MCTP2 is a dosage-sensitive gene required for cardiac outflow tract development

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Coarctation of the aorta (CoA) and hypoplastic left heart syndrome (HLHS) have been reported in rare individuals with large terminal deletions of chromosome 15q26. However, no single gene important for left ventricular outflow tract (LVOT) development has been identified in this region. Using array-comparative genomic hybridization, we identified two half-siblings with CoA with a 2.2 Mb deletion on 15q26.2, inherited from their mother, who was mosaic for this deletion. This interval contains an evolutionary conserved, protein-coding gene, MCTP2 (multiple C2-domains with two transmembrane regions 2). Using gene-specific array screening in 146 individuals with non-syndromic LVOT obstructive defects, another individual with HLHS and CoA was found to have a de novo 41 kb intragenic duplication within MCTP2, predicted to result in premature truncation, p.F697X. Alteration of Mctp2 gene expression in Xenopus laevis embryos by morpholino knockdown and mRNA overexpression resulted in the failure of proper OT development, confirming the functional importance of this dosage-sensitive gene for cardiogenesis. Our results identify MCTP2 as a novel genetic cause of CoA and related cardiac malformations.

INTRODUCTION

Obstructive defects of the left ventricular outflow tract (LVOT) form an important spectrum of cardiovascular malformations that include coarctation of the aorta (CoA), aortic valve stenosis (AVS), hypoplastic left heart syndrome (HLHS), complicated mitral valve stenosis with HLHS and CoA (Shone complex), interrupted aortic arch type A and bicuspid aortic valve (BAV). LVOT defects account for ~14–20% of all medically significant cardiovascular malformations (1). Familial aggregation of these defects is well-recognized (2,3) and diverse genetic mechanisms with extensive locus and allelic heterogeneity are likely. The existence of families with multiple occurrences of the full range of LVOT lesions strongly suggests the existence of common mechanisms underlying the different anatomic forms (4,5). Mutations in NOTCH1 have been described in a subset of families with calcific AVS and HLHS (6,7); however, the underlying genetic basis of CoA in the majority of patients remains undetermined. In a review of known cytogenetic associations with cardiovascular malformations, we observed that many individuals with large deletions of 15q26 (MIM # 612626) predominantly have LVOT lesions,
including CoA and HLHS (8–14) (Supplementary Material, Fig. S1). Congenital diaphragmatic hernia (CDH), also observed in 15q terminal deletions (11–13,15), is known to be associated with hemodynamically significant cardiac anomalies (16,17). However, this association of CDH with significant heart defects is not understood. The role of COUP-TFII gene, encoding a transcription factor regulated by the retinoid signaling pathway, within this region on 15q26.2 has been established in angiogenesis, lymphatic development and atrial malformations in animal models (18,19). While COUP-TFII is implicated in both CDH (20) and cardiac defects in humans (8,21), it is unclear if the disruption of the gene is indeed responsible for the OT lesions in 15q26 segmental aneuysmy. In COUP-TFII mouse mutants, the sinus venosus and atria fail to develop past the primitive tube stage (18). Based on the animal histological data and the review of the mapped breakpoints from various published reports (Supplementary Material, Fig. S1), we hypothesized that other dosage-sensitive gene(s) within this region may be responsible for left heart developmental defects in humans. Using array-comparative genome hybridization (CGH) in two half-siblings with CoA with normal dosage of COUP-TFII, we identified microdeletion of the centromeric gene, MCTP2, and confirmed the functional importance of this gene in the OT formation in Xenopus.

