MEDNIK syndrome: a novel defect of copper metabolism treatable by zinc acetate therapy

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MEDNIK syndrome—acronym for mental retardation, enteropathy, deafness, neuropathy, ichthyosis, keratodermia—is caused by AP1S1 gene mutations, encoding α1A, the small subunit of the adaptor protein 1 complex, which plays a crucial role in clathrin coat assembly and mediates trafficking between trans-Golgi network, endosomes and the plasma membrane. MEDNIK syndrome was first reported in a few French-Canadian families sharing common ancestors, presenting a complex neurocutaneous phenotype, but its pathogenesis is not completely understood. A Sephardic-Jewish patient, carrying a new AP1S1 homozygous mutation, showed severe perturbations of copper metabolism with hypocupremia, hypoceruloplasminemia and liver copper accumulation, along with intrahepatic cholestasis. Zinc acetate treatment strikingly improved clinical conditions, as well as liver copper and bile-acid overload. We evaluated copper-related metabolites and liver function retrospectively in the original French-Canadian patient series. Intracellular copper metabolism and subcellular localization and function of copper pump ATP7A were investigated in patient fibroblasts. Copper metabolism perturbation and hepatopathy were confirmed in all patients. Studies in mutant fibroblasts showed abnormal copper incorporation and retention, reduced expression of copper-dependent enzymes cytochrome-c-oxidase and Cu/Zn superoxide dismutase, and aberrant intracellular trafficking of Menkes protein ATP7A, which normalized after rescue experiments expressing wild-type AP1S1 gene. We solved the pathogenetic mechanism of MEDNIK syndrome, demonstrating that AP1S1 regulates intracellular copper machinery mediated by copper-pump proteins. This multisystem disease is characterized by a unique picture, combining clinical and biochemical signs of both Menkes and Wilson’s diseases, in which liver copper overload is treatable by zinc acetate therapy, and can now be listed as a copper metabolism defect in humans. Our results may also contribute to understand the mechanism(s) of intracellular trafficking of copper pumps.

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Introduction

In humans, copper is required for numerous cellular processes, including mitochondrial respiration, antioxidant defence, neurotransmitter synthesis, connective tissue formation and skin pigmentation (de Bie et al., 2007). Until recently, two inherited disorders of copper metabolism were known: Menkes disease (MIM 309400) and Wilson’s disease (MIM 277900), caused by mutations in two distinct but closely related copper ATPases that markedly differ in their tissue expression (Bull et al., 1993; Chelly et al., 1993). ATP7A, the Menkes protein, is present in most non-hepatic tissues (Paynter et al., 1994), whereas the Wilson’s disease protein ATP7B is predominantly expressed in liver (Hung et al., 1997). Both are localized at the level of the trans-Golgi network, where they transport copper to cuproenzymes synthesized within the secretory compartments (Petris et al., 2000). When intracellular copper level rises, both determine its efflux (La Fontaine et al., 2001), trafficking copper to the plasma membrane (ATP7A) or relocating it to cytoplasmic vesicles associated with bile ducts canalicular membrane in the liver (ATP7B) (Hung et al., 1997). Menkes disease, a severe encephalopathy with multisystem manifestations affecting bones, hair, skin and vessels, and its milder allelic variant, occipital horn syndrome (MIM 304150), cause copper accumulation in enterocytes and severe deficiency of copper and ceruloplasmin in plasma and tissues, impairing activities of several copper-dependent enzymes [lysyl oxidase, tyrosinase, superoxide dismutase (SOD), dopamine beta-monooxygenase and cytochrome oxidase (COX)] (Kaler, 2011). Recently, unique missense ATP7A mutations impairing its intracellular trafficking have been found in patients with distal motor neuropathy showing normal plasma copper and ceruloplasmin (Kennerson et al., 2010; Yi et al., 2012). In Wilson’s disease, tissue copper overload, mainly in brain and liver, causes neurological, ocular (Kayser–Fleisher ring) and psychiatric symptoms, along with hepatic cirrhosis and liver failure caused by impaired copper export into the bile (Bull et al., 1993).

