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ORIGINAL INVESTIGATION

Molybdenum cofactor deficiency type B knock-in mouse models carrying patient-identical mutations and their rescue by singular AAV injections

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Abstract
Molybdenum cofactor deficiency is an autosomal, recessively inherited metabolic disorder, which, in the absence of an effective therapy, leads to early childhood death due to neurological deterioration. In type A of the disease, cyclic pyranopterin monophosphate (cPMP) is missing, the first intermediate in the biosynthesis of the cofactor, and a biochemical substitution therapy using cPMP has been developed. A comparable approach for type B of the disease with a defect in the second step of the synthesis, formation of molybdopterin, so far has been hampered by the extreme instability of the corresponding metabolites. To explore avenues for a successful and safe gene therapy, knock-in mouse models were created carrying the mutations c.88C>T (p.Q30X) and c.726_727delAA, which are also found in human patients. Recombinant adeno-associated viruses (rAAVs) were constructed and used for postnatal intrahepatic injections of MoCo-deficient mice in a proof-of-concept approach. Singular administration of an appropriate virus dose in 60 animals prevented the otherwise devastating phenotype to a variable extent. While untreated mice did not survive for more than 2 weeks, some of the treated mice grew up to adulthood in both sexes.

Introduction

Molybdenum cofactor (MoCo) deficiency is an ultra-rare autosomal, recessively inherited disease, which is characterized by the simultaneous loss of xanthine oxidoreductase and sulfite oxidase (Atwal and Scaglia 2016). Neurotoxic sulfite accumulates and triggers a progressive neurological disease, which is lethal if untreated. According to Mechler et al. (2015) the median survival is 36 months. Age at onset of the disease is the first day of life and initial cardinal disease features are intractable seizures and feeding difficulties. Elevated sulfite levels in the urine and a lowered plasma level of uric acid confirm the diagnosis (Johnson et al. 1980). Nevertheless there is a diagnostic delay of 3 months on average (Mechler et al. 2015), which is probably due to the extremely low incidence and the resulting unawareness of the disease. Only around 100 patients are described worldwide (Hinderhofer et al. 2017).

The biosynthetic pathway leading to active MoCo is conserved among bacteria, plants and animals and can be divided into three steps (Schwarz et al. 2009). In the first step, Guanosine triphosphate (GTP) is converted to cyclic pyranopterin monophosphate (cPMP) by the two proteins encoded by the gene *MOCS*1. Mutations in this gene lead to MoCo deficiency type A (OMIM#252150), the most frequent type of the disease with approximately two-thirds of cases (Reiss and Hahnewald 2011). cPMP can be isolated effectively from recombinant bacteria and then used in a substitution therapy for type A of the disease, as shown first for mice (Schwarz et al. 2004) and then in humans (Hitzert et al. 2012; Schwahn et al. 2015; Veldman et al. 2010).

In the second step, cPMP is further transformed to molybdopterin by molybdopterin synthase consisting of two small (MOCS2A) and two large (MOCS2B) subunits, which are the products of the *MOCS*2 gene (Schwarz et al. 2009). Prior to this reaction, the small subunit has to be sulfurated by the *MOCS*3 gene product (Matthies et al. 2004). Patients with mutations in the genes *MOCS*2 or *MOCS*3 are categorized

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as type-B patients (OMIM#252160 and OMIM#609277), with only a single report describing disease-causing *MOCS*3 mutations (Huijmans et al. 2017). Type-B patients represent the second largest group with approximately one-third of cases (Reiss and Hahnewald 2011).

In the third step, molybdenum is incorporated into molybdopterin via an adenylated intermediate (Schwarz et al. 2009). This is catalyzed by a two-domain protein called Gephyrin, which is also required for receptor clustering (Kins et al. 2000). Pathogenic mutations in the encoding *GPHN* gene lead to MoCo deficiency type C (OMIM#615501). Only two cases of this type have been described (Reiss et al. 2001, 2011) and I am not aware of a living patient of type C.

Molybdopterin or active MoCo would be a hypothetical drug for MoCo deficiency type B, but both molecules contain oxygen-sensitive sulfhydryl-groups and thus cannot be isolated in sufficient amounts and quality for a biochemical substitution therapy (Johnson et al. 1984). In the mouse model, however, we have already demonstrated the feasibility of a gene therapy for MoCo deficiency type A using a recombinant Adeno-associated virus (rAAV) with a *MOCS*1 expression cassette in a preclinical model (Kugler et al. 2007; Hahnewald et al. 2009). Gene therapy via rAAVs is generally considered a safe procedure and already has been approved as a standardized treatment in humans (Russell et al. 2017).

