ATP7A-Regulated Enzyme Metalation and Trafficking in the Menkes Disease Puzzle

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Abstract: Copper is vital for numerous cellular functions affecting all tissues and organ systems in the body. The copper pump, ATP7A is critical for whole-body, cellular, and subcellular copper homeostasis, and dysfunction due to genetic defects results in Menkes disease. ATP7A dysfunction leads to copper deficiency in nervous tissue, liver, and blood but accumulation in other tissues. Site-specific cellular deficiencies of copper lead to loss of function of copper-dependent enzymes in all tissues, and the range of Menkes disease pathologies observed can now be explained in full by lack of specific copper enzymes. New pathways involving copper activated lysosomal and steroid sulfatases link patient symptoms usually related to other inborn errors of metabolism to Menkes disease. Additionally, new roles for lysyl oxidase in activation of molecules necessary for the innate immune system, and novel adapter molecules that play roles in ERGIC trafficking of brain receptors and other proteins, are emerging. We here summarize the current knowledge of the roles of copper enzyme function in Menkes disease, with a focus on ATP7A-mediated enzyme metalation in the secretory pathway. By establishing mechanistic relationships between copper-dependent cellular processes and Menkes disease symptoms in patients will not only increase understanding of copper biology but will also allow for the identification of an expanding range of copper-dependent enzymes and pathways. This will raise awareness of rare patient symptoms, and thus aid in early diagnosis of Menkes disease patients.

Keywords: ATP7A; Menkes disease; symptomatology; copper enzyme; copper trafficking

1. ATP7A-Related Copper Disorders

ATP7A-related X-linked genetic disturbances exhibit dysfunction of multiple copper-dependent processes resulting in a broad spectrum of disease phenotypes. Three clinical groups are described: Menkes disease (MNK), occipital horn syndrome (OHS), and X-linked distal spinal muscular atrophy 3 (SMAX3) but overlapping intermediate forms (Table 1) confuse grouping [1,2]. MNK is characterized by neurodegeneration, fair skin, kinky hair, connective tissue abnormalities, and short life span. OHS presents with connective tissue symptoms, develops pathognomonic occipital bony exostosis (horns), and has reduced life expectancy. SMAX3 comprises a yet limited group of adult-onset progressive motor neuron disease, minimal copper disturbance, normal fertility, and long lifespan. Grouping into three phenotypes is arbitrary, and the spectrum is better described as a clinical continuum from severe disease with many affected enzyme systems to very mild affection with few enzyme systems involved. The best nosology should refer to ATP7A-related disturbances as the main pointer [2]. To best explain patients’ symptoms, we have here focused on the severest form, i.e., MNK.
| Clinical Type * | Abreviation | OMIM   | Age of Onset | Diagnostic Pointer                                                                 | Connective Tissue Involvement                                                                 | Motor Function * | Mental Function * | Age of Death § |
|----------------|-------------|--------|--------------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|------------------|-------------------|-----------------|
| Menkes Disease | MNK         | 309400 | 0–8 months   | Kinky Hair; floppy infant; mimicry of severe metabolic disorders of lysosomes, mitochondria, and peroxisomes; dysautonomia | Severe; osteoporoses; hemorrhages; bladder and bowel diverticulae                            | Poor mobility; no head control | Severe mental retardation | <3 years       |
| Long Surviving Menkes | LS       | 309400 | 0–8 months  | Hair changes; dysautonomia; floppy; initial symptoms similar to MNK                  | Severe to moderate                                                                          | Poor mobility; limited head control         | Severe mental retardation | <15 years      |
| Moderate Menkes | MOD        | 309400 | 3–12 months  | Coarse hair; dysautonomia; initial course milder; clinical diagnosis difficult       | Present, but not obvious; skeletal dysplasia may occur                                      | Wheelchair bound; cannot sit unsupported | Mentally retarded     | <40 years       |
| Mild Menkes    | MILD       | 309400 | 2–3 years    | Coarse hair; dysautonomia; initial course mild; clinical diagnosis difficult         | Minimal to moderate; skeletal dysplasia;                                                   | Walk with difficulty and with use of aid         | Moderately retarded; Slow and dull   | >40 years       |
| Occipital Horn Syndrome * | OHS     | 304150 | Childhood    | Coarse hair; X-linked family history; connective tissue problems; muscle affection; dysautonomia, exostoses on occipital bones; skeletal dysplasia; cutis laxa; hyperextensible joints; vascular complications | Walk independently; appear clumsy                                                            | Slow or dull to normal IQ                  | 40–60 years     |
| X-linked distal spinal muscular atrophy 3 | SMAX3 | 300489 | Adulthood    | X-linked family history of muscle wasting; dysautonomia with prevalent adrenergic involvement | Minor connective tissue involvement; minor occipital horns may occur                        | Walk independently; progressive distal motor neuropathy | Normal IQ; normal fertility | <80 years       |

* clinical types are poorly delineated with overlapping symptoms and functions within and between groups; # occipital horns depend on head control and can occur from two years; § connective tissue complications shorten lifespan; Cu treatment prolongs lifespan.
The basic defect in MNK is deficient copper transfer to secretory pathway, the intracellular sorting station for proteins, which affects metalation and trafficking of copper-dependent enzymes and diminishes copper extrusion from cells. Insufficient functional ATP7A results in abnormal body copper distribution with high values in tissues, but severe lack in blood, brain, and liver [1]. Current understanding of copper dependent processes does not account for all neurological symptoms [3], and a broad update is needed. Substrates are included with each enzyme to better define key symptoms in MNK.

Clinical features found in MNK patients can be explained by deficiencies of copper-dependent enzymes, amine oxidases (biodegradation of histamine and polyamines), lysyl oxidases (cross-linking of elastin, collagen, and collectin), cytochrome c oxidase (energy formation), peptidyl α-amidating enzyme (activation of neurohormones and neuropeptides), dopamine β-hydroxylase (catecholamine production), tyrosinase (pigment formation and free radical defense), superoxide dismutase (free radical detoxification), and ferroxidase (iron mobilization and free radical defense). In addition, newly discovered copper-activated enzymes, including several lysosomal and steroid sulfatases, provide further insight into MNK pathophysiology [4] and will be discussed herein.

2. Copper Enzymes

Copper serves as redox cofactor in a wide range of reactions including electron transfer, oxidation, reduction, and disproportionation [4] (Table 2). Copper is regulated by redox shifts at several cellular and subcellular membranes and is further implicated in redox shifts of iron. In addition, copper acts as allosteric regulator at binding sites spatially apart from catalytic centers (Table 2). In enzymes, copper acts as integral electron modulator, but roles in formation and coupling of cofactors are important for understanding MNK clinical spectrum [4] and copper chaperone activation of enzymes without catalytic copper sites is emerging [5]. Copper enzymes contain active centers shuttling electrons from one molecule to another, and copper changes reversibly between oxidation states during catalytic cycles. Nature uses a variety of copper centers to facilitate electron transfer, and enzymes are grouped accordingly. Often catalytic metal sites are made up of metal coordinating residues located far apart in primary structure, but in the folded structure, they create a cluster of closely spaced amino acids forming metal-binding sites. The protein’s tertiary structure is often stabilized by coordination of the metal [6,7].

Copper enzymes are widely distributed both intracellularly and extracellularly. Intracellular copper enzymes are located in subcellular compartments and organelles such as cytoplasm, secretory pathway, peroxisomes, nucleus, and mitochondria, and metalation occurs at different sites. It is an important question to ask when and where the polypeptide meets the metal for copper-dependent enzymes. Newly synthesized apoenzymes are directed to the secretory pathway, and during passage from endoplasmic reticulum (ER) to trans-Golgi Network (TGN), maturation, assembly, glycosylation, and metalation occur. Copper transfer uses different mechanisms involving specific copper chaperones and combine metal coordination chemistry with protein-protein interactions for donor-acceptor docking. Specific copper chaperones for all copper-dependent enzymes have not been identified, and additional ones are likely to be discovered in the future. Cellular copper redox states and concentrations are strictly controlled, and free copper ions are kept at low, non-toxic levels. Within secretory pathway pH is gradually shifted towards an acidic environment and from oxidizing to reducing milieu changing the strength of copper binding [8,9]. Centers with tight metal coordination are preserved during secretory passage, while centers with low avidity can lose copper, and chaperones may be needed to protect their lability. Several copper enzymes possess labile copper sites, such as SOD1, SOD3, DBH, PAM, and TYR, while CP and HEPH have more stable copper sites.

This review centers on function and biogenesis of copper-dependent enzymes, which are activated/matured by copper loading at various sites in cells, which relates to protein trafficking. Cellular copper homeostasis is extensively covered in other reviews, e.g., [10–12], and will not be discussed here unless pertinent for understanding activation of copper enzymes.
| Enzyme | OMIM | EC-no. | Cofactor | Cu Donor | Cu Loading Site | Cu Chaperone | Subcellular Localization | ATP7A-Linked Cu Deficiency Symptoms |
|--------|-------|--------|----------|----------|-----------------|--------------|--------------------------|-------------------------------------|
| ATOX1 regulated Copper Pumps | | | | | | | | |
| ATP7A | 300011 | 7.2.2.8 | Mg; ATP Cu-ATOX1 allosteric | Cu-ATOX1 Cu-GSH | Cytosol | ATOX1 piggy-backing | SP, TGN, PM | Cu storage in tissues, low in brain, liver, plasma; S-Cu diagnostic after 1.5 mo |
| ATP7B | 606882 | 7.2.2.8 | Mg; ATP Cu-ATOX1 allosteric | Cu-ATOX1 Cu-GSH | Cytosol | ATOX1 piggy-backing | SP, TGN, secretory vesicles | Low activity in brain and liver; Fe accumulation; icterus, steatosis |
| Copper Reductases | | | | | | | | |
| STEAP1 | 604415 | 1.16.1.- | Heme; NAD | Cu-His | EC redox | NAp | PM, endosomes | Cu and Fe accumulation on plasma membranes and vesicles; hypochromic anemia |
| STEAP2 | 605094 | 1.16.1.- | Heme; NAD | Cu-His | EC redox | NAp | PM, Golgi | |
| STEAP3 | 609677 | 1.16.1.- | Heme; NADP | Cu-His | EC redox | NAp | PM, endosomes | |
| STEAP4 | 611098 | 1.16.1.- | Heme; NADPH | Cu-His | EC redox | NAp | PM, ER, Golgi endosomes nucleus, MIT | |
| CYBDR | 605745 | 1.-.- | Heme; ascorbate | Cu-His | EC redox | NAp | PM | |
| Copper Oxidases | | | | | | | | |
| CP | 117700 | 1.16.3.1 | Cu; ascorbate | ATP7B | ERGIC | NK | EC | CP low in plasma; diagnostic after 1½ month; Cu and Fe storage |
| FV+VIII | 612309 | 300841 | Cu; ascorbate | ATP7B | ERGIC | NK | EC | Mild clotting deficiency |
| HEPH | 300167 | 1.16.3.1 | Cu; ascorbate | ATP7A | ERGIC | NK | Vesicles | Cu and Fe intracellular storage; AMD |
| HEPHTL | 618455 | 1.16.3.1 | Cu; ascorbate | ATP7A | ERGIC | NK | cis-Golgi | Cutis laxa |
| COX | | | | | | | | |
| CuA (II) | 516040 | 1.9.3.1 | Cu | Redox | SC01; SCO2; COA6 COX11 | IMS | COX17 | Low COX in brain and liver due to low Cu availability; High lactate in blood; Leigh-like symptoms; COX defects; ragged red fibers; hypotonia |
| CuB (I) | 516030 | | Cu;Heme | | | IMS | COX17 | |