RESULTS

Copy-number variations involving MCTP2 in LVOT obstructive defects

The diagnostic array-CGH analysis in subject 1 with CoA, developmental delay and dysmorphic features (Fig. 1A and B and Supplementary Material, Table S1) identified an interstitial genomic loss of copy number in the 15q26.2 region, confirmed by fluorescence in situ hybridization (FISH) analysis (Fig. 1E and F). His maternal half-sister, subject 2 with CoA and facial dysmorphism, was also found to be deleted for this region (Fig. 1C, D and G). Using bacterial artificial chromosome (BAC) clone RP11-4F5, mosaicism for this deletion was identified in the mother by FISH, with 26% of lymphocytes showing normal hybridization signals (Fig. 1H) and 74% of the cells showing a single hybridization signal for this clone (Fig. 1I). Maternal echocardiogram showed no abnormalities of the LVOT. To address whether the identified deletion was a benign copy-number polymorphism, we reviewed the copy-number variation data in 40 200 individuals subjected to the clinical array-CGH at Baylor Medical Genetics Laboratories. No similar size deletions were identified in the clinical database. In addition, no large variants corresponding to the deleted region were identified in the Database of Genomic Variants (http://projects.tcag.ca/varia tion/), indicating that there were no significant copy-number polymorphisms in this genomic region. To further characterize the deletion in subject 1, we used the Illumina HumanHap300 array and found copy-number loss of 2.2 Mb encompassing an evolutionary conserved gene, MCTP2 and a hypothetical protein, LOC145820 (chr15:92,282,797-94,500,921-hg18), within the deleted interval (Fig. 1E).

We then studied 146 individuals with LVOT cardiac malformations for genomic copy-number alterations involving MCTP2, COUP-TFII and other flanking genes, using a customized high-definition CGH microarray (Agilent Technologies) with oligonucleotide probes in the chr15:89212888-99847710 genomic region (hg18). This cohort (group 2) included 137 patients with isolated LVOT obstructive defects and 9 individuals with syndromic LVOT, with additional features of developmental delay and/or other congenital malformations. While no genomic alterations were observed for COUP-TFII in the 146 subjects studied, the array-CGH revealed an intragenic duplication (Fig. 2A) within MCTP2 in patient 3, with non-syndromic complex cardiac malformation consisting of HLHS, mitral atresia, CoA, BAV and muscular ventricular septal defect. The 41 kb duplication, spanning from intron 10 to intron 17 of the gene, was confirmed by PCR and interphase FISH analysis (Fig. 2B and C) and was not identified in the unaffected parents (Fig. 2A and C). A 670 bp junction fragment was amplified (Fig. 2C) and sequenced. If fully spliced, predicted mRNA and translation product would yield a protein with premature truncation at p.F697X (Fig. 2D–F). MCTP2 spans 180 kb of genomic DNA, with 22 coding exons, encoding an 878 residue protein with three C2 domains and two transmembrane regions (TMRs). The nonsense allele predicted to result from the intragenic duplication causes truncation within the first TMR of the protein.

Knockdown of Mctp2 in Xenopus laevis embryos

The results from these three subjects strongly suggested that MCTP2 had an important function in cardiac development. Using reverse transcription-polymerase chain reaction (RT-PCR) and RNA harvested from the developing mouse heart at embryonic day 8.5 (E8.5), E9.5, E10.5, E11.5 and E12.5, we determined that Mctp2 is expressed during critical phases of primary heart tube maturation and morphogenesis (Supplementary Material, Fig. S2A). The expression of Mctp2 in the atrioventricular (AV) canal was also confirmed by RT–PCR, using endocardial cushion primitive valve tissue isolated from E14.5 embryos (Supplementary Material, Fig. S3). To functionally evaluate the role of Mctp2 in cardiac development, we carried out loss of function experiments in X. laevis embryos. Two morpholino antisense oligonucleotides (MO) were created to target the splice donor of exon 5 (MO1) or splice acceptor of exon 6 (MO2). The in vivo efficiency of splice blocking was tested by harvesting RNA from three stage 25 control or Mctp2 morphant embryos (MO1 or MO2), and demonstrating decreases in transcript levels (Supplementary Material, Fig. S2B).