We have also described a *Mocs*2 knockout mouse and its phenotype, which resembles that of MoCo deficiency type-B patients (Jakubiczka-Smorag et al. 2016). In this knockout model both open reading frames for MOCS2A and MOCS2B were destroyed. Attempts to rescue the phenotype by injection of rAAVs with bicistronic expression cassettes proved unsuccessful (data not shown). Since almost all patients carry point mutations or small insertions or deletions (indels) in one of the two frames, this situation was now simulated in two knock-in models with the *Mocs*2A mutation c.88C>T (p.Q30X) and the *Mocs*2B mutation c.726_727delAA, respectively, with the latter being the most frequent mutation found in our type-B patient cohort (7 of 72 alleles).

No compound heterozygous type-B patients have been described in the literature, who carry one mutation in the MOCS2A region and a second mutation in the MOCS2B region (Reiss and Johnson 2003). This is indirect evidence that two alleles with such heterozygous mutations in trans would complement each other to a normal phenotype. The correctness of this assumption, however, is a prerequisite for a successful gene therapy with monocistronic expression cassettes encoding either the small or the large subunit of molybdopterin synthase. The two murine knock-in mouse models were used to examine the independent expression of the two subunits and whether they could be delivered via rAAVs to be functional, thus exploring the feasability of an AAV-mediated gene therapy for MoCo deficiency type B.

Materials and methods

Construction of the knock-in mice

Knock-in mice carrying either the *Mocs*2A mutation c.88C>T or the *Mocs*2B mutation c.726_727delAA were constructed on a C57Bl6N background by homologous recombination commercially (Genoway). The corresponding targeting vectors are depicted in Fig. 1. In case of the *Mocs*2B mutation, the deletion of the two nucleotides 726 and 727 in the mouse sequence would result in the replacement of the last nine amino acids (aa) by seven de novo aa, while in the human sequence the same deletion results in the addition of 30 de novo aa. To resemble the pathological state in humans as closely as possible, the last 27 coding nucleotides in the murine sequence were replaced by the human sequence coding for the 30 de novo aa expected for the c.726_727delAA mutation ("Human CDS" in Fig. 1). Recombination was confirmed by Southern blot analysis and verified by sequencing, genotyping was performed by PCR (data not shown). Homozygous mice were always derived from matings of two heterozygotes.

Verification of the in trans complementation ability

To test the production of functional molybdopterin synthase from small and large subunits encoded on different alleles, heterozygous animals of both knock-in lines were mated and the resulting offspring genotyped for both alleles according to the assays developed by the manufacturer (Genoway, data not shown).

Construction of the expression cassettes and AAV packaging

Using the human coding sequences for MOCS2A (NM_176806.3) and MOCS2B (NM_004531.4), two plasmids were constructed commercially as shown in Fig. 2 (VectorBuilder). Large-scale preparations of these plasmids were used for packaging the two expression cassettes in AAV serotype 2 (Vigene).

Virus injections

Based on previously published dosage studies (Hahnewald et al. 2009), 2×10^{10} genome copies (GC) were injected intrahepatically using phosphate-buffered saline (PBS, ph 7.0) and a 0.3 mm needle at days 2, 3 or 4 after birth. In both lines (Mocs2A p.Q30X and Mocs2B c.726delAA,