**Table 2.** Copper-dependent Enzymes and basic properties.
Table 2. Cont.

| Enzyme                  | OMIM   | EC-no. | Cofactor | Cu Donor | Cu Loading Site | Cu Chaperone | Subcellular Localization | ATP7A-Linked Cu Deficiency Symptoms                                                                 |
|-------------------------|--------|--------|----------|----------|-----------------|--------------|--------------------------|------------------------------------------------------------------------------------------------------|
| Copper Quinone Amine Oxidases |        |        |          |          |                 |              |                          | Numerous connective tissue abnormalities: tortuous vessels, aortic aneurisms, and dissections, umbilical or inguinal hernias, bladder and bowel diverticulae, loose joint and skin, osteoporosis, lymphedema, lung infections; collectin defects with protein trafficking problems, and deficient activation of complement pathway; NAI-like; cataract |
| LOX                     | 153455 | 1.4.3.1| Cu; LTQ  | ATP7A    | cis-Golgi       | HEPHIL redox | EC                       |                                                                                                       |
| LOXL1                   | 153456 | 1.4.3.1| Cu; LTQ  | ATP7A    | cis-Golgi       | Redox loading # | EC                       |                                                                                                       |
| LOXL2                   | 606663 | 1.4.3.1| Cu; LTQ  | ATP7A    | cis-Golgi       | Redox loading # | ER, nucleus *            |                                                                                                       |
| LOXL3                   | 607163 | 1.4.3.1| Cu; LTQ  | ATP7A    | cis-Golgi       | Redox loading # | ER, nucleus *            |                                                                                                       |
| LOXL4                   | 607318 | 1.4.3.1| Cu; LTQ  | ATP7A    | cis-Golgi       | Redox loading # | EC                       |                                                                                                       |
| LOXL5                   |        |        |          |          |                 |              |                          |                                                                                                       |
| AOC1                    | 104610 | 1.4.3.22| Cu; TPQ  | ATP7A    | cis-Golgi       | Redox loading # | EC                       | Ichthyosis, alopecia, inflammation, conjunctivitis, atopy, photophobia, keratitis, diarrhoea, gastrointestinal polyps |
| AOC2                    | 602268 | 1.4.3.21| Cu; TPQ  | ATP7A    | cis-Golgi       | Redox loading # | PM                       |                                                                                                       |
| AOC3                    | 603735 | 1.4.3.21| Cu; TPQ  | ATP7A    | cis-Golgi       | Redox loading # | PM                       |                                                                                                       |
| FGly Generation         | SUMF1  | 1.8.3.7| Cu; Ca   | ATP7A    | ER              | SUMF2         | SP                       | GAG accumulation in tissues and urine; metachromasia, Alder Reilly anomaly; overlapping clinical features of multiple sulfatase deficiency (MSD) mimicking metachromatic leukodystrophy, mucopolysaccharidosis (MPS), mucolipidosis (MLP), chondrodysplasia punctata, hydrocephalus |
| FGly Activated Sulfatases | ARSA   | 607574 | 3.1.6.8  | FGly; Ca  | NAp             | NAp           | NAp                      | Lysosomes                                                                                           |
| ARSB                    | 611542 | 3.1.6.12| FGly; Ca | NAp      | NAp             | NAp           | Lysosomes                |                                                                                                       |
| ARSD                    | 300002 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | Lysosomes                |                                                                                                       |
| ARSF                    | 300003 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | EC                       |                                                                                                       |
| ARSE                    | 300180 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | Golgi                    |                                                                                                       |
| ARSG                    | 610008 | 3.1.6.1 | FGly; Ca | NAp      | NAp             | NAp           | Lysosomes                |                                                                                                       |
| ARSH                    | 300586 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | PM                       |                                                                                                       |
| ARSI                    | 610009 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | EC                       |                                                                                                       |
| ARSJ                    | 610010 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | EC                       |                                                                                                       |
| ARSK                    | 610011 | 3.1.6.- | FGly; Ca | NAp      | NAp             | NAp           | EC                       |                                                                                                       |
| GALNS                   | 612222 | 3.1.6.4 | FGly; Ca | NAp      | NAp             | NAp           | Lysosomes                |                                                                                                       |
| GNS                     | 607664 | 3.1.6.14| FGly; Ca | NAp      | NAp             | NAp           | Lysosomes                |                                                                                                       |
| IDS                     | 300823 | 3.1.6.13| FGly; Ca | NAp      | NAp             | NAp           | Lysosomes                |                                                                                                       |
| STS                     | 300747 | 3.1.6.2 | FGly; Ca | NAp      | NAp             | NAp           | ER                       | Ichthyosis, seborrhoea, hair changes                                                                 |

Notes: # = Not documented; SP = Specific Proteins; EC = Enzyme Complex.
| Enzyme                          | OMIM   | EC-no. | Cofactor       | Cu Donor | Cu Loading Site | Cu Chaperone | Subcellular Localization | ATP7A-Linked Cu Deficiency Symptoms                                                                 |
|--------------------------------|--------|--------|----------------|----------|----------------|--------------|---------------------------|-------------------------------------------------------------------------------------------------------|
| **Copper Amine Oxidases**      |        |        |                |          |                |              |                           |                                                                                                       |
| DBH                            | 609312 | 1.14.17.1 | Cu; ascorbate  | ATP7A    | ER             | MOXD1        | Vesicles; EC             | High DA/NE; vomiting, hypotension, hypothermia, hypoglycemia                                          |
| PAM                            | 170270 | 1.14.17.3 4.3.2.5 | Cu; ascorbate Za; Ca | ATP7A | TGN | NAp | NK | NAp | Golgi Vesicles | Pain, seizure, anxiety, impaired wakening, temperature, weight and fluid balance |
| PHM                            |        |        |                | ATP7A | TGN | NAp | NK | NAp |                                                                                       |
| PAL                            |        |        |                | ATP7A | ER  | -  | ER | -  | ~DBH deficiency |
| TYR                            | 606933 | 1.14.18.1 | Cu; ascorbate  | ATP7A | ER  | TYRP1 TYRP2 | Melanosomes | Albinism, visual and hearing problems                                                                 |
| MOXD1                          | 609000 | 1.14.17- | Cu; ascorbate  | ATP7A | ER  | -  | ER | -  |    |
| **Cu/Zn Superoxide Dismutases**|        |        |                |          |                |              |                           |                                                                                                       |
| SOD1                           | 147450 | 1.15.1.1 | Cu; Zn Cu-CSS allosteric | GSH ATP7A matrix NK | Cytosol ER IMS NK | CCS redox and piggy-backing CCS redox CCS | Cytosol peroxisomes IMS nucleus | Low SOD1 activity in nerve tissue and liver due to poor Cu availability; peroxisomal pathologies; motor neuron disease |
| SOD3                           | 185490 | 1.15.1.1 | Cu; Zn ATP7A | SP | NK | EC | Lung disease, angiopathy |                                                                                                       |
| CCS                            | 603864 | NA     | Cu; Zn ATP7A matrix nucleus | GSH ATP7A matrix nucleus | Cytosol ER IMS nucleus | NAp | Cytosol peroxisomes IMS nucleus | Purkinje cell pathologies; ALS-like phenotype                                                                 |
| APP                            | 104760 | NA     | Cu; Zn ATP7A matrix nucleus | GSH ATP7A matrix nucleus | Cytosol ER IMS nucleus | NAp | Cytosol peroxisomes IMS nucleus | Purkinje cell pathologies; ALS-like phenotype                                                                 |
| APLP1                          | 104775 | NA     | Cu; Zn ATP7A matrix nucleus | GSH ATP7A matrix nucleus | Cytosol ER IMS nucleus | NAp | Cytosol peroxisomes IMS nucleus | Purkinje cell pathologies; ALS-like phenotype                                                                 |
| APLP2                          | 104776 | NA     | Cu; Zn ATP7A matrix nucleus | GSH ATP7A matrix nucleus | Cytosol ER IMS nucleus | NAp | Cytosol peroxisomes IMS nucleus | Purkinje cell pathologies; ALS-like phenotype                                                                 |
| **Cu-CSS Regulated Enzyme**    |        |        |                |          |                |              |                           |                                                                                                       |
| BACE1                          | 649252 | 3.4.23.46 | Cu-CSS allosteric | CCS | SP  | CCS | TGN | Poor neuronal growth                                                                 |

*: indicated; #: histone biology; EC: extracellular; ER: endoplasmic reticulum; ERGIC: ER–Golgi intermediate compartment; GAG: glycoamino glycans; GHS: glutathione; IMM: inner mitochondrial membrane; IMS: intra mitochondrial space; MIT: mitochondria; NA: not assigned; NAI: non-accidental injuries; NAp: not applicable; NK: not known; PM: plasma membrane; SP: secretory pathway; TGN: trans-Golgi network.
3. Copper-Dependent ATPases

Copper-transporting ATPases, α (ATP7A) and β (ATP7B), are homologous P-type ATPases utilizing energy for pumping copper across a membrane. They are ion gated channels crucial for cellular and whole-body copper homeostasis. They have similar but distinct functions, and supplement and complement each other to fine tune equilibrium by transporting copper in different tissues and by coordinating activity in specific cells [11,13]. Both enzymes pump copper from cytoplasm into compartments with higher copper concentration [14]. ATP7A moves copper out of cytosol and across the basolateral membrane in extra-hepatic tissues [15], while ATP7B moves copper out of cytosol and across the apical membrane in liver, brain, and kidney [11,16]. ATP7A controls transport across the gut mucosa and the blood–brain barrier (BBB).

ATP7A and ATP7B are multi-domain enzymes that undergo profound changes during pumping [17]. They share highly conserved domain structure and basic mechanism with other P-type ATPases. Eight membrane-spanning helices constitute a pore-forming transmembrane domain for copper translocation. The channel is linked to three cytoplasmic ATP hydrolytic domains plus six metal binding domains (MBD) with copper-specific motifs (GMXCXXC) [18]. MBD’s initiate pumping through ATOX1 copper activation [11]. Each MBD possesses a compact fold linked by a flexible loop, enabling independent and cooperative action [19]. During pumping conformation undergoes a flip-flop movement with sequential changes allowing unidirectional transfer of copper from the entry site, through the channel by two embedded sites, and release from an exit site. A kinked transmembrane segment at the cytosolic interface forms an electronegative platform for electrostatic ATOX1 docking, initiating opening of the entry gate [11,20].

Pump domain interactions depend on conformation and position during the catalytic cycle, and energy for pumping stems from ATP-dependent transient phosphorylation of the cytoplasmic part [13]. Disruption of the cycle at any point reduces copper transfer.

Regulatory mechanisms are slowly unraveled. ATP7A/B pumping activity is via MBD’s controlled by copper. Docking of ATOX1 on the kinked platform, filling and packing of MBD’s serve as metal sensor besides allosteric regulation. Exact molecular mechanisms that modulate ATP7A/B activity still remain unclear [21].

ATP7A/B trafficking is copper regulated. At basic homeostatic levels copper is pumped into the secretory pathway, but at high levels the pumps relocate to excrete surplus. At low copper, a high free GSH pool secure glutathionylation of MBD’s and retention, while high copper results in low glutathionylation and trafficking [22,23]. ATP7A/B contain a histidine and methionine rich luminal loop located between TM1 and TM2 that may function as ER retention signal [20,24–26]. Ca-pumps possess similar regulatory motifs at corresponding locations to secure Ca-guided ER retention [27]. The ATP7A loop motif may act as copper donor in metalation of certain enzymes [28]. ATP7A and ATP7B show distinct copper transport kinetics, where ATP7A is faster than ATP7B [11,29], but underlying reasons for differences are not clear [21].

