. Preparations were mounted in fluorescence mounting medium (Dako) and were examined under a confocal LSM510 laser-scanning microscope (Zeiss).

Protein blots. Fibroblasts were lysed with RIPA buffer (Invitrogen), and proteins were separated by SDS-PAGE on an 8% gel and transferred to nitrocellulose membrane (Protran BA85, Whatman). Membranes were blocked at room temperature for 1 h with 5% nonfat dry milk in PBS containing 0.1% Tween-20 and were incubated overnight with a polyclonal rabbit antibody to RFP (R10377, Invitrogen). Visualization of protein bands was performed using a goat secondary antibody against rabbit conjugated with horseradish peroxidase (Jackson ImmunoResearch) and enhanced chemilluminescence (ECL) reagent according to the manufacturer's instructions (Perbio). PageRuler Plus Prestained Protein Ladder (Fermentas) was used as a protein molecular size marker.

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