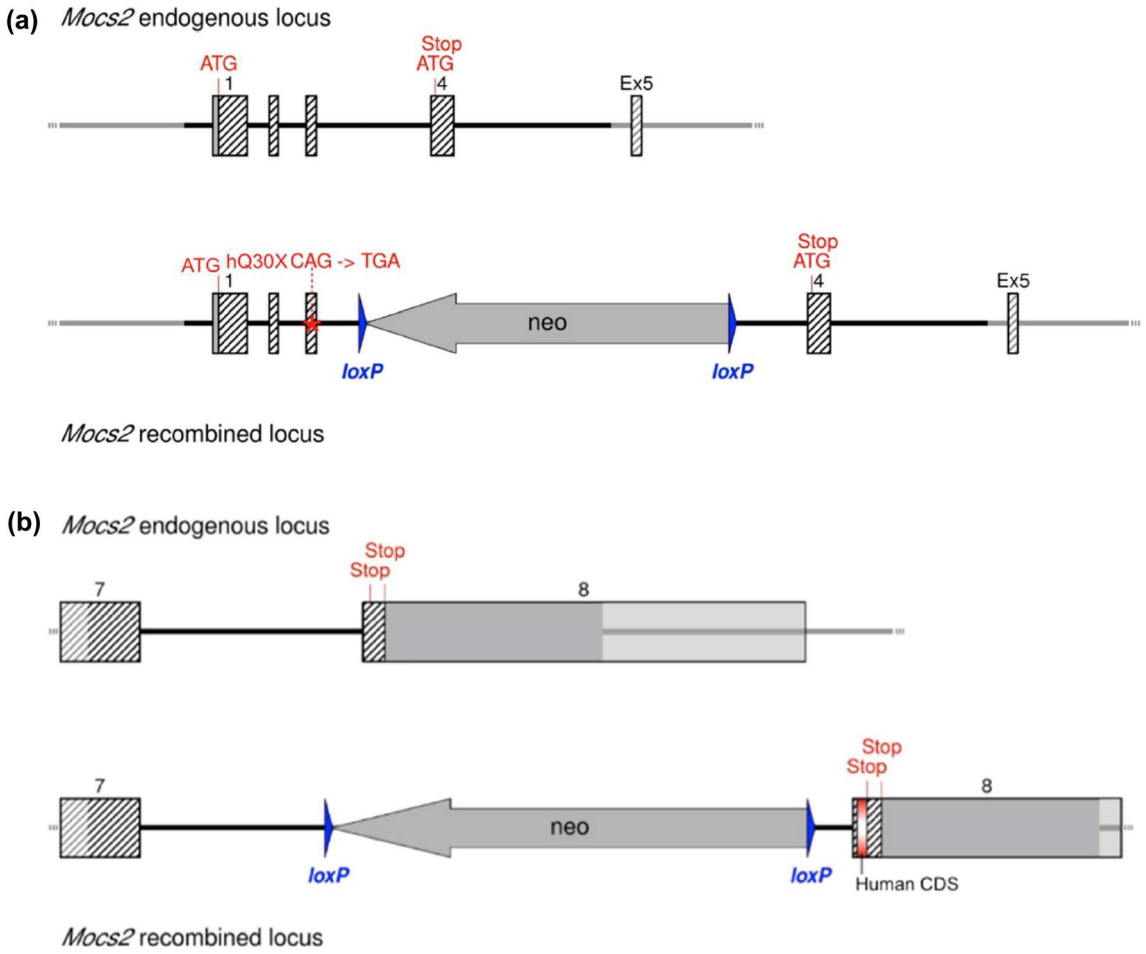

Fig. 1 Strategy for the construction of the *Mocs*2 knock-in mutations. Exon numbering here is consecutively from 1 to 8 rather than using the human exon 1a and exon 1b designation (Reiss and Johnson 2003). **a** Construction of the Mocs2A Q30X mutant. **b** Construction of the Mocs2B c.726_727del2 mutant. The structure of the human CDS is explained in the text

respectively) 10 homozygous animals were injected at each time point in a proof-of-concept approach.

Phenotype assessment

Treated and untreated mice were inspected visually on a daily basis, weighed and—if applicable—tested on a self-made rotating rod.

Sulfite oxidase assay

Sulfite oxidase activity was determined in liver tissue by a standard method (Macleod et al. 1961). In short, 100–200 mg of tissue after freezing was homogenized mechanically and centrifuged. Aliquots of the supernatant were incubated with sodium sulfite and cytochrome C in a cuvette at room temperature and the absorption difference at 550 nm was measured photometrically.

Results

Phenotype of the untreated MoCo Type-B mouse models

The phenotype of both homozygous Mocs2A-deficient mice as well as homozygous Mocs2B-deficient mice resembled, what already had been described extensively for homozygous *Mocs*1- or *Mocs*2-knockout mice (Jakubiczka-Smorag et al. 2016; Lee et al. 2002). Essentially, animals appeared healthy at birth but failed to thrive within their 1st week after birth with the parallel development of seizures (Supplementary Material Videos 1 and 2). Absence of MoCo was confirmed by the lack of sulfite oxidase activity (Fig. 3). Untreated homozygous Mocs2A-deficient mice died between day 2 and day 11 after birth with a mean life span of 8.7 days ($n = 14$), untreated Mocs2B-deficient mice between days 9 and day 12 with a mean life span of 10.6 days ($n = 11$). In both lines, heterozygous animals were without symptoms.