3.1. Copper-Transporting ATPase 1 (ATP7A)

ATP7A regulates tissue copper levels and is expressed in most tissues except postnatal liver. At basal levels ATP7A transports copper into lumen of secretory pathway to load secreted and vesicular copper enzymes. At standard tissue culture conditions ATP7A reside in TGN and when exposed to excess copper, the pump relocates to the plasma membrane to export copper [30]. Some enzymes require metalation in ER, and removal of TGN signal by skipping of the alternatively spliced exon 10 retains the protein in ER [20,24] and may be of functional significance. ATP7A is rate limiting in gut uptake and import to the brain.

ATP7A interacts with a range of adaptor molecules, some affect nerve development [31]. ATP7A contains several N-glycosylation sites [32] and need ERGIC trafficking by a carbohydrate-recognition domain (CRD) and an adaptor complex like LMAN1 [33].
3.2. Copper-Transporting ATPase 2 (ATP7B)

ATP7B regulates whole-body copper homeostasis by excreting surplus into bile [34]. ATP7B is expressed in numerous tissues, and plays a role in copper regulation in liver, brain, placenta, and kidneys [4]. ATP7B supplies copper in liver to ceruloplasmin, and clotting factors V and VIII. Gene defects cause Wilson disease (WND) with copper accumulation in liver and brain.

ATP7B is not N-glycosylated [32] and not sugar sorted to the apical canicular membrane but uses a subset of secretory lysosomes. Trafficking is directed by a motif of aromatic amino acids between MBD4 and MBD5 with loose copper binding [15,35] requiring an acidic milieu to secure high free copper for activation. Consistently ATP7B uses an acidic lysosomal pool for excretion. Inactivation of the trafficking signal directs ATP7B to the sinusoidal membrane [35] to mobilize hepatic stores into circulation [36]. In the brain, ATP7B likely traffics by other mechanisms, though not described. ATP7B is also regulated by alternative splicing of the loop motif [13,37].

4. Redox Shifting Enzymes

Intracellularly cupro ions, Cu(I) predominate, while higher extracellular oxidation potential results in cupric ions, Cu(II) [11,38]. A redox shift is needed at copper uptake and export, and organelle membranes likely also require copper redox shifts for transfer. This mimics conclusions for iron transport [39] and at sites the two metals share redox enzymes [40,41]. Copper and iron are reduced at plasma membranes by a heme reductase [42]. Before release iron uses a multicopper oxidase also having copper oxidizing capacity [43]. Iron has higher reduction potential than copper, while copper is superior in oxidative reactions [44,45]. Iron prosthetic groups are involved in a broad range of biological processes. Iron utility depends on careful control of redox state, and specific redox enzymes are found widespread also at subcellular levels. Iron–copper interactions have emerged as crucial, and copper is critical for normal handling of iron [46].

Mitochondria have significant iron and copper stores, securing biogenesis of two iron prosthetic groups, heme and iron-sulfur (Fe-S). Heme is tightly interconnected with copper metabolism and dependent on Fe-S availability [47]. Fe-S clusters are found at several subcellular sites including mitochondrial respiratory chain. Fe-S biogenesis is complex involving numerous steps, some occur in mitochondrial matrix, others in cytoplasm. Fe-S biosynthesis is interconnected with heme biosynthesis [48]. Iron trafficking is not well understood [49], and redox changes critical in organelle metal homeostasis are less known. Copper deficiency leads to low heme-iron which in turn gives insufficiency of enzymes needed for mitochondrial iron membrane translocation [50]. Deficient heme affects copper through dysfunction of membrane uptake, conferring a gatekeeping role for copper in translocation of both iron and copper.

4.1. Heme Copper Reductases

Six-transmembrane epithelial antigen of prostate (STEAP) comprises a family of metalloreductases with ability to reduce iron and copper [51]. STEAP4 shows physiological Km values for both metals [52]. Reducing sites use heme and cofactors like NAD, NAD(P)H, and ascorbate as electron donor [53,54]. The STEAP family is widely expressed [51], but tissue-specific expressions suggest distinct roles [53]. STEAP proteins locate at plasma membrane for copper and iron uptake but are also implicated in trafficking by modulating redox states in endocytotic and secretory pathway, and in mitochondria. STEAP1 acts at tight junctions, gap junctions, and cellular adhesion, and is hormone regulated [53]. STEAP2 regulates iron and copper absorption in gastrointestinal tract [53] and flux across BBB [55]. STEAP2 is expressed in most tissues primarily at plasma membrane and Golgi complex, possibly regulating metal availability in secretory pathway [53]. STEAP3 is an endosomal reductase required for efficient iron uptake into erythroid precursor cells. STEAP3 is highly expressed in liver, placenta, and bone marrow [53], and is located at plasma membrane, near nucleus, and in vesicular tubular structures [53]. STEAP4
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is ubiquitously expressed at plasma membrane, ER, and TGN, suggesting a trafficking role [53,56]. STEAP4 also localizes at early endosomes and mitochondria [57,58], and splice variants localize to nucleus [59].

Cytochrome B reductase 1 (CYBRD1) is a di-heme reductase with iron and copper reducing capacity located at intestinal brush border to reduce iron and copper for mucosal uptake [60]. It may act also as a reductase in airway epithelium. CYBRD1 reduces iron and copper, uses ascorbate, and possesses 6TM, hence naturally grouped among STEAP.

4.2. Multicopper Oxidases

Multicopper oxidases (blue copper oxidases) contain six copper in two complex sites with different redox properties, oxidizing sequentially without formation of free radicals. Cupro and ferro ions are strong pro-oxidants, and multicopper oxidases scavenge radicals by preventing Fenton chemistry [11,43].

4.2.1. Ceruloplasmin (CP)

Ceruloplasmin (CP) is a major redox buffer in blood converting highly toxic ferro ions to less toxic ferri ions. CP mobilizes iron from stores in liver and other tissues, and copper by securing oxidation state [11,61], not by moving copper from one binding site to another; CP is not part of the exchangeable copper pool. Fet3p, a yeast homologue, exhibits cuperoxidase activity at same site having ferroxidase activity [61]. CP is synthesized in liver, but a glycosylphosphatidylinositol-linked form (GPI-CP) is found in astrocytes and choroid plexus [62,63]. GPI is attached in ER and serves as O-glycosylation signal for trafficking to the apical membrane [62,64]. CP is ascorbate dependent and has two deeply embedded catalytic centers [6,65,66], copper loaded in liver by ATP7B, and released to circulation from the sinusoidal membrane. Apo-CP passes to Golgi though exact metalation site is not identified [65,67]. The apoform is secreted into blood, but rapidly degraded [68]. It lacks ferroxidase activity [65], and cannot be copper activated later [66], underlining that CP is not a transport form for tissue copper exchange [11]. Aceruloplasminemia leads to iron accumulation in brain and Parkinson-like ataxia and progressive dementia [69]. In ATP7A-related disturbances, CP metalation is not affected, but low hepatic copper results in low plasma holoenzyme activity. Ferroxidase activity is also low in other tissues, especially brain GPI-CP. Low plasma CP is well-documented in MNK, and some patients show moderate hypochromic anemia, and iron accumulates in kidney and nerve tissue [70]. Poor glycosylation may contribute to poor hepatic secretion, and MNK plasma CP is lower (half) than nutritional copper deficiency and WND [71]. Low ceruloplasmin is a well-established marker for MNK, OHS, and intermediate forms, though less depressed in OHS; but it is not applicable for diagnosis of SMAX3 [2].

4.2.2. Hephaestin (HEPH)

Hephaestin (HEPH) is a membrane-anchored homologue oxidizing iron before release with main role in gut and BBB [64]. Copper oxidizing capacity is likely [11]. The iron metabolome contains large functional redundancy and potentially CP and HEPH can substitute for each other [72], and CP may in absence of HEPH promote iron efflux from enterocytes [73].

HEPH and CP both appear critical for CNS-iron homeostasis [74], and HEPH is abundantly expressed in neurons [75]. CP and HEPH are co-expressed in retina [76] and combined loss lead to age-related macular degeneration (AMD) [72]. Double-knockout Heph and Cp mice, but neither alone, lead to kidney iron deposition and toxicity [77], and iron accumulation in liver, brain, pancreas, and adipocytes [74,78,79], with significant deficiencies in serum and neurons [80]. Knockout mice exhibit neurodegeneration and retinal degeneration similar to aceruloplasminemic patients. HEPH sites have similar architecture as CP but are loaded by ATP7A [43]. The apoform is rapidly degraded, and by analogy cannot be loaded after biosynthesis [81]. HEPH is regulated by copper and iron; iron induces translocation from intracellular sites to basolateral membrane [82]. HEPH is
ubiquitously expressed, most strongly in small intestine followed by kidney [43]. HEPH is N-glycosylated [81], sorted and anchored to vesicles at basolateral membrane [64,81,83].

4.2.3. Hephaestin-Like Protein 1 (HEPHL1)

Hephaestin-like protein 1 (HEPHL1) is a membrane-bound homologue (zyklopen) with similar oxidation functions [84]. Immunostaining shows expression in brain, kidney, testes, and retina, but not liver and intestine [84]. Further expressed in placenta, reproductive tract, and mammary glands and suggested to act in placental iron transport [84]. HEPHL1 requires copper for stability [84], but is less investigated [41,43,64]. Molecular modeling on CP crystal structure indicates preserved HEPHL1 copper sites [84], and by analogy likely involved in copper mobilization. Recent proof is gained by a compound heterozygous patient with abnormal hair, joint laxity, and developmental delay (HJDD) [85]. Hair changes (pili torti and trichorrhexis nodosa) are similar to MNK, and muscular affection similar to SMAX3 [85]. LOX deficiency explains connective tissue involvement and indicates disturbed copper metabolism. HEPHL1 likely acts as redox chaperone in LOX cofactor formation and metalation. Lack of copper affects N-linked glycosylation sites, but none of the O-linked, indicating that copper is loaded before N-glycosylation [85].

4.2.4. Factor V+VIII Clotting Factors

Factor V+VIII clotting factors belong to blue copper oxidases with complex catalytic sites requiring ascorbate as cofactor [86,87]. Both are glycoproteins loaded by ATP7B before secretion to circulation. Within ER, FV and FVIII are guided by mannose binding lectin 1 (LMAN1) for trafficking and secretion [86,88,89]. LMAN1 receptor complex defects cause combined FV+VIII deficiency and mild coagulation disorder [90], underlining importance of mannose binding lectins in glycosylated protein trafficking. LMAN1 is a collectin requiring LOX activation, and in MNK low hepatic copper can affect maturation and trafficking leading to mild clotting deficiency [91].

4.3. Cytochrome c Oxidase

Cytochrome c oxidase (COX) uses copper and heme for reduction of oxygen, making mitochondrial copper and iron homeostasis indispensable for life. COX is the terminal oxidase of the electron transport chain comprising four complexes (numbered I, II, III, and IV), each with increasing reduction potential. The inner membrane contains respiratory complexes and copper chaperones; inter-membrane space (IMS) contains Cu/Zn superoxide dismutase 1 and soluble copper chaperones; matrix stores a mobilizable copper pool [92,93]. Copper delivery to COX requires an elaborate machinery of cytoplasmic guiding molecules, outer and inner membrane translocators, and embedded membrane chaperones [94]. Soluble and membrane anchored copper chaperones take different routes: (1) direct uptake via outer membrane into IMS using the redox import machinery [93], and (2) via matrix and redirection. Uptake of complex IV assembly factors is covered in reviews [94–96]. Matrix copper is routed to IMS for metalation and activation of enzymes and chaperones. Knowledge about copper exchange between mitochondrial compartments with different redox potentials is limited [97,98]. ATP7A dysfunction affects mitochondrial redox balance [99], and in MNK with low copper in liver and brain, COX deficiency becomes severe. COX is a multimeric complex containing two catalytic copper centers, CuA/heme and CuB, requiring assembly of numerous subunits. Formation of catalytic sites takes place within mitochondria and requires uptake of copper (and iron) into IMS from matrix pools, making insertion after biosynthesis unlikely. Assembly is complicated and assisted by several factors and copper chaperones [93–96]. The COX assembly will not be covered in detail. However, SCO1, SCO2, and COA6 are inner membrane embedded thiol-disulfide oxidoreductases utilizing copper to modulate redox state, before delivery to CuA and CuB [100–102]. These copper chaperones are yet examples of redox-dependent copper transfer.
Respiratory chain secures cellular energy production, and disruption affects high energy-demanding tissues like CNS, liver, heart, and skeletal muscles. MNK shows numerous clinical signs of compromised mitochondrial activity \[103–105\]. Patients are hypotonic and floppy, often leading to suspicion of mitochondrial disturbance, and milder cases show myopathy. High lactate in blood or cerebrospinal fluid further strengthens a suspicion. Late disease stages develop deficiency of several respiratory chain complexes \[105,106\], and muscle ragged red fibers, a sign of mitochondrial dysfunction \[103,106\].