We hypothesized that Mctp2 is a dosage-sensitive gene important for cardiac development and performed morpholino dose titration and synergy experiments to demonstrate the specificity of the morpholino effect (Fig. 3A). Viability was assessed at neurula, late tailbud and stage 46 using different concentrations of morpholino and cardiac phenotypes of surviving embryos were assessed. Synergism experiments, in which two morpholinos are injected at amounts that singly would not cause a phenotype but together demonstrate a specific phenotype, indicated the specificity of the morpholino effect (Fig. 3A). A majority of morphant embryos analyzed at stages 44–46 showed grossly normal development but demonstrated variable degrees of edema, suggesting defects of the cardiovascular system (Fig. 3B–D).
Cardiac defects with altered Mctp2 dosage in X. laevis

To further investigate the role of Mctp2 in cardiac development, expression levels of Mctp2 were altered using single morpholinos, combined morpholinos or Mctp2 mRNA (Fig. 4). Embryos were scored for cardiac looping and OT position and the development of pericardial edema (Fig. 4A–C). Embryos injected with a sub-effective dose of single Mctp2 morpholinos did not have increased rates of cardiac defects, but when morpholinos were combined, 70% demonstrated grossly abnormal hearts (Fig. 4A–C). Whole-mount in situ hybridization using cardiac troponin was used to better visualize the hearts (Fig. 4B and C). Embryos were subsequently injected with effective doses of single Mctp2 morpholinos or Mctp2 mRNA (Fig. 4D–H) for the evaluation of cardiac histology. Serial transverse sections were collected through the entire cardiac compartment for 53 gain or loss of function Xenopus embryos and compared with control embryos and published Xenopus cardiac histology (22). MO1-injected embryos were analyzed at stage 44/45 and compared with controls for endocardial cushion formation (Fig. 4D–G). The thin walled atria and trabeculated ventricle were morphologically distinct. The ventricular myocardium was of normal thickness with well-formed trabeculae in MO1-injected embryos (Fig. 4E), indicating that myocardial differentiation was intact. In control embryos, the endocardial cushion of the developing spiral valve filled the OT throughout its proximal to distal length (Fig. 4D and F). In contrast, sections of Mctp2 morphant embryos (n = 17) showed no evidence of endocardial cushion formation at any level of the developing OT (Fig. 4E and G) in a subset, confirming the critical functional role of Mctp2 in cardiac OT development. Quantitative analysis showed that 41% of MO1 morphants (n = 7/17), 44% of MO2 morphants (n = 7/16) and 45% of Mctp2 mRNA-injected embryos (n = 9/20) showed abnormalities of OT development when assessed by histologic analysis of transverse sections (Fig. 4H). Phenotypic defects with both knock down and overexpression of Mctp2 suggest the dosage-sensitive nature of the gene product.

DNA sequencing of MCTP2 in 146 individuals with LVOT obstructive defects

The Xenopus studies indicated that decreases in Mctp2 may perturb cardiac development, leading us to hypothesize that alterations in MCTP2 protein function in humans might increase susceptibility to congenital heart defects. To investigate this hypothesis, we sequenced the coding exons of MCTP2 in the same cohort of 146 subjects studied for MCTP2 dosage. We found heterozygosity for single base changes within MCTP2 in 7% of the subjects, with a total of 14 distinct mutations identified. Among these, seven were novel missense mutations, and six were polymorphisms that had been previously described in the literature. The remaining three mutations were unclassified variants. Overall, these results indicate that genetic variation in MCTP2 may contribute to the development of LVOT obstructive defects in humans.
subjects with CoA (Supplementary Material, Table S2). In all the cases where parental samples were available, the corresponding variant was identified in one apparently unaffected parent. The variants were also compared against the Exome Variant Server (version ESP6500, accessed at eversvs.gs.washington.edu), the 1000 Genome Project data (May 2012 update, accessed at 1000genomes.org) and the single nucleotide polymorphism database (build 137, accessed at www.ncbi.nlm.nih.gov). All variants were found in at least one of those databases, albeit at very low minor allele frequencies (Supplementary Material, Table S3). The variants were also assessed in a cohort of 192 apparently unaffected, ethnically matched controls. In this cohort, only variants A60T and G203D were identified, at the heterozygous state in one individual each. A comparison of amino acid sequences in among MCTP2 orthologs showed that glycine203 is highly conserved among the human, rat, mouse, chicken, Xenopus, Caenorhabditis elegans and zebrafish proteins (Supplementary Material, Fig. S4). The variant G203D, observed in subject 7, with isolated CoA and AVS, was predicted to have deleterious effects by multiple prediction programs, including PolyPhen-2 and SIFT (Supplementary Material, Table S3). This variant was found to be present in his mother. We have previously shown that there is a higher rate of asymptomatic LVOT anomalies including the occurrence of BAV in first-degree relatives of the affected subjects (23); thus, we cannot rule out clinically asymptomatic defects in the parent with this variant. The other variant, Y235C, observed in subject 8 with CoA and mitral stenosis, was found to be paternal in origin and involved amino acid residues that were conserved among human, rat and mouse proteins. Predicted to be damaging...
by PolyPhen-2, this variant was not present in our control cohort or the exome variant server but was found in the 1000 genomes database at a very low frequency (SNP ID rs191271656, minor allele frequency 0.0009). Both variants, G203D and Y235C, were studied further by functional analysis.