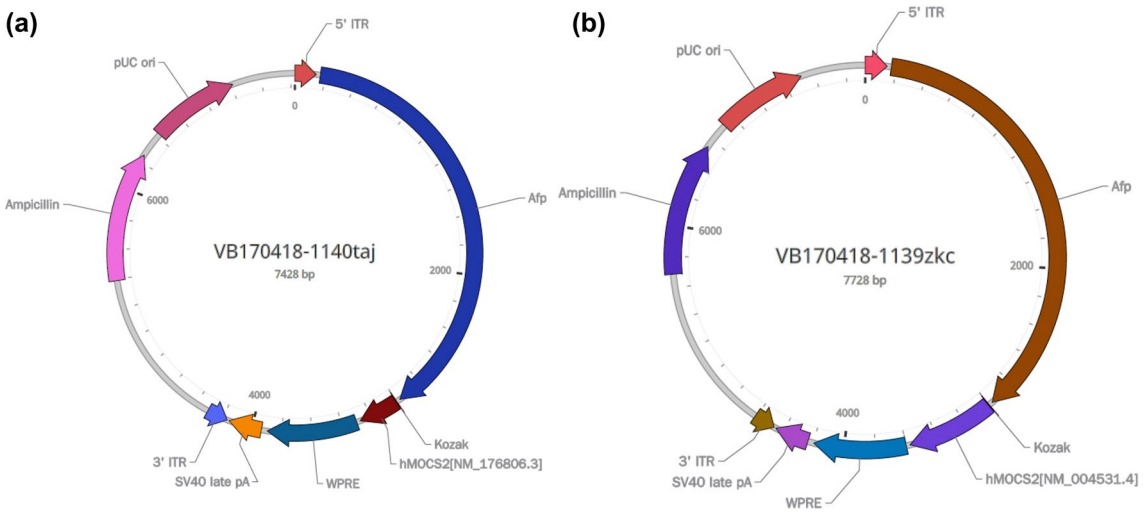

Fig. 2 Architecture of the AAV packaging plasmids. ITR, inverse terminal repeats (packaging signals). The Ampicillin resistance gene and the pUC origin of replication were used for the propagation of the plasmids in *E. coli*. The expression cassettes consist of the following elements: Afp, mouse alpha-fetoprotein enhancer II fused with human beta globin promotor (liver-specific). Kozak, translation initiation. WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. SV40 late pA, Simian virus 40 late polyadenylation signal. **a** hMOCS2[NM_176806.3], coding sequence for human MOCS2A. **b** hMOCS2[NM_004531.4], coding sequence for human MOCS2B

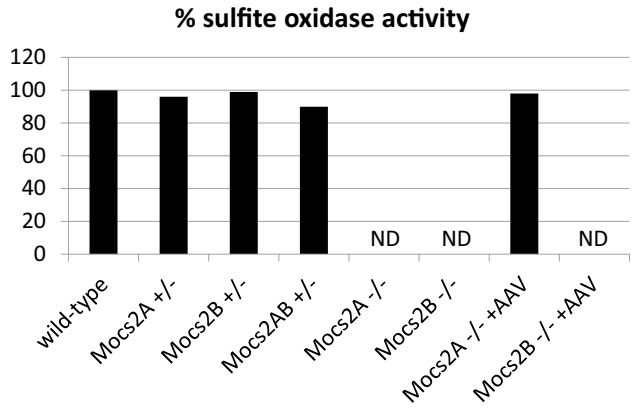

Fig. 3 Sulfite oxidase activity in liver tissue of different mice. Each column represents the average of at least two animals. Wild-type activity was defined as 100% activity. *ND* not detectable

In trans complementation in compound heterozygous mice

Heterozygous animals of both knock-in lines were mated with each other and compound heterozygous offspring identified. These animals showed no respective phenotype (see also Fig. 3) and grew up to adulthood and fertility in both sexes ($n = 7$). Matings of two compound heterozygote animals resulted in offspring with homozygous Mocs2A-deficient and homozygous Mocs2B-deficient mice according to the Mendelian rules. These homozygous animals of the F2 generation showed the pathological phenotype as described above. Thus, the two mutated alleles complement each other in trans in the F1 generation.