5. Copper-Catalyzed Cofactor Containing Enzymes

This enzyme group needs copper for cofactor activation. Each subclass has its unique activation, some are formed using copper during enzyme biogenesis, while others are attached to holoenzymes by a copper dependent process. SUMF1 dependent enzymes comprise a large new group of mainly lysosomal enzymes only recently joined with copper homeostasis

5.1. Copper Quinone Amine Oxidases

Copper quinone amine oxidases contain two subgroups, lysyl oxidases and copper amine oxidases, based on their specific internal cofactors that are formed post-translationally \[107,108\]. Copper-dependent monoamine oxidases are covered separately below, as they do not use an internal copper cofactor for catalysis. Notably, MAO A and B are not copper catalyzed.

5.1.1. Lysyl Oxidases (LOX)

Lysyl oxidases (LOX) comprise amine oxidases initiating extracellular matrix (ECM) formation by catalyzing oxidative deamination of an epsilon-amino group in lysine and hydroxylysine residues in first step of elastin and collagen cross-linking \[108\]. Importantly LOX cross-links a variety of triple helical proteins including collectins, significantly expanding LOX functions \[109\].

All members share a highly conserved catalytic site made up of three His \[110\] in close proximity to a cofactor created by copper catalyzed cross-linking of two internal amino acids \[107,111\]. Formation of the internal cofactor, LQT is described as an autocatalytic process, but new evidence points to a need for a redox process \[85\]. Copper LOX metalation occurs before N-glycosylation \[85\], and failure to acidify Golgi affects glycosylation resulting in cutis laxa \[112,113\].

Collagens (COL) and elastin (ELN) provide stability and connectivity among tissues and organs, and fiber building by trimerization and intra- and intermolecular cross-linking is crucial. Other components of ECM, mucopolysaccharides and mucolipids interact with fibers to form connective tissue. Lack of fiber tensile strength affects flexibility and integrity, and results in connective tissue disorders. Tissues affected are bones, tendons, ligaments, joints, muscles, blood vessels, heart, and eyes, with symptoms spanning from osteoporosis, loose joints, lung emphysema, aneurysms, glaucoma to pelvic organ prolapse or rupture \[114\]. New collectin symptoms are emerging. Numerous COL proteins exist, but type I is most abundant. Clinical defects span from osteogenesis imperfecta to osteoporosis and Ehlers-Danlos syndrome. ELN provides flexibility to fibers that rapidly expand and return to original shape. Deficiency results in joint laxity, wrinkling of skin, aneurysms and emphysema. Gastrointestinal and bladder diverticulae are common. Gly-X-Y triple repeats, hallmarks of COL are versatile and widespread in proteins adapted to a range of functions \[115\], and an increasing number of proteins with COL-like domains are identified \[109\]. Trimerization is crucial and needs LOX \[115–117\].

Amongst triple repeat proteins is complement Q1 (C1q), first member of the complement-cascade. Dysregulation of C1q is characterized by recurrent skin lesions, susceptibility to infections, increased risk of autoimmune disease, and chronic kidney disease \[118,119\]. C1q belongs to collectins \[109,120\] comprising a superfamily of lectins with a COL-like stretch fused with CRD. Collectins are present in plasma and on cell
surfaces acting in first line of defense [109]. Lung surfactant collectins lubricate alveoli, besides being acute phase reactants.

A well described CRD receptor protein is mannose-binding lectin (lectin mannose binding 1) (LMAN1) that triggers the lectin pathway of complement activation. LMAN1 (often named ERGIC-53) is membrane bound and cycles between ER, ERGIC, and cis-Golgi [121–123]. LMAN1 and related proteins form complexes with lectin chaperones and is a rate-limiting step in maturation of secreted glycoproteins [89]. Compromised glycoprotein trafficking leads to incorrect localization, and mutations in LMAN1 receptor complex lead to combined deficiency of FV+VIII and hepatic accumulation of α-1-antitrypsin [90]. LMAN1 deficiency leads to susceptibility to meningitis and infections of upper respiratory tract, and as a trafficking factor for neuroreceptors will lead to CNS dysfunction [124,125]. More LMAN1 substrates are being identified also affecting immunobiology [123].

Numerous effector proteins exist, we include only a few here that are most important for MNK. Collagen-like tail of endplate acetylcholinesterase (COLQ) is acetylcholinesterase with a triple-helical membrane anchor to rapidly regulate muscle activation. If deficient, neuromuscular signaling causes muscle weakness [126]. Clinical signs are muscle fatigueability affecting limb muscles, ocular muscles (ptosis and ophthalmoplegia), and facial and mouth musculature (poor sucking and swallowing) as seen in MNK. Collagen and calcium binding EGF domains 1 (CCBE1) protein is important for lymphatic vessel formation, and deficiency results in lymphedema, and mutations in CCBE1 causes Hennekam syndrome [127]. MNK often encounter severe puffiness of face and feet, and a dough-like skin. Collectin defects are accompanied by susceptibility to infections [109], and distinctive facial features also seen in MNK, including widely spaced eyes (hypertelorism), narrowing of eye opening (blepharophimosis), droopy eyelids (ptosis), and high arched (cupid) eyebrows [128,129].

The LOX family is important in relation to MNK and contains five members, lysyl oxidase (LOX) and four lysyl oxidase-like (LOXL) enzymes working on different biological substrates [108,130,131]. LOX is secreted and cross-links ELN and COL. The proenzyme is activated attached to its extracellular substrates [131]. LOX plays a role in aortic wall formation, and deficiency predisposes to aortic aneurisms and dissections [132,133], a prevalent cause of death in MNK. Copper is loaded during Golgi passage, before final N-glycosylation, and a redox step is required [85]. LOX structure has been determined by homology modeling using LOXL2 [134] demonstrating copper coordination by three His and LQT oxygen [110] explaining a redox need in formation of the active center [85]. LOXL1 preferably cross-links ELN [135] and is linked to glaucoma, cataract [136,137], and lens zonule weakness eventually leading to lens subluxation. It is a major risk factor for pseudoexfoliation syndrome. Preferred substrate for LOXL2 is Type IV COL [138], a basement membrane component scaffolding other ECM molecules [130,139]. LOXL2 oxidizes histone and localizes in pericentromeric region [140]. Defects are associated with diseases of muscle, neural, ocular, cutaneous, vascular, lung and kidney tissues [141,142]. Substrates of LOXL3 are not clearly defined, but defects have been associated with early onset myopia [143] and Stickler syndrome that is a group of connective tissue defects with variable facial features, eye abnormalities, hearing loss, and joint problems [144]. LOXL3 localizes in nucleus and is involved in histone biology [145]. LOXL4 is expressed in cartilage and many tissues, the highest levels found in skeletal muscle, testis, and pancreas.

MNK shows numerous LOX and LOXL deficiency symptoms [146,147], and non-accidental injury (NAI) is often suspected [148–150], and an important differential diagnosis. LOX deficiency is also well established in OHS [1].

5.1.2. Copper-Containing Amine Oxidases (AOC)

Copper-containing amine oxidases (AOC) comprise a family of both diamine (DAO) and polyamine (PAO) oxidases. AOC participates together with numerous transporters and enzymes to precisely regulate polyamine pathways in CNS and periphery. Functions regulated are wakefulness, inflammation, and neurotransmitter release [108]. In addition,
a catalytic role, copper is required for biogenesis of the internal topaquinone cofactor (TPQ) \[108\]. By analogy, a copper oxidase is likely required for formation of the active catalytic site where copper bridges three His and oxygen of TPQ. Histamine (monooamine), putrescine (diamine), spermidine (triamine), and spermine (tetramine) are ubiquitous AOC regulated amines, involved in proliferation, differentiation, and apoptosis, and in modulation of neurotransmitter receptors \[151\]. Polyamines play important roles in rapidly dividing cells like immune cells and enterocytes, and in regulation of membrane potentials in excitable tissues. Interactions with ligand-gated ion channels and tight-junctions are emerging as crucial polyamine regulated functions \[151,152\].

Polyamines are stable compounds present in mM amounts as free (minor), bound, and conjugated forms. Polyamine homeostasis is precisely regulated by de novo synthesis, extracellular catabolic control by AOC, intracellular regulatory feed-back loops, and membrane transfer by solute carriers (SLC) to balance intracellular and extracellular pools \[153\]. Physiological functions of charged polycations are not fully understood \[153\]. We focus on accumulation of specific polyamines, the result of deficient AOC catabolic control.

Histamine is the best studied amine, expressed at numerous sites including mast cells, gastrointestinal tract, and neurons \[154\]. Histamine regulates gastric acid secretion and CNS neurotransmission, in addition to a range of inflammatory reactions \[155,156\] mediated by specific histamine receptors \[156,157\].

Abnormal spermidine catabolism results in skin (ichthyosis), hair (alopecia), and eye (conjunctivitis) problems \[158,159\]. Allergic reactions (atopy), light intolerance (photophobia), and cornea inflammation (keratitis) may occur. High polyamine levels trigger persistent diarrhea with gastrointestinal polyps \[154\]. All these symptoms are found in MNK patients.

Humans have three AOC: AOC1, diamine oxidase, histaminase, or amiloride-binding protein 1 (ABP1), is mainly expressed in kidney, placenta, intestine, thymus, and seminal vesicles \[108\], and released at plasma membranes in response to external stimuli. AOC2, or retina-specific amine oxidase, is expressed on cell surfaces in many tissues with a particular high expression in retina \[160\]. AOC3, also named vascular adhesion protein 1 (VAP1), is widely distributed with highest expression in peripheral lymph nodes, hepatic endothelia, appendix, lung, and small intestine \[108\]. AOC3 has been implicated in lung inflammation, asthma, psoriasis, and vascular stroke \[108\]. Expression is also high in white fat tissue where it may be implicated in adipocyte differentiation and metabolism \[161\].

### 5.2. Formylglycine Activated Sulfatases

Sulfation/desulfation regulate numerous pathways, and sulfatases are responsible for break down and recycling of both complex sulfated sugars and hormones \[162,163\]. Sulfatases share a post-translationally formed internal cofactor, FGly essential for activity \[164,165\]. Cofactor generation requires sulfatase-modifying factor 1 (SUMF1) or formylglycine generating enzyme (FGE) \[163,166\]. SUMF1 oxidises cysteine in target enzymes using a highly conserved sequence, CXPSR \[166,167\], and recently copper was found to be required \[167\]. SUMF1 is an ER located soluble glycoprotein acting on native sulfatase polypeptides \[168\]. ER resident SUMF2 \[169,170\], a non-copper binding paralog acts as chaperone and retains SUMF1 by heterodimerization while activating sulfatases \[171\]. SUMF1 interacts with numerous trafficking factors including LMAN1, and lack of activation and trafficking leads to proteasomal degradation of SUMF1 \[172\]. Sulfatases localize to subcellular sites such as lysosomes, Golgi, and ER \[170\], where they break-down complex mucopolysaccharides, mucolipids, and steroid hormones. Lysosomal glycosaminoglycan (GAG) sulfatases comprise a major group \[173\]. GAGs are complex sugar polymers and important components of bone and cartilage, joint lubricants, and cell surface initiating growth factor activity and first line of defense against microorganisms. Recycling of GAGs starts by removal of sulfated groups and defective recycling results in GAG accumulation. Deficiencies present as mimicry of mucopolysaccharidoses (MPS) and mucolipidoses (MLP).
affecting multiple organ systems [162,170,173]. Sulfation/desulfatation are crucial for cartilage formation, and defects are often accompanied by bone dysplasias [173].