Functional analysis of MCTP2 variants

Since it is known that the C2A domain of MCTP2 has a high affinity for Ca$^{2+}$ \((24)\), we asked if the two heterozygous base substitutions within the C2A domain, G203D and Y235C, altered the calcium-binding affinity of the protein. We used terbium (lanthanide metal) fluorescence to compare the Ca$^{2+}$-binding affinity of expressed wild-type or variant-containing fragments. Terbium directly competes for calcium-binding sites in natural calcium-binding proteins and is intrinsically fluorescent when complexed to protein, with a broad excitation spectrum and an emission maximum at \(\approx 545\) nm. To assess the differences in relative Ca$^{2+}$ affinity, we used Ca$^{2+}$ (5–50 nM) to decrease the terbium(III) fluorescence at 545 nm (Fig. 5). The competition curves were biphasic for both the wild-type C2A fragment and G203D and Y235C mutants suggesting two Ca$^{2+}$-binding sites. The Ca$^{2+}$ IC50 s for wild-type C2A domain were 524 nM and 27.5 \(\mu\)M; for G203D, 60 nM and 99 \(\mu\)M and for Y235C, 104 nM and 35 \(\mu\)M. Both G203D and Y235C mutants had altered relative affinity for Ca$^{2+}$ with a lower relative affinity at the first Ca$^{2+}$-binding site (IC50-1, wild-type [wt] versus G203D \(P < 0.0001\); wt versus Y235C \(P = 0.0173\)). These results suggest that the G203D and Y235C mutations alter the calcium-binding affinity of the MCTP2 protein.

DISCUSSION

Several individuals have been reported with congenital heart defects in 15q26-ter deletion syndrome (MIM# 612626). Individuals with terminal deletions disrupting COUP-TFII often present with septal defects (21,25,26), while OT defects are observed more often when deletions extend centromeric to the gene (8–14). Our findings indicate that disruption of MCTP2, which is \(\approx 1.9\) Mb centromeric to COUP-TFII, contributes to congenital left heart obstructive cardiac defects in humans. The following lines of evidence support this conclusion: (i) the 2.2 Mb microdeletion on 15q26.2 involving the MCTP2 gene with normal dosage of COUP-TFII in two individuals with CoA and the absence of this deletion in 40,200 individuals studied by the clinical array-CGH; (ii) a de novo intragenic duplication of MCTP2 predictive of protein truncation in a non-syndromic individual with HLHS and CoA; (iii) functional knockdown of Mctp2 expression in Xenopus resulting in abnormal cardiac development and (iv) identification of rare MCTP2 missense variants in patients with CoA, which alter the Ca$^{2+}$-binding affinity of the protein. It is evident from the morpholino experiments that despite its important role in cardiac development, the penetrance for the cardiac phenotype is not complete with Mctp2 knockdown. This is furthermore apparent from the normal echocardiographic findings of the mother of subjects 1 and 2, who had the MCTP2 heterozygous deletion in 74% of