Rescue of the p.Q30X mutation by AAV-mediated delivery of the MOCS2A expression cassette

In the Mocs2A line (p.Q30X), 10 homozygous deficient mice each were injected with the respective rAAV on days 2, 3 or 4 after birth. This resulted in a total of ten animals reaching the age for weaning (3 weeks), while the remaining animals showed no change of phenotype and mortality (Table 1). In the cohort injected at day 3 after birth, one animal survived for 66 days before dying without showing the typical signs of MoCo deficiency (i.e., seizures and weight loss).

In the two other cohorts one animal each survived for 4 months (one male injected at day 2 after birth and one female injected at day 4 after birth) without showing symptoms (Supplementary material Video 3). These animals were sacrificed for enzyme analysis without recognizable disease phenotype at that time (Fig. 3).

Rescue of the c.726_727delAA mutation by AAV-mediated delivery of the MOCS2B expression cassette

In the Mocs2B line (c.726_727delAA) 30 animals were injected as described above, but only 4 animals survived for longer than the untreated animals without phenotype (Table 1). Although their development appeared normal (Supplementary material Video 4), none of these animals

Table 1 Survival of homozygous animals after rAAV injections at different time points

MOCS2A (p.Q30X)	Injected	Survival beyond weaning	Death at day (pp)
Injected at day 2 pp	10	4	26, 28, 31, 122*
Injected at day 3 pp	10	3	21, 27, 66
Injected at day 4 pp	10	3	22, 30, 125*
MOCS2B (c.726delAA)	*n*	Extended survival without phenotype	
Injected at day 2 pp	10	4	9*, 11*, 13, 20
Injected at day 3 pp	10	0	
Injected at day 4 pp	10	0	

pp post partem

*Sacrificed for enzyme analysis

survived for longer than 20 days. Two of such mice with a normal development were sacrificed at days 9 and 11 after birth for enzyme analysis. Surprisingly, no sulfite oxidase activity could be detected (Fig. 3).

Discussion

The mutation detection rate in MoCo deficiency nowadays is almost 100 percent involving mutations in the genes *MOCS*1, *MOCS*2, *MOCS*3 and *GPHN* (Reiss and Hahnewald 2011; Huijmans et al. 2017).

In roughly one-third of secured cases of MoCo deficiency, homozygosity or compound heterozygosity for *MOCS*2 mutations is found resulting in a type-B MoCo deficiency due to the lack of molybdopterin (Reiss and Hahnewald 2011). MoCo-deficient patients with this type of mutations thus represent the largest group for which hitherto no effective therapy is available.

This study describes the simulation of patient-identical mutations in murine models for both the MOCS2A and the MOCS2B subtype. The murine phenotype was as expected from the two previously described models (Lee et al. 2002; Jakubiczka-Smorag et al. 2016). This and measurement of sulfite oxidase activity in liver tissue confirmed absence of active MoCo (Fig. 3).

The architecture of both the human and the murine *MOCS*2 gene with two overlapping open reading frames is unusual for eukaryotes. In trans complementation of the two lines, being defective in different subunits of the molybdopterin synthase, was confirmed as a prerequisite for a gene therapy approach by breeding the two mouse lines among each other resulting in compound heterozygous animals without symptoms and normal levels of sulfite oxidase activity (Fig. 3).

Intrahepatic injections of rAAVs carrying expression cassettes for either the small or the large subunit of molybdopterin synthase (Fig. 2) could rescue the phenotype in both lines, but only in a fraction of the injected animals. A possible explanation for the lower success rate as compared to previous studies with *Mocs*1-deficient mice might be the route of application, which is the main drawback of this study. In our studies with *Mocs*1-deficient mice we could use cPMP to raise homozygous animals up to a size where also intravenous injections via the tail vein or intraperitoneal delivery were possible (Kugler et al. 2007; Hahnewald et al. 2009). Here, only intrahepatic injections at a very young age could be used and since the depth of the injection is difficult to control, the reproducibility of the virus delivery is diminished. Further preclinical studies might be improvable, e.g., using the cre-loxP-mediated recombination system to construct inducible mouse models (McLellan et al. 2017). However, we have already demonstrated the feasibility of intravenous AAV delivery to cure MoCo deficiency in the type A mouse model and this certainly will be the method of choice for a human application. Other examples of AAV-mediated gene therapy have been reviewed by Naso et al. (2017).