Most steroids, e.g., cholesterol, pregnenolone, and estrone, are sulfated after biosynthesis [162], and sulfation is vital for endocrine function. Cholesterol is crucial for neurotransmission, myelination, and synaptogenesis [174], and desulfatation provides a copper link. Dysregulation is associated with numerous pathologies, including faulty regulation of GABA receptor function [175,176]. Niemann-Pick C disease may be accompanied by copper disturbance likely secondary to poor steroid sulfatase activity and disrupted trafficking of cholesterol [177].

Combined impairment of all sulfatases, multiple sulfatase deficiency (MSD), are clinically heterogeneous disorders caused by mutations in SUMF1 or SUMF2 [169,170]. Symptoms present features of metachromatic leukodystrophy, mucopolysaccharidosis, chondrodysplasia punctata, hydrocephalus, ichthyosis, neurological deterioration, and developmental delay.

ATP7A-related disturbances may mimic MSD and present with overlapping clinical features from a complex interplay between SUMF1 and the LOX family. Sulfated molecules build up in lysosomes, resulting in necrosis and metachromasia, a sign noted early in MNK [178], but forgotten when the copper disturbance was discovered. Morphologic changes with vacuoles in myeloid cells, termed Alder Reilly anomaly are seen in patients with mucopolysaccharidoses (MPS) and have also been reported in MNK [179,180]. Skin problems in MNK may be related to deficient steroid sulfatase (ichthyosis) [181,182] also affecting keratinocyte biogenesis and hair development [183]. Build-up of cholesterol sulfate in the outermost layer of epidermis causes hyperkeratosis with scaling [184].

6. Copper-Dependent Mono-Amine Oxidases

Copper monooxygenases catalyze reactions in catecholamine and hormone pathways. The group consists of four enzymes that are free or membrane attached within vesicles of same embryonic origin: adrenal chromaffin vesicles (DBH), synaptic vesicles of the sympathetic nervous system (DBH), secretory vesicles of the pituitary gland (PAM), and melanocytes in periphery and CNS (TYR). Enzymes travel to their final destination, but trafficking is not completely understood and depend on metalation and N-glycosylation. Crystal structure of DBH shows two copper sites, one (CuH) coordinated by three His, the other (CuM) by two His and one Met [185]. All sites have similar copper avidity [28].

6.1. Dopamine β-Hydroxylase (DBH)

Dopamine β-hydroxylase (DBH) is an ascorbate-dependent monooxygenase converting dopamine (DA) to norepinephrine (NE) [187,188]. DBH localizes in synaptic vesicles in noradrenergic and adrenergic nerve terminals of central and peripheral nervous system, as well as adrenal medulla [189]. DBH is targeted to secretory granules by ER glycosylation [185].

DBH contains three N-glycosylation sites [185], and may show trafficking problems [190], and misfolding is suggested to cause DBH deficiency [190]. ATP7A supplies copper to DBH both centrally and in the periphery [191–193] and is needed during formation and maturation of the holoenzyme, though copper can likely be loaded later. Met-His-rich lumenal loop of ATP7A can experimentally transfer copper to DBH [28]. NE controls mood, attention, and overall arousal, as well as stress, learning, and memory [185], and the adrenal system is important in maintaining blood pressure, glucose, and sodium levels [194]. Congenital NE deficiency shows profound autonomic failure [188], and perinatal period may be complicated by vomiting, dehydration, hypotension, hypothermia, and severe hypoglycemia all seen in early MNK. Later symptoms are dizziness upon standing (orthostatic hypotension), blurred vision, and difficulty in exercising. Other symptoms are droopy eyelids (ptosis), nasal congestion, muscle pain, and weakness, symptoms well recognized in MNK. DA/NE ratio is increased in plasma and CSF, and dopaminergic
imbalance is an early discriminatory marker for MNK [195], and milder forms also show abnormal values [2].

6.2. Peptidyl α-Amidating Enzyme (PAM)

Peptidyl α-amidating enzyme (PAM) activates a vast amount of neuroendocrine hormones involved in regulation of numerous processes. PAM is a bifunctional enzyme, consisting of two distinct catalytic domains working sequentially, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl–α-hydroxyglycine α-amidating lyase (PAL). Copper containing PHM catalyzes hydroxylation of a glycine, subsequently cleaved by PAL to generate C-terminal amidation in activated peptide hormones. PHM has copper-binding sites similar to DBH and also requires ascorbate as cofactor [185,186]. Lack of metalation does not alter passage through secretory pathway, and the apoenzyme is not degraded [196], though not directed to correct vesicular location [186]. Copper required for enzyme activity is not tightly bound [7] and can be lost, but secreted apoenzyme can be activated [197]. This likely also apply for trafficking and metalation of related enzymes, DBH and TYR. The first luminal loop of ATP7A involved in release of copper contains an amino acid stretch rich in His and Met acting as potential copper donor for metalation of PAM in secretory pathway [186]. Functionally PAM and DBH overlap, and neuropeptides and neurotransmitters participate in a large number of processes related to feeding and body weight, fluid balance, pain, anxiety, memory, circadian rhythms, and reward [186,198]. PAM is essential for activation of numerous neuroendocrine peptide hormones such as cholecystokinin, gastrin, vasoactive intestinal peptide, thyrotropin-releasing hormone, calcitonin, corticotropin-releasing hormone, and vasopressin [186].

Biological significance of PAM is not fully understood, but deficiency results in widespread effects. Brindled mice, a genetic model of ATP7A-related copper disturbances, fail to produce normal levels of α-amidated peptides [198,199]. PAM deficient mice show CNS problems, e.g., impaired vasoconstriction and thermoregulation, increased seizure susceptibility, anxiety, and increased response to noise [198].

6.3. Monooxygenase, DBH-Like 1 (MOXD1)

Monooxygenase, DBH-like 1 (MOXD1) is structurally similar to other ascorbate requiring copper-containing monooxygenases, but with unknown substrate. MOXD1 lacks signal sequence and localizes throughout ER in both endocrine and non-endocrine cells [200]. MOXD1 is membrane-associated and oligomerize. MOXD1 is predicted to hydroxylate a substrate in ER, and possibly acts as enzyme chaperone for DBH [200].

6.4. Tyrosinase (TYR)

Tyrosinase (TYR) catalyzes the first two steps in melanogenesis from tyrosine to DOPA and to dopaquinone. Tyrosine oxidation is rate-limiting followed by ER polymerization reactions [201,202] catalyzed by two members of tyrosinase-related proteins TYRP1 and TYRP2 [203].

TYR is membrane anchored and possesses two copper centers resembling DBH though entirely made up of His [185,204]. Copper is acquired during maturation in secretory pathway, but apo-TYR can be activated later by addition of copper [205,206]. TYR localizes to specialized endosomes termed melanosomes and undergoes maturation and sorting before reaching integration site [207,208]. Intracellular sorting and polymerization steps from ER through Golgi to melanosomes is tightly regulated including metalation and N-glycosylation [203,209,210]. During sorting in ER, TYR interacts with lectins normally associated with LMAN1 [207,209]. Metalation likely occurs in ER before action of TYRP1 and TYRP2, but can take place later in melanosomes, and TYR becomes fully functional only at its final destination [210,211]. TYRP1 and TYRP2 belong to the same protein family and have similar metal binding sites though using zinc. TYR substrates play a conformational role as molecular chaperones to enhance folding and ERGIC trafficking [208]. The Met-His-
rich first luminal loop of ATP7A possibly metalates TYR [28]. TYR may lose copper during passage of acidic TGN but is reloaded in melanosomes with neutral pH [210,212].

Melanosomes originate from distinct, though related, embryonic stem cells: (1) neural tube derived retinal pigment epithelium and pineal gland melanocytes; (2) neural crest derived melanocytes of inner ear, skin, hair-bulbs, and iris [213]. Highest TYR expression is in pigment epithelium of retina. Skin melanosomes are transferred to keratinocytes where melanins protect against UV sun radiation [214]. Melanins are negatively charged, polymerized and hydrophobic pigments working as capacitor to absorb and dissipate energy to neutralize radiation. In case of high energy absorption, output occurs as heat and reactive oxygen species (ROS), eventually resulting in sun burn and necrosis. Complex neuromelanins are synthesized mainly in dopaminergic neurons of substantia nigra and noradrenergic neurons of locus coeruleus [215]. Midbrain catecholaminergic neurons of basal ganglia network are crucial for brain cognitive functions. Biosynthesis and regulation of neuromelanins are poorly understood [216,217] as is their role in smell, vision, and hearing. Deficient development of inner ear melanocytes causes deafness [218,219]. TYR mutations result in hypopigmentation disorders and sensitivity to UV radiation, visual problems like nystagmus, strabismus, and reduced visual acuity with photophobia [220]. Transduction overload may lead to local oxidative stress and accumulation of waste products in central and peripheral ganglions and increased risk of melanoma [217]. Pigment and cell debris accumulation in CNS may increase susceptibility to Parkinson [217].

In MNK visual problems are early onset nystagmus, iris trans-luminescence, hypopigmented fundus, and reduced visual acuity [221]. Hearing may be impaired, but often not investigated. In accord with above, copper replacement therapy in MNK shows darkening of hair and skin [206].

7. Copper/Zinc-Containing Superoxide Dismutases (Cu/Zn-SODs)

Superoxides are products of normal aerobic metabolism and crucial in oxidative burst of innate immune responses [222], but in need of strict control. Superoxide dismutase (SOD) disproportionate the reactive radicals into molecular oxygen and less reactive hydrogen peroxide. Uncontrolled, ROS will attack unsaturated fatty acids, and SOD is of particular importance for a healthy brain, and of the most abundant enzymes underlining importance of ROS control. SOD1 is compartmentalized into distinct cellular and minor extracellular pools. SOD3 is attached to extracellular matrix, and often named extracellular SOD (EC-SOD). SOD1 activity is copper regulated at protein level, while SOD3 activity is copper regulated at gene level. Amyloid-β precursor protein (APP) family consists of Cu/Zn proteins with a SOD-like structure and possible dismutase activity [223] and is copper regulated through Cu-CCS activated cleavage of β-secretase 1 (BACE1). A manganese form (SOD2) in mitochondrial matrix is interconnected with IMS-SOD1 [224] and SOD3 [225].

7.1. Superoxide Dismutase 1 (SOD1)

Superoxide dismutase 1 (SOD1) is the master SOD and sole cytosolic and peroxisomal cuproenzyme. SOD1 mainly localizes in cytosol, an almost equal fraction in peroxisomes [226], and minor pools in mitochondrial intermembrane space (IMS), and nucleus. SOD1 comprise a large copper pool, earlier viewed as copper buffer [227], substantiated by labile metal binding by a cluster of four imidazole groups [11]. Some cell types secrete SOD1 [228]. SOD1 is unusual by having a labile copper site in cytoplasm abundant in GSH, and likely needs shielding by vesicular structures [229]. Copper chaperone for SOD1 (CCS) participates in maturation and activation of SOD1 at all subcellular locations. CCS is a member of the Cu/Zn-SOD family and acts as an enzyme chaperone to catalyze an intramolecular disulfide bond, stabilizing correct SOD1 conformation for incorporation of copper and zinc. CCS also functions as molecular chaperone and contains three domains having different roles: N-terminus possesses a copper binding site (MXCXXC), similar to ATOX1 [230], also with potential allosteric role in copper activation. The homologous middle part interacts with SOD1, and C-terminus contains a copper catalytic CXC site
needed for intramolecular S-S bridge formation [231,232]. Nascent SOD1 and CCS polypeptides devoid of metal enter IMS individually and with essential sulfides reduced while traversing outer mitochondrial membrane [93,233]. In IMS, SOD1 meets CCS, and is folded and activated as in cytosol, hereby retained in IMS as functional enzyme. SOD1 and CCS are both taken up by the CHCHD4 (~MIA40) redox import machinery [234].