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Figure 3. Mctp2 morphant analyses. (A) Embryos were scored for viability at the indicated stages. Sub-effective doses of morpholinos had no effect on phenotype or viability when compared with controls when injected individually [Ctrl, MO1 1:50 (0.54 ng), MO2 1:50 (0.54 ng)]. Morphants injected with both morpholinos at sub-effective doses showed significant decreases in viability. The number of embryos analyzed in each group is represented above each group of bars. (B–D) Control and Mctp2 morphant embryo morphology at stage 44/45. Qualitatively similar phenotypic results were achieved using both morpholinos and (C) and (D) are representative images using MO1. (C) Mctp2 morphants have grossly normal external morphology with the exception of moderate to severe edema in a subset of embryos (arrows). (D) High power view of embryo with severe edema showing accumulation of fluid (arrowhead) in the cardiac region (arrow at heart).
lymphocytes. The observed incomplete penetrance is in keeping with several other genomic disorders that cause cardiovascular malformations (27,28). We note that deletions encompassing MCTP2 could, in principle, affect regulatory sequences that influence COUP-TFII expression. It is possible that the cardiovascular malformations observed in the subjects described here could be the result of combined effects on MCTP2 and COUP-TFII. However, the data provided here support the hypothesis that MCTP2 can make a contribution independent of COUP-TFII.

MCTPs are composed of a variable N-terminal sequence, three C2 domains, two TMRs and a short C-terminal sequence (24). Proteins with multiple C2 domains and a single TMR such as synaptotagmins (29), ferlins (30) and E-Syts (31) are known to be involved in the Ca^{2+}-dependent regulation of exocytosis. The MCTP2 protein is unusual in that unlike other families of trafficking proteins containing multiple C2 domains and a single TMR, the C2 domains of MCTP2 bind to Ca^{2+} in the absence of phospholipids with a high apparent affinity (24). We show that two rare missense variants in the C2A domain of MCTP2 identified in individuals with sporadic CoA alter the calcium-binding affinity of the protein. Future studies focusing on the cell-type-specific role of MCTP2 during cardiac morphogenesis, including its role in Ca^{2+} binding and intracellular trafficking, should provide important insights into novel mechanisms underlying OT development.

The primitive heart tube of the early embryo (E8.0 in mouse) is comprised of an inner layer of endocardial cells and an exterior layer of myocardial cells. As cardiac development proceeds, cells from the second heart field migrate in at the anterior and posterior poles and contribute to the development of the right ventricle, conotruncus and part of the atria. Subsequently, cardiac neural crest cells migrate into the heart following rightward looping (32–34). As development proceeds (at stage 14 in chick and 9.5 ED in mouse embryos), some of the endothelial cells in the OT and AV regions lose their epithelial phenotype, transform into migratory mesenchymal cells and invade the adjacent extracellular matrix, resulting in the formation of
valvuloseptal endocardial tissue (35). The morphogenesis of the endocardial cushion is induced by regionalized myocardial signals in the AV canal and OT regions, causing a subset of endocardial cells to undergo epithelial–mesenchymal transformation (EMT) and invade the cardiac jelly. Endocardial cushions are precursors of the heart valves and contribute to the septation of the atria and ventricles and the remodeling of the OT to form the aorta and the pulmonary artery. In Mctp2 morphants, the intact myocardial and endocardial layers of the OT in the presence of cardiac jelly suggest failure of the endocardial cells to undergo EMT and highlight an important potential mechanistic basis for the development of structural heart defects that warrants additional investigation. Interestingly, Notch1, which is implicated in LVOT abnormalities, such as familial aortic valve anomalies and BAV (6), has been shown to be essential for EMT during endocardial cushion formation (36). Notch1-deficient mouse embryos fail to form endocardial cushions and demonstrate an absence of mesenchymal cells migrating from the endocardium. These results are very similar to the phenotypes identified in Mctp2 morphants. Future studies will determine whether Notch signaling is perturbed with Mctp2 haploinsufficiency.

Despite the large number of animal models demonstrating defects in cardiac development, single gene causes of human cardiovascular malformations have been more difficult to identify. This study highlights the importance of a novel gene in human cardiovascular malformations. Whether the haploinsufficiency of MCTP2 causes a broader pleiotropic effect pertaining to neurocognitive and language development, as observed in individuals with 15q26 segmental aneusomies and subjects 1 and 2 in this study remains to be determined as additional cases are identified. Future mechanistic studies of Mctp2 function will provide an opportunity to identify new pathways important for cardiac development. In conclusion, these experiments identify MCTP2 as a novel dosage-sensitive gene important for human LVOT development and highlight an important role of MCTP2 in cardiac development.