The central question of this study was, whether the two subunits of molybdopterin could—in principle—be delivered via rAAVs to be functional. The results summarized in Fig. 3 show that heterozygous and compound heterozygous animals show nearly wild-type levels of sulfite oxidase, and also the Mocs2A-deficient mice treated with the respective rAAV. The most surprising result, however, is the absence of detectable sulfite oxidase activity in the injected Mocs2b-deficient mice—even when their development is clearly improved as compared to untreated homozygous animals. Using liver material derived from a patient with a homozygous *MOCS*2A initiation mutation, we have observed a strikingly diminished level of MOCS2B protein and we have suggested a reduced half-life of this subunit in the absence of MOCS2A protein (Hahnewald et al. 2006). Since in the injected Mocs2b-deficient mice the AAV-encoded Mocs2B protein clearly is the limiting factor in the production of active MoCo, an additionally reduced

half-life of this component until it combines with the in trans encoded Mocs2A subunit might be a possible explanation for the lower success rate of AAV-mediated therapy of the c.726_727delAA strain. If this assumption is correct, it might be possible to overcome this drawback by increasing the rAAV dosage massively.

Although the effectiveness within this study appeared low, the results described here should encourage the pursuit of more detailed preclinical studies. These should address questions such as (1) optimization of virus dosage and delivery, (2) comparison of different AAV serotypes and (3) optimization of the expression cassette.

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Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

Statement of human/animal rights This article does not contain any studies with human participants.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Nds. Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Dezernat 33, Röverskamp 5, 26203 Wardenburg).

References

Atwal PS, Scaglia F (2016) Molybdenum cofactor deficiency. Mol Genet Metab 117:1–4

Hahnewald R, Leimkuhler S, Vilaseca A, Acquaviva-Bourdain C, Lenz U, Reiss J (2006) A novel MOCS2 mutation reveals coordinated expression of the small and large subunit of molybdopterin synthase. Mol Genet Metab 89:210–213

Hahnewald R, Wegner W, Reiss J (2009) AAV-mediated gene therapy for metabolic diseases: dosage and reapplication studies in the molybdenum cofactor deficiency model. Genet Vaccines Ther 7:9

Hinderhofer K, Mechler K, Hoffmann GF, Lampert A, Mountford WK, Ries M (2017) Critical appraisal of genotype assessment in molybdenum cofactor deficiency. J Inherit Metab Dis 40:801–811

Hitzert MM, Bos AF, Bergman KA, Veldman A, Schwarz G, Santamaria-Araujo JA, Heiner-Fokkema R, Sival DA, Lunsing RJ, Arjune S, Kosterink JG, van Spronsen FJ (2012) Favorable outcome in a newborn with molybdenum cofactor type A deficiency treated with cPMP. Pediatrics 130:e1005–e1010

Huijmans JGM, Schot R, de Klerk JBC, Williams M, de Coo RFM, Duran M, Verheijen FW, van Slegtenhorst M, Mancini GMS (2017) Molybdenum cofactor deficiency: identification of a patient with homozygote mutation in the *MOCS3* gene. Am J Med Genet A 173:1601–1606

Jakubiczka-Smorag J, Santamaria-Araujo JA, Metz I, Kumar A, Hakroush S, Brueck W, Schwarz G, Burfeind P, Reiss J, Smorag L (2016) Mouse model for molybdenum cofactor deficiency type B recapitulates the phenotype observed in molybdenum cofactor deficient patients. Hum Genet 135:813–826

Johnson JL, Waud WR, Rajagopalan KV, Duran M, Beemer FA, Wadman SK (1980) Inborn errors of molybdenum metabolism: combined deficiencies of sulfite oxidase and xanthine dehydrogenase in a patient lacking the molybdenum cofactor. Proc Natl Acad Sci USA 77:3715–3719

Johnson JL, Hainline BE, Rajagopalan KV, Arison BH (1984) The pterin component of the molybdenum cofactor. Structural characterization of two fluorescent derivatives. J Biol Chem 259:5414–5422

Kins S, Betz H, Kirsch J (2000) Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. Nat Neurosci 3:22–29