Peroxisomes enclosed by a single lipid bilayer use special import of membrane proteins and matrix enzymes [235]. Most contains a peroxisomal targeting signal (PTS) and are taken up via peroxin (PEX) membrane receptors [236,237] and delivered through direct contact between ER and peroxisomes. Folded, co-factor bound, and oligomeric proteins can be imported [238]. A major SOD1 route through ER has been discovered, securing high peroxisomal matrix content [239]. SOD1 does not contain PTS and is piggy-backed into peroxisomes by its chaperone [237,239]. CCS-PTS is in ER recognized by PEX5 receptor, shuttling CCS-SOD1 into peroxisomal matrix [235]. SOD1 rapidly enters nucleus in response to increased H$_2$O$_2$ levels and is potentially piggy-backed via ER by CCS. Peroxisomes are present in all tissues catalyzing a wide range of anabolic and catabolic reactions. SOD1 generates H$_2$O$_2$, and catalase uses H$_2$O$_2$ to oxidize substrates. SOD1 dysfunction leads to ROS accumulation that eventually damage the peroxisomal membrane, and release catalase to cytosol [240]. Severe pathologies result from peroxisomal dysfunction showing multi-systemic symptoms referred to as peroxisome biogenesis disorders (PBD). Neurological dysfunction is prominent usually accompanied by brain malformations, myelin abnormalities, and neuronal degeneration [241]. Systemic manifestations often include dysmorphic features, liver dysfunction, and skeletal abnormalities [241].

In MNK brain, both CCS and SOD1 polypeptides are taken up into mitochondrial IMS, but SOD1 is not properly folded and activated due to lack of copper. ROS are expectedly high, and matrix SOD2 induced as compensation [224]. The ER-peroxisomal route is also compromised creating a deficit of peroxisomal matrix SOD1 and enhanced peroxisomal stress in turn affecting nerve development. Still CCS accumulates [224,242] indicating faulty heterodimerization when copper is low. Deficiencies affect cerebellar maturation and axonal integrity, and lead to Purkinje cell pathologies with “weeping willow”, a well-recognized sign in MNK [242,243]. Low hepatic copper results in low SOD1, and oxidative stress plays a role in the pathogenesis of steatosis.

If nascent SOD1 is not correctly processed, it will remain inactive, potentially misfold, dimerize or tetramerize, as is the case in some neurodegenerative diseases [244]. Genetic disturbances of SOD1 lead to motor neuron disease, amyotrophic lateral sclerosis (ALS). Most SOD1 mutations affects heterodimerization and piggy-backing into peroxisomes [245]. Like MNK, lack of peroxisomal uptake of SOD1 will in ALS lead to oxidative stress and development of varying motor neuron affection as part of the PBD spectrum.

7.2. Superoxide Dismutase 3 (SOD3)

Extracellular superoxide dismutase (SOD3) is anchored to heparan sulfate in ECM [246]. SOD3 is structurally closely related to SOD1 and also contains copper in its catalytic center and zinc to stabilize structure. The central part of SOD3 is homologous to SOD1, the metal binding sites preserved and with similar copper avidity, but structures vary at ends. SOD3 contains a signal peptide plus three N-glycosylation sites for GAG guidance [247]. The enzyme is copper loaded in secretory pathway, but no specific copper chaperone has been identified. ATOX1 regulates protein expression through copper dependent binding to SOD3 promoter [248]. C-terminus contains a heparan binding domain securing attachment to ECM [246]. After secretion SOD3 forms tetramers stabilized by intermolecular disulfide bonds. SOD3 is secreted by fibroblasts and glial cells and protects cell membranes against ROS; about 1% is free in plasma, lymph, and cerebrospinal fluid [249]. SOD3 levels are high in vasculature, heart, lungs, kidney, and placenta [250]. Low SOD3 activity is linked to lung disease such as acute respiratory distress syndrome or chronic obstructive pulmonary disease [251] and deficiency may result in angiopathy.
8. Cu/Zn-SOD-Related Proteins Regulated by β-Secretase 1 (BACE1)

Amyloid-β precursor protein (APP), and amyloid-like proteins APLP1 and APLP2 contain a dismutase fold resembling Cu/Zn-dismutases and are regulated by copper through protease cleavage [11,224]. They bind copper and zinc primarily through His coordination [252] but an enzymatic role has not been established, though APP redox capacity has been demonstrated [253,254]. APP and its processed forms appear to have a growth-factor-like role and promotes neuronal proliferation and division [255]. Thus, the APP family is important for synaptic development and plasticity of central and peripheral nervous systems [256]. We will point to the relationship between the APP family and the Cu/Zn-SOD family to emphasize remote regulation by CCS-Cu.

β-secretase 1 (BACE1) is a membrane-bound protease, catalyzing first step of extracellular release of soluble amyloid β peptide (Abeta) from APP. BACE1 is rate-limiting in neuronal Abeta generation and also cleaves numerous other substrates important in formation of myelin. BACE1 contains a CCS-Cu regulatory site spatially separated from the protease site [5,11]. N-terminal CCS-MXCXXC binds to a cysteine rich area in C-terminal cytoplasmic tail of BACE1 regulating numerous brain functions including PAM [257]. BACE1 is expressed at high levels in brain and pancreas. Expression is highest in substantia nigra, locus coeruleus and medulla oblongata [258]. Abrogated cleavage in BACE1 knockout mice shows a role in neuronal migration, axonal growth, and muscle spindle function [259]. BACE1 is N-glycosylated [260] implying poor ERGIC trafficking in addition to poor protease activity secondary to low brain copper in MNK.

9. Conclusions

The main objective of this review is tying enzymes, substrates, and key symptoms together in a unified hypothesis to explain Menkes disease symptoms and pathologies (Table 3). We also wish to shed light on crucial steps in biogenesis of copper-dependent enzymes (dysfunctional in MNK) by focusing on metalation sites in cells, metal chaperoning and trafficking of enzymes in the secretory pathway.

ATP7A disturbances result in complicated copper disorders starting by poor uptake at intestinal brush border, aggravated by poor release from enterocytes, further affecting all barriers in the body, underlining that the basic defect is not a simple copper insufficiency. Defects in reduction (STEAP) before cellular uptake and in oxidation (HEPH) before release contribute to a complex copper transport defect resulting in complex clinical traits. Intracellular organelle deficiencies develop, combined with copper accumulation in unavailable pools. Copper pumping into secretory pathway and enzyme metalation are clinically significant, and ERGIC enzyme trafficking is also emerging as a copper regulated step (LOX). MNK diagnosis is often missed until hair changes are obvious, and the delay may leave many undiagnosed cases. To improve diagnostic awareness, focus should be shifted from hair as the main diagnostic pointer to more subtle symptoms. We found no evidence of a copper specific sulfhydryl oxidase, and hair and skin changes likely result from combined lack of steroid sulfatase (SUMF1), copper amine oxidase (AOC), and defective mitochondrial Fe-S biogenesis. SUMF1 is a new player in Menkes disease linking faulty cholesterol biology to the clinical picture and a whole new group of GAG sulfatases, which may lead to mimicry of lysosomal storage disorders (Table 3).

Symptoms secondary to LOX dysfunction have been expanded and shed light on their role in activation of receptor and adapter collectin molecules. Though important, it is an overlooked component of Menkes disease pathology. In liver, LMAN1 deficiency affects coagulation factors V+VIII and alpha-1-antitrypsin, and in brain leads to poor trafficking of numerous neuroreceptors explaining nervous symptoms in MNK. Other receptors with a collagen-like stretch, COLQ and CCBE1, explain muscle weakness and lymphedema. Cq1 deficiency add problems with innate immunity, and lung surfactant defects. Unexpectedly the peroxisomal SOD1 pool requires ER for metalation, and Zellweger-like symptoms are becoming part of the MNK symptom spectrum. Interestingly, motor neuron disease is a characteristic of the mildest disease form, SMAX3.
Trafficking and post-translational modifications of copper enzymes, including metalation, begin in the endoplasmic reticulum (ER) and continues in Golgi before proteins are sorted and sent to their final destinations. Sugar tags guide enzymes during folding, proof-reading, refolding, and holoenzyme trafficking [261]. In ER nascent polypeptides are core glycosylated, and the added sugar tags are used for cargo receptor recognition by LMAN1 and other lectins. Adaptor sugar recognition is important for correct folding and trafficking, and GAG defects lead to multiple tissue and organ failures as well as abnormal physiognomy. Proteins with a CRD domain constitute a distinct class of adaptor molecules of which collectins [109] require LOX for correct conformation and stability.

At present, the extent of glycosylation and trafficking defects in Menkes disease is unclear, and copper's significance for sugar sorting is an emerging field. LMAN1 is one of several homologous mannose binding adapter molecules securing protein trafficking in the secretory pathway. Further glycosylation modifications occur in the Golgi complex where an array of enzymes modifies the sugar tags for their final destination but may require metalation to expose N-glycosylation sites correctly [261]. Lack of copper can result in distorted conformation and lead to normally unexposed N-glycosylation sites being exposed or the opposite, resulting in integration at wrong membrane sites [261].

Copper metalation is most often cited as taking place in TGN, but we found clear evidence in the literature of metalation in ER. Possibly ATP7A delivers copper in ER, in ERGIC, and in Golgi. SUMF1 is resident and metalated in ER and requires an ER-resident homologue devoid of copper; SUMF2 as molecular chaperone. Strong indication exists that TYR and DBH are metalated in ER, also making ER-metalation of PHM likely. TYR sites have low avidity and if pH is low, often loses copper, but is reloaded in melanocytes with a neutral pH. Potentially ATP7A also provides copper here. TYR is used as molecular chaperones, TYP1 and TYP2 as molecular chaperones. TYP1 and TYP2 contain zinc and are both ER located. DBH is suggested to use the ER-resident homologue, MOX1D as molecular chaperone.

Blue copper oxidases (CP, HEPH, and HEPHL1) appear to be metalated in cis-Golgi. Cofactor formation of LOX and AOC happens by use of a redox process, before N-glycosylation in Golgi, though the process is normally cited as autocatalytic. However, required reactions will likely not rely on chance, but is facilitated by an enzyme reaction. HEPHL1 is needed for metalation of LOX, and AOC likely use the same or a similar redox chaperone.

SOD1 is metalated at several cellular sites and depends on ER for metalation of the peroxisomal pool. CCS does not load SOD1 but is needed as redox chaperone for S-S bridges stabilizing the conformation for proper metalation and subsequent piggy-backing of the CCS-SOD1 complex to peroxisomes. CCS provides allosteric regulation of SOD1 and BACE1, similarly to ATOX1 that allosterically regulates MBPs to initiate ATP7A/B pump activity.

ATP7A contains a Golgi localization signal and locates in ER when the signal is removed by alternative splicing. The first luminal loop may help retain the protein in ER by binding of copper to Met-His-rich sequences similar to calcium ATPases using corresponding sites for calcium regulated ER retention. Metalation of DBH, PAM, and TYR may be facilitated by the Met-His-rich luminal loop of ATP7A.