MATERIALS AND METHODS

Human subjects

Written informed consents were obtained from the research subjects and the study was performed in accordance with the institutional guidelines for human research with approval by the Institutional Review Board at Baylor College of Medicine. Our patients consisted of two groups; group 1 included two maternal half-siblings, subjects 1 and 2 with syndromic CoA and their mother. Group 2 consisted of 146 patients with LVOT obstructive defects, primarily enrolled at Texas Children’s Hospital in Houston, TX (included 83 Caucasians, 48 Hispanics, 9 African Americans, 3 Asians and 3 mixed Caucasian/Hispanics). This cohort had 137 patients with isolated cardiac defects, with additional features of developmental delay and non-cardiac malformations. Each enrolled family was interviewed in person for detailed information on the prenatal course, diagnosis and family history of heart defects. Additional clinical information was obtained through review of the medical records.

Array-CGH, genome-wide SNP genotyping and FISH analyses

Peripheral blood samples from subject 1 in group 1 were submitted for clinical testing to the Baylor Medical Genetics Laboratories for BAC array-CGH analyses. Reciprocal dye reversal experiments were performed as described previously (37). Data analysis was performed by a web-based software platform with the capability of displaying the raw, normalized and integrated data of all clones to detect genomic copy-number changes for patient relative to a gender-matched control. A copy-number loss was determined by a combined log2 ratio of test/control < −0.2 and a P-value of < 0.05. FISH experiments on metaphase cells were performed to confirm the deletion of the region corresponding to BAC clone, RP11-4F5 in subject 1, identified by the clinical array-CGH. Miniprep BAC DNA (100 ng) was labeled with Spectrum Orange-dUTP or Spectrum Green-dUTP (Vysis, Downers Grove, IL, USA), according to the manufacturer’s instructions, and used as probes for FISH analysis using the standard protocols (38). To precisely map the deletion breakpoints, genome-wide analysis for DNA copy-number alterations was performed in subject 1 using Illumina HumanHap300 genotyping BeadChip array (37K, Illumina, San Diego, CA, USA) according to the manufacturer’s specifications, as described previously (39).

In group 2, 146 patients with LVOT obstructive lesions were studied with a custom high-definition CGH microarray (Agilent Technologies). These arrays included 1004 oligonucleotide probes in the chr15:89212888-99847710 genomic region (hg18), in a 4 × 4k format, spanning exonic, intronic and 5’ and 3’ regions of candidate genes within this interval, including MCTP2 and COUP-TFII. Array data were analyzed using Feature Extraction and CGH Analytics software (Agilent Technologies). The MCTP2 intragenic duplication of 41 kb was confirmed in patient 3 by PCR and FISH analysis, using fosmid G248P86659A10.

Identification of X. laevis Mctp2

Transcript information for Xenopus tropicalis Mctp2 was derived from the Joint Genome Institute database. Primers were designed in regions highly conserved across species to attempt to amplify X. laevis genomic DNA. Primers were designed in the first and second coding exons (177 bp product) and fourth and sixth coding exons (234 bp product; homologous to the fifth and seventh exons in human) for PCR and RT–PCR: exon 1F 5’AGCATTGGTTCAAGAGGA 3’; exon 2R 5’TGAACTTCTTACAGCTTTT 3’; exon 4F 5’CTTCTGCAAAAGCAAGGTCA 3’; exon 6F 5’CCATGTCTCCACCAGTCA 3’; exon 7F 5’CCATGTCTCCACCAGTCA 3’. Genomic DNA was isolated from X. laevis and used with exon 4 and exon 6 primers for PCR amplification using an annealing temperature of 54°C. The resulting 1038 bp product was PCR purified (Qiagen), sequenced and compared with the X. tropicalis database (sequence available upon request). No pseudoalleles were identified within the region sequenced. RNA was prepared from stage 25 X. laevis embryos using Trizol as per the manufacturer’s protocol. RT–PCR was performed as described previously (37).
performed using Titan One Tube RT–PCR (Roche) with 50 ng template RNA and a post reverse transcription annealing temperature of 54°C. The 234 bp product was purified (Qiagen PCR purification) and sequenced.