Kugler S, Hahnewald R, Garrido M, Reiss J (2007) Long-term rescue of a lethal inherited disease by adeno-associated virus-mediated gene transfer in a mouse model of molybdenum-cofactor deficiency. Am J Hum Genet 80:291–297

Lee HJ, Adham IM, Schwarz G, Kneussel M, Sass JO, Engel W, Reiss J (2002) Molybdenum cofactor-deficient mice resemble the phenotype of human patients. Hum Mol Genet 11:3309–3317

Macleod RM, Farkas W, Fridovich I, Handler P (1961) Purification and properties of hepatic sulfite oxidase. J Biol Chem 236:1841–1846

Matthies A, Rajagopalan KV, Mendel RR, Leimkuhler S (2004) Evidence for the physiological role of a rhodanese-like protein for the biosynthesis of the molybdenum cofactor in humans. Proc Natl Acad Sci USA 101:5946–5951

McLellan MA, Rosenthal NA, Pinto AR (2017) Cre-loxP-mediated recombination: general principles and experimental considerations. Curr Protoc Mouse Biol 7:1–12

Mechler K, Mountford WK, Hoffmann GF, Ries M (2015) Ultra-orphan diseases: a quantitative analysis of the natural history of molybdenum cofactor deficiency. Genet Med 17:965–970

Naso MF, Tomkowicz B, Perry WL, Strohl WR (2017) Adeno-associated virus (AAV) as a vector for gene therapy. BioDrugs. 31, 317–334

Reiss J, Hahnewald R (2011) Molybdenum cofactor deficiency: mutations in GPHN, MOCS1, and MOCS2. Hum Mutat 32:10–18

Reiss J, Johnson JL (2003) Mutations in the molybdenum cofactor biosynthetic genes MOCS1, MOCS2, and GEPH. Hum Mutat 21:569–76

Reiss J, Gross-Hardt S, Christensen E, Schmidt P, Mendel RR, Schwarz G (2001) A mutation in the gene for the neurotransmitter receptor-clustering protein gephyrin causes a novel form of molybdenum cofactor deficiency. Am J Hum Genet 68:208–213

Reiss J, Lenz U, Aquaviva-Bourdain C, Joriot-Chekaf S, Mention-Mulliez K, Holder-Espinasse M (2011) A GPHN point mutation leading to molybdenum cofactor deficiency. Clin Genet 80:598–599

Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, Tillman A, Wittes J, Pappas J, Elci O, McCague S, Cross D, Marshall KA, Walshire J, Kehoe TL, Reichert H, Davis M, Raffini L, George LA, Hudson FP, Dingfield L, Zhu X, Haller JA, Sohn EH, Mahajan VB, Pfeifer W, Weckmann M, Johnson C, Gewaily D, Drack A, Stone E, Wachtel K, Simonelli F, Leroy BP, Wright JF, High KA, Maguire AM (2017) Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet 390:849–860

Schwahn BC, Van Spronsen FJ, Belaidi AA, Bowhay S, Christodoulou J, Derks TG, Hennermann JB, Jameson E, Konig K, McGregor TL, Font-Montgomery E, Santamaria-Araujo JA, Santra S, Vaidya

M, Vierzig A, Wassmer E, Weis I, Wong FY, Veldman A, Schwarz G (2015) Efficacy and safety of cyclic pyranopterin monophosphate substitution in severe molybdenum cofactor deficiency type A: a prospective cohort study. Lancet 386:1955–1963

Schwarz G, Santamaria-Araujo JA, Wolf S, Lee HJ, Adham IM, Grone HJ, Schwegler H, Sass JO, Otte T, Hanzelmann P, Mendel RR, Engel W, Reiss J (2004) Rescue of lethal molybdenum cofactor deficiency by a biosynthetic precursor from *Escherichia coli*. Hum Mol Genet 13:1249–1255

Schwarz G, Mendel RR, Ribbe MW (2009) Molybdenum cofactors, enzymes and pathways. Nature 460:839–847

Veldman A, Santamaria-Araujo JA, Sollazzo S, Pitt J, Gianello R, Yaplito-Lee J, Wong F, Ramsden CA, Reiss J, Cook I, Fairweather J, Schwarz G (2010) Successful treatment of molybdenum cofactor deficiency type A with cPMP. Pediatrics 125:e1249–e1254