At experimental tissue culture conditions, ATP7A is found in TGN. However, most tissue culture experiments use fibroblasts, and the principal enzyme in this cell type is LOX, which is metalated in the late secretory pathway. Fet3 models will misinterpret ER activity as it is a CP/HEPH homologue metalated in Golgi. Thus, experiments using tissue culture may not represent the full picture of what takes place in vivo. We hypothesize that if ER metalation is diminished, all enzymes including downstream metalated enzymes may be affected leading to the severest phenotype. Milder phenotypes may preserve ER metalation of enzymes but show Golgi metalation problems. However, enzymes with low copper avidity may lose the metal during Golgi passage, and the enzyme may integrate at a faulty
site resulting in deficient function, as may be the case for, e.g., DBH and TYR. Notably, all ATP7A-related phenotypes except SMAX3 show pale skin color and dysautonomia.

Table 3. Menkes Disease Symptoms.

| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| Preterm                   | AOC (histaminase) | One-third before 37 weeks |
| Premature rupture of fetal membranes | LOX |          |
| Weight                    | -                 | One-third less than 2500 g |
| Length                    | -                 |          |
| Head circumference        | -                 |          |
| Apgar score               | -                 | Quick test at 1 and 5 min, in rare cases, also 10 min after birth |
| Denver scale              | -                 | Developmental score for milestones in young children according to age |
| Bayley score              | -                 | Cognitive, language, and motor developmental infants and toddlers score |
| Hydrops fetalis           | LOX               | Severe swelling (oedema) |
| Intrauterine growth retardation | LOX, SUMF1 | Small for gestational age |
| Decreased fetal movements | -                 |          |
| Neonatal onset            | -                 | Rarely recognized before hair changes at 2–3 month |
| Neonatal death            | -                 |          |
| Early death               | -                 | Usually before three years |
| Failure to thrive         | -                 |          |
| Feeding difficulties      | DBH, LOX          | Poor sucking and swallowing |
| Floppy infant             | COX, LOX          |          |
| Poor head control         | COX               |          |
| Dysautonomy               | DBH               |          |
| Infantile spasm            | COX               | Shivers or a small jerks in series |
| Irritability              | DBH, PAM, SUMF1   |          |
| Babinski reflex           | DBH, PAM, SUMF1   | Upward movement of the big toe sign of pyramidal dysfunction |
| Anxiety                   | DBH, PAM, SUMF1   |          |
| Increased response to noise | DBH, PAM, SUMF1   |          |
| Lethargy                  | DBH, PAM, SUMF1   | Decreased alertness |
| Respiratory distress      | LOX, SOD3         |          |
| Icterus/jaundice          | CP, LOX           | Photo therapy resistant |
| **External features**     |                   |          |
| **- Head and neck**       |                   |          |
| Face lacking in expression | DBH                | Low mimic |
| Pallor                    | TYR               | Light skin color |
| Hypertelorism             | LOX, SUMF1        | Widely spaced eyes |
| Nystagmus                 | DBH, LOX, TYR     | Difficulty in controlling eye movements |
| Blepharophimosis          | LOX               | Narrowing of eye opening |
| Photophobia               | AOC, TYR          | Light intolerance |
| Keratitis                 | AOC, LOX          | Cornea inflammation |
| Conjunctivitis            | AOC, LOX          | Eye inflammation |
### Table 3. Cont.

| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| Ptosis                    | DBH, LOX          | Droopy eyelids |
| Miosis                    | DBH               | Excessive constriction of pupils |
| High arched (cupid) eyebrows | LOX, SUMF1       |          |
| Ophthalmoplegia           | DBH, LOX          |          |
| Cherubic appearance       | LOX, SUMF1        |          |
| Microcephaly              | LOX, SUMF1        | <2 SD for age |
| Brachycephaly             | LOX, SUMF1        |          |
| Frontal bossing           | LOX, SUMF1        |          |
| Occipital bossing         | LOX, SUMF1        |          |
| Long philtrum             | LOX, SUMF1        |          |
| High forehead             | LOX, SUMF1        |          |
| High-arched palate        | LOX, SUMF1        |          |
| Small chin                | LOX, SUMF1        |          |
| Pudgy cheeks              | SUMF1             |          |
| Flat central face         | LOX, SUMF1        |          |
| Depressed nasal bridge    | LOX, SUMF1        |          |
| Nasal congestion          | LOX               |          |
| Hypoplastic mandibles     | LOX, SUMF1        |          |
| Micrognathia              | LOX, SUMF1        |          |
| Retrognathia              | LOX, SUMF1        |          |
| Drooping jaws             | SUMF1             |          |
| Low set ears              | LOX, SUMF1        |          |
| Large ears                | LOX, SUMF1        |          |
| Occipital exostoses       | LOX               | Calcified exostoses palpable from occiput, uncommon |
| Internal jugular phlebectasia | LOX             |          |
|                          | **Chest**         |          |
| Pectus excavatum          | LOX               |          |
| Pectus carinatum          | LOX               |          |
|                          | **Neurological symptoms** |          |
| Corpus callosum agenesis  | SUMF1             | Absence of brain structure that connects the two hemispheres |
| Dysautonomia              | DBH               |          |
| Cerebellar hypoplasia     | LOX               |          |
| Mental retardation        | COX, PAM, SOD1    |          |
| Motor retardation         | COX, PAM, SOD1    |          |
| Loss of milestones        | -                 | Progressive neurologic defects |
| Hypothermia               | DBH, PAM          | Subnormal body temperature |
| Hypoglycemia              | DBH, PAM          | Subnormal sugar values |
| Nasal congestion          | DBH               |          |
| West syndrome             | COX               | Epileptic encephalopathy |
| Seizures                  | COX               | Refractory and early onset |
## Table 3. Cont.

| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| Clonic seizures           | COX               |          |
| Myoclonic seizures        | COX               |          |
| Tonic seizures            | COX               |          |
| Motor dysfunction          | DBH               |          |
| Ataxia                    | DBH, PAM, SOD1, SUMF1 |          |
| Spasticity                | COX               |          |
| Hypertonia                | DBH               |          |
| Hypotonia                 | DBH               |          |
| **Eye symptoms**           |                   |          |
| Cataract                  | LOX               |          |
| Myopia                    | LOX               |          |
| Nystagmus                 | DBH, LOX, TYR     | Difficulty in controlling eye movements |
| Strabismus                | TYR               |          |
| Blepharophimosis          | LOX               | Narrowing of eye opening |
| Photophobia               | AOC, TYR          | Light intolerance |
| Keratitis                 | AOC, LOX          | Cornea inflammation |
| Conjunctivitis            | AOC, LOX          |          |
| Ptosis                    | DBH, LOX          | Droopy eyelids |
| Miosis                    | DBH               | Excessive constriction of pupils |
| Reduced visual acuity     | TYR               |          |
| Optic discs palor         | TYR               |          |
| Optic atrophy             | TYR               | Abnormal electroretinogram (ERG) |
| Visual loss               | TYR               | Visual evoked potential (VIP) |
| Retinal and iris depigmentation | TYR              |          |
| Iris trans-luminescence   | TYR               |          |
| Iris microcysts           | SUMF1, TYR        |          |
| Hypopigmented fundus      | TYR               | Fundoscopy |
| **Ear symptoms**          |                   |          |
| Hearing loss              | LOX, PAM, TYR     | Brain stem auditory evoked potential (BAEP) |
| **Hair and skin symptoms** |                   |          |
| Fine, silvery and brittle hair | AOC, TYR, SUMF1 | Short, stubby, friable |
| Depigmented scalp hair    | TYR, SUMF1        | Lusterless, silvery, steel wool |
| Sparse hair               | AOC, SUMF1        | Rubbing against pillow may feel like unshaven stubbles |
| Alopecia                  | AOC, SUMF1        | Lack of hair |
| Fetal hair may be unaffected | -                | Soft |
| Pili torti                | SUMF1             | Hair twisted about their own axis |
| Trichorhesis nodosa       | SUMF1             | Frying and splitting of hair ends |
| Monilethrix               | SUMF1             | Varying diameters of the shafts |
| Cupid eyebrows            | LOX, SUMF1        | Eyebrows with a high arch |
| Sparse eyebrows           | AOC, SUMF1        | Look like old man’s eyebrows |
Table 3. Cont.

| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| Sparse eyelashes          | AOC, SUMF1        | Breaks easily |
| Seborrhea                 | AOC, SUMF1        | Dry and scaly skin |
| Erythroderma              | AOC, SUMF1        | Generalized exfoliative dermatitis with redness and scaling |
| Cutis laxa                | HEPHL1, LOX       | Lax and wrinkled skin may give a progeria like appearance |
| Pale skin                 | PAM, TYR          | Almost like an albino |
| Anhydrosis                | DBH, LOX          | Inability to sweat normally |
| Doughy skin               | LOX               | Swelling of subcutaneous tissue |
| Lymphedema                | LOX               | Swelling due to poor lymphatic system |
| **Dentation**             |                   |          |
| Hyperplastic gums         | LOX               | Prominent gums |
| Dental abnormalities      | LOX               |          |
| Enamel defects            | LOX               |          |
| Delayed eruption          | LOX               |          |
| Biconically shaped incisors| LOX          |          |
| **Lung symptoms**         |                   |          |
| Acute respiratory distress syndrome | AOC, LOX, SOD3, SUMF1 |          |
| Chronic obstructive pulmonary disease | AOC, LOX, SOD3, SUMF1 |          |
| Emphysema                 | LOX, SOD3, SUMF1  | Damaged air sacs (alveoli) with breathing difficulty |
| **Cardiovascular symptoms**|                   |          |
| Congenital heart disease  | COX, LOX          | About 5% |
| Angiopathy                | AOC, APP, LOX, SOD3 | Disease of arteries, veins, and capillaries |
| Tortuous blood vessels    | LOX               | Twisted with frayed and split inner walls |
| Bleeding tendency         | FV+VIII, LOX      |          |
| Mild coagulation deficiency| FV+VIII         |          |
| Hematomas                 | LOX               |          |
| Subdural hematomas        | LOX               |          |
| Intracranial hemorrhage   | LOX               |          |
| Cephalohematomas          | LOX               | Prevalent at birth |
| **Gastrointestinal symptoms** |                 |          |
| Chronic diarrhea          | AOC               |          |
| Vomiting                  | AOC               |          |
| Bowel dysfunction         | AOC               |          |
| Gastrointestinal polyps   | LOX               |          |
| Hiatal hernia             | LOX               |          |
| **Hepatic symptoms**      |                   |          |
| Hepatomegaly              | COX, SOD1, SUMF1  | Low hepatic copper gives low enzymatic activity |
| Icterus                   | CP, ATP7B         | Yellowish color of skin and eyes |
| Steatosis                 | COX, SOD1         | Fatty liver |
| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| **Genitourinary symptoms**|                   |          |
| Bladder diverticula       | LOX               |          |
| Bladder rupture           | LOX               |          |
| Ureteral obstruction      | LOX               |          |
| Glomerulonephritis        | LOX               |          |
| Urinary tract infection   | LOX               |          |
| Vesico-ureteral reflux    | LOX               |          |
| Hydronephrosis            | LOX               | Partial urinary tract blockage |
| Diaphragmatic hernia      | LOX               |          |
| Umbilical hernias         | LOX               |          |
| Inguinal hernia           | LOX               |          |
| Cryptorchidism            | LOX, SUMF1        | Undescended testicles |
| **Connective tissue symptoms**|                 |          |
| Loose/hypermobile joints  | LOX               |          |
| Tortuous vessels          | LOX               |          |
| Wrinkled and loose/extensible skin | LOX |          |
| Soft skin / edema         | LOX               |          |
| **Musculoskeletal symptoms**|                 |          |
| - Skeletal—neck and chest |                   |          |
| Cervical spine anomalies  | LOX               | Mimics non-accidental lesions |
| Short, broad clavicles    | LOX               |          |
| Flaring of the ribs       | LOX               |          |
| Short, broad ribs         | LOX               |          |
| Pectus excavatum          | LOX               | Sunken breastbone |
| Pectus carinatum          | LOX               | Protruding breastbone; “pigeon chest” |
| - Skeletal—limbs          |                   |          |
| Congenital bone fractures | LOX               | Symmetrical uncommon in “battered child”/NAI |
| Long-bone fractures       | LOX               |          |
| Metaphyseal spurring      | LOX               | Can resemble scurvy |
| Diaphyseal periosteal reaction | LOX |          |
| Cortical thickening       | LOX               |          |
| Short humeri              | LOX               |          |
| - Skeletal—others         |                   |          |
| Wormian bones             | LOX               | Intrasutural supernumerary bones, not found in child abuse |
| Spondyloysis              | LOX, SUMF1        | Fractures of vertebra |
| Osteoporosis              | LOX, SUMF1        | Brittle bones |
| Osteopenia                | LOX, SUMF1        |          |
| Cartilage malformation    | LOX, SUMF1        |          |
| Joint laxity              | LOX               |          |
| Limb dislocations         | LOX               |          |
Table 3. Cont.

| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| Metaphyseal widening      | LOX               |          |
| Osteochondrodysplasia     | LOX, SUMF1        |          |
| Occipital horn exostoses  | LOX               | Uncommon in MNK, but can be observed from 2 years |

- **Muscles**

| Motor neuron disease | SOD1, LOX |

**Investigations**

| Activity measured |
|-------------------|
| MR MRI and MRA    | Neuroimaging     |
| CT                | Neuroimaging     |
| EEG               | Brain activity   |
| Radiography Bone  | Symmetrical metaphyseal flaring and spurring of ribs, and cervical fractures may mimic non-accidental trauma, but these are not symmetrical; skull wormian bones are not seen in child abuse |
| Arteriography      | Vasculature      |
| Ultrasoundography   | Bladder, bowel   |
| Light Microscopy    | Hair examination |
| Echocardiography    | Heart            |
| ERG                | Electroretinogram |
| VIP                | Visual evoked potential |
| Fundoscopy         | Eye background, macula |
| BAEP               | Brain stem auditory evoked potential |
| Cell culture Radioactive copper test | Increased accumulation and retention |
| Tissue copper      | ICPMS; AA        |

**Biomarkers**

| Boy ATP7A          | X-linked          |
| Family history of male infant death ATP7A | X-linked |
| Hyperbilirubinemia ATP7B | Transient, but prolonged and light therapy resistant |
| Low plasma copper ATP7A | Diagnostic from 4–6 weeks |
| Low free Cu ATP7A | Diagnostic from birth |
| Low ceruloplasmin CP | Diagnostic from 4–6 weeks |
| High RBC (Red Blood Cells) Cu SOD1 | Erythrocyte SOD1 in neonates |
| Anemia HEPH, CP | May be hypochrome |
| Neutropenia LOX | Decreased neutrophils |
| Thromboembolism FV+VIII | Blood clot breaking loose and plugs other vessels |
| Urinary Cu low to normal ATP7A | MT |
| Low liver Cu ATP7A | Diagnostic from birth |
| High placenta Cu ATP7A | Diagnostic from birth; CVS diagnostic prenatally |
| High metallothionein levels ATP7A | MT1 and MT2 (diagnostic?) |
## Table 3. Cont.

| Birth and Neonatal Period | Enzyme Deficiency | Comments |
|---------------------------|-------------------|----------|
| Plasma DA/NE ratio increased | DBH | Diagnostic from birth |
| Urinary HVA/VMA ratio increased | DBH | Diagnostic from birth |
| Hypoglycemia | SUMF1, PAM, DBH | Transient |
| High blood lactate | COX | CSF, intermittent |
| Pyruvate | COX | Intermittent |
| Hyperammoniemia | COX | Intermittent |
| High plasma glutamic acid | COX | Intermittent, alpha-ketogluterate conversion |
| Respiratory chain deficiencies | COX | Indicative |
| Intracellular Cu accumulation | ATP7A | Diagnostic, tissue culture |
| Molecular screening of ATP7A | ATP7A | Definitive diagnosis |

### Pathology

| Pathology | Enzyme Deficiency | Comments |
|-----------|-------------------|----------|
| Purkinje cell pathologies | SOD1 | Faulty arborization and “weeping willow” |
| Ragged red fibers | COX | Subsarcolemmal aggregates of mitochondria in muscle fiber |
| Alder Reilly anomaly | SUMF1 | Vacuolization of blood cells; observed in GAG deficiencies |
| Metachromasia | SUMF1 | Color staining change of accumulated tissue sugar sulfatides |
| Pili torti | SUMF1 | Hair twisted about their own axis |
| Trichorhexis nodosa | SUMF1 | Frying and splitting of hair ends |
| Monilethrix | SUMF1 | Varying diameters of the shafts |

### Differential diagnosis:

| Differential diagnosis | Enzyme Deficiency | Comments |
|------------------------|-------------------|----------|
| NAI | LOX | Non-accidental injuries; >10% symmetric changes think MNK |
| Osteogenesis imperfecta | LOX | Brittle bones and bone dysplasias |
| Mitochondrial disorder | COX | Compromised energy production affecting all organs and tissues |
| Organic acid uria | COX | Defective mitochondrial matrix metabolism |
| Cutis laxa | LOX | Loose and wrinkled skin in an infant |
| Progeria | LOX, SUMF1 | Old age syndrome in young people |
| Syndromes with hair abnormalities | SUMF1, AOC | Multiple sulfatase deficiency |
| Glutamine defects | COX | Defective mitochondrial matrix metabolism |
| MSD | SUMF1 | Multiple sulfatase deficiency |
| MPS | SUMF1 | Mucopolysaccaridoses |
| MLP | SUMF1 | Mucolipidoses |
| Leukodystrophy | SUMF1 | E.g., metachromatic leukodystrophy |
| DBH deficiency, congenital | DBH | CNS Cu deficiency |

### 10. Future Directions

Numerous copper chaperones and adaptor molecules regulate copper-dependent enzyme passage in the secretory pathway, but the specific guiding molecules are only known for a fraction of copper enzymes. We hypothesize that more copper-dependent enzymes need specific copper chaperones for metal activation, and chaperoning roles may emerge for copper binding proteins for which today there is no known function. For example, the APP family may act as redox-active copper chaperones similarly to CCS. More copper-dependent enzyme reactions are likely to be unraveled, e.g., mitochondrial enzymes controlled by lipoic acid may also depend on copper. Finally, the iron–copper
connection needs to be further explored on molecular, cellular, and organelle levels. Despite the new cellular/molecular connections outlined here for copper-dependent processes, the Menkes disease enzyme puzzle, linking consequences of ATP7A dysfunction in cells and tissue to MNK patients’ clinical symptoms, is not yet complete.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ABP1         | Amiloride-binding protein 1 |
| ALS          | Amyotrophic lateral sclerosis |
| AMD          | Age-related macular degeneration |
| AOC          | Copper-containing amine oxidases |
| APP          | Amyloid-β precursor protein |
| APLP         | Amyloid-like protein |
| ARS          | Arylsulfatase |
| ATOX1        | Antioxidant 1 copper chaperone |
| ATP          | Adenosine triphosphate |
| ATP7A        | Copper-transporting ATPases, α |
| ATP7B        | Copper-transporting ATPases, β |
| BACE1        | β-secretase 1 |
| BBB          | Blood–brain barrier |
| C            | Cysteine |
| C1q          | Complement Q1 |
| CCBE1        | Collagen and calcium binding EGF domains 1 |
| CCS          | Copper chaperone for SOD1 |
| CHCHD        | Coiled-coil-coiled-coil domain |
| CNS          | Central Nervous System |
| COA6         | COX Assembly Factor 6 |
| COL          | Collagen |
| COLQ         | Collagen-like tail of endplate acetylcholinesterase |
| COX          | Cytochrom c Oxidase |
| CP           | Ceruloplasmin |
| CRD          | Carbohydrate-recognition domain |
| CSF          | Cerebrospinal Fluid |
| Cu           | Copper |
| Cys          | Cysteine |
| CYBRD1       | Cytochrome b reductase 1 |
| DA           | Dopamine |
| DAO          | Diamine Oxidase |
| DBH          | Dopamine β-hydroxylase |
| DOPA         | Dihydroxyphenylalanine |
| ECM          | Extracellular Matrix |
| EC           | Extracellular |
| ELN          | Elastin |
| ER           | Endoplasmic reticulum |
| ERGIC        | ER–Golgi intermediate compartment |
| FAD          | Flavin adenine dinucleotide |
| FV+VIII      | Clotting factors V and VIII |
| Abbreviation | Full Name |
|--------------|-----------|
| Fe-S          | Iron sulfur site |
| FGE          | Formylglycine generating enzyme |
| FGly         | Formylglycine |
| G            | Glycine |
| GABA         | Gamma aminobutyric acid |
| GAG          | Glycosaminoglycan |
| GALNS        | Galactosamine-6-sulfate sulfatase |
| Gly          | Glycine |
| GMXCXXC      | Amino acid sequence of ATP7A/B MBD |
| GNS          | N-acetylglucosamine-6-sulfatase |
| GPI          | Glycosylphosphatidylinositol |
| GSH          | Glutathione |
| HEPH         | Hephaestin |
| HEPHL1       | Hephaestin-like protein 1 |
| His          | Histidine |
| H$_2$O$_2$   | Hydrogen peroxide |
| IDS          | Idurunate 2-sulfatase |
| IMM          | Inner mitochondrial membrane |
| IMS          | Inter-membrane space |
| LMAN         | Lectin mannose binding |
| LOX          | Lysyl oxidase |
| LOXL         | Lysyl oxidase-like |
| LTQ          | Lysine tyrosylquinone |
| M            | Methionine |
| MNK          | Menkes disease |
| MBD          | Metal binding domain |
| MXCXXC       | Amino acid sequence of CCS-MBD |
| Met          | Methionine |
| MIA40        | Mitochondrial IMS assembly 40 |
| MND          | Motor neuron disease |
| MLP          | Mucolipidose |
| MPS          | Mucopolysaccharidase |
| MSD          | Multiple sulfatase deficiency |
| MOXD1        | Monoxygenase, DBH-Like 1 |
| NAD(P)H      | Nicotinamide adenine dinucleotide phosphate |
| NE           | Norepinephrine |
| NLS          | Nuclear localization sequence |
| OHS          | Occipital horn syndrome |
| OMIM         | Online mendelian inheritance in man |
| PAL          | Peptidyl-α-hydroxyglycine α-amidating lyase |
| PAM          | Peptidyl α-amidating enzyme |
| PAO          | Polyamine oxidase |
| PEX          | Peroxin |
| PHM          | Peptidylglycine α-hydroxylating monoxygenase |
| PTS          | Peroxisomal targeting signal |
| ROS          | Reactive oxygen species |
| SCO          | Synthesis of COX |
| SMAX3        | X-linked distal spinal muscular atrophy 3 |
| SOD          | Superoxide dismutase |
| SP           | Secretory pathway |
| S-S          | Disulfide bridge |
| STEAP        | Six-transmembrane epithelial antigen of prostate |
| STS          | Steroid sulfatase |
| SUMF         | Sulfatase-modifying factor |
| TGN:         | Trans Golgi Network |
| TM           | Transmembrane |
| TPQ          | Trihydroxyphenylalanine quinone |
| TYR          | Tyrosinase |
| Reference                                                                 | Title                                                                 | Journal                                                                 | Page Numbers | Year | URL                                                                 |
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**TYRP** Tyrosinase related protein  
**VAP-1** Vascular adhesion protein 1  
**PBD** Peroxisome biogenesis disorders  
**Zn** Zinc
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