**Mctps antisense morpholino oligonucleotide and mRNA expression construct**

For *X. laevis*, two splice site Mctp2 antisense morpholino oligonucleotides were designed (Gene Tools, LLC). The first MO (MO1) was located at the splice donor site at the junction of exon 5–intron 5; the sequence was 5′ GCG GAA ATA ATT TGA TCA TAC TTG T 3′. The second MO (MO2) was located at the splice receptor site at the junction of intron 5–exon 6 with a sequence 5′ TCT GTA GTT CAT TAG GAA GGA A 3′. Both MOs were fluorescein tagged. A control antisense morpholino was also obtained from Gene Tools, LLC, with the following sequence 5′ CCT CTT ACC TCA GTT ACA ATT TAT A 3′. All morpholinos were resuspended and stored according to the manufacturer’s protocol (Gene Tools, LLC). To verify effective knockdown of the Mctp2 transcript by each morpholino, three control or morpholino-injected embryos were selected at stage 25 and pooled for homogenization in Trizol. Only grossly normal frog embryos were selected for use. Sections were prepared at 8–10 μm using a Leica microtome. Subsequently, slides were dewaxed in xylene, rehydrated through successive ethanol washes and stained with Gill’s Haematoxylin and Eosin Y.

**Whole-mount in situ hybridization**

*Xenopus* embryos were fixed at the desired stage in MEMFA for 2 h at room temperature. Following fixation, embryos were dehydrated in 100% ethanol and stored at –20°C. Whole-mount *in situ* hybridization was performed as described previously (41). The pBSK–Cardiac Troponin plasmid was kindly provided by the Zorn Laboratory and was used to generate antisense riboprobes.

**MCTP2 sequencing**

Mutation analysis of MCTP2 was performed in all 146 subjects in group 2, studied for MCTP2 dosage alteration. The protein-coding exons of MCTP2 were amplified from genomic DNA (sequence of primers and amplification conditions available on request). The PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced with a dye terminator cycle-sequencing system (Applied Biosystems 3730xl).

**Expression and purification of recombinant wild-type and mutant proteins**

We performed functional analyses of the two heterozygous base substitutions, G203D and Y235C, within the C2A domain in two individuals with CoA. The DNA encoding the human MCTP2-C2A (residue 195-286) was PCR amplified, subcloned into pGEX-6p-1 vector (GE Healthcare, NJ, USA) and verified by DNA sequencing. This construct was used for the site-directed mutagenesis. Two missense variants (G203D and Y235C) were constructed using the QuickChange site-directed mutagenesis kit (Stratagene, CA, USA) and verified by DNA sequencing. These plasmids were transformed into *Escherichia coli* strain and expressed as glutathione-S-transferase (GST) fusion proteins, refolded with Rapid GST Inclusion Body Kit (Cell Biolabs, Inc., CA, USA) and purified with Glutathione Sepharose 4 Fast Flow (GE Healthcare). Recombinant GST protein was expressed and purified as control using the same method.

**Fluorescence spectroscopy**

The relative Ca$^{2+}$-binding affinity of the three recombinant proteins was assessed by monitoring the changes in terbium(III) fluorescence binding. Buffer (50 mM Tris, pH 7.4, 0.1 mM NaCl) was incubated with Chelex resin 100 (Bio-Rad) for 15 min at room temperature to remove trace levels of Ca$^{2+}$. The Chelex resin was removed by centrifugation before lanthanide metal ion terbium(III) (Sigma) binding. Fluorescence due to terbium(III) (1 mM) binding was recorded on an ISS PCI photon-counting spectrophotometer (Champaign, IL, USA) excited at 285 nm (UV solar pass filter) and the emission spectra (520–570 nm). Ca$^{2+}$ was titrated from 5 to 50 mM. For each recombinant protein, the experiment was repeated three to six times. Emission
maxima (545 nm) at each Ca\textsuperscript{2+} concentration were corrected by subtracting background at 530 nm. These data were normalized, their percent change from 0 Ca\textsuperscript{2+} calculated, and data fit with a two site competition equation using SigmaPlot 10.0.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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**REFERENCES**