Recently, a new copper metabolism disorder, characterized by congenital cataracts, sensorineural deafness, and low serum copper and ceruloplasmin, has been associated with mutation in SLC33A1, a gene required for acetylation and secretion of ceruloplasmin (Huppke et al., 2012). However, some patients with copper metabolism abnormalities do not meet the diagnostic criteria of these well-defined entities.

In a child with MEDNIK syndrome (mental retardation, enteropathy, deafness, neuropathy, ichthyosis, keratoderma), a rare autosomal recessive neurocutaneous disease, we detected relevant copper metabolism changes not reported so far. MEDNIK syndrome has been described in a few patients from French-Canadian families sharing common ancestors from the Kamouraska region living in Quebec, but its pathogenesis is not yet completely understood (Saba et al., 2005; Montpetit et al., 2008). Patients who presented an atypical form of erythrokeratodermia variabilis (EKV3, MIM 609313) with mild increased plasma very long chain fatty acids, carried a homozygous mutation in AP1S1 (MIM 603531), coding for the small subunit σ1A of the adaptor protein 1 (AP-1) complex, which plays a crucial role in clathrin coat assembly (Montpetit et al., 2008).

Our patient, who carried a novel homozygous AP1S1 mutation, showed marked improvement of clinical signs and biochemical copper abnormalities on zinc acetate therapy. Re-evaluation of the whole series of original French-Canadian patients (Saba et al., 2005; Montpetit et al., 2008) confirmed that MEDNIK syndrome is caused by a perturbation of copper metabolism.

Patients and methods

Case series

We studied a total of six patients, one Italian and five from the original French-Canadian cohort (Saba et al., 2005; Montpetit et al., 2008). The study was approved by our institutions’ ethic committees and written informed consent from patients’ caregivers was obtained.

Sequence and expression analysis of AP1S1

Genomic DNA was extracted from peripheral blood lymphocytes following standard methods and the whole AP1S1 gene coding regions and splice sites were sequenced with BigDye® Terminator system on an automated sequencer (Life Technologies).

For expression analysis, AP1S1 messenger RNA from Patient 1 fibroblasts and five normal control subjects were measured by quantitative real-time PCR in an ABI PRISM 7500 Sequence Detection System (Life Technologies) using Power SYBR® Green I dye chemistry, as previously described (Zanni et al., 2011).

Western blotting

For western blotting analysis, 50 μg of proteins from cultured fibroblasts were subjected to SDS PAGE and probed with the following antibodies: AP1S1 (Abcam), β-Actin (Abcam), complex II–70kDa subunit (SDH-70), complex IV–subunit II (COX-II) and complex IV subunitIV (COX-IV), VDAC (porin) (all purchased from MitoSciences), and Cu/Zn superoxide dismutase (Cu-SOD) (StressGen). Reactive bands were detected using the Immobilon Western Chemiluminescent HRP substrate detection kit (Millipore).

Fibroblast copper determination, copper-64 accumulation and retention studies

Copper content, uptake and retention were measured in mutant fibroblasts from Patient 1 and compared with cell lines from two patients...
with Menkes disease and two healthy control subjects, as previously described (Rachidi et al., 2003).

Cell culture, cytochemistry and epifluorescence studies

Human fibroblasts were grown in Dulbecco’s modified Eagle medium supplemented with 10% foetal bovine serum, 4.5 mg/ml glucose, penicillin (100 U/ml) and streptomycin (100 μg/ml). For peroxisomal visualization, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% TritonX-100 and incubated with rabbit anti-PMP70 primary antibody (Sigma) and anti-rabbit IgG secondary antibody conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories). Nuclei were stained with DAPI (Molecular Probes/Invitrogen). Fluorescence was visualized using an inverted fluorescence microscope (Olympus IX70), and images were captured with a RT Color Spot (Diagnostic Instrument) camera system and LAS 2000 acquisition software. COX activity of the cells was detected cytochemically as previously reported (Tiranti et al., 2000 acquisition software).

Cells transfections and confocal laser microscopy analysis

Full-length AP1S1 was constructed by reverse transcription PCR using total RNA as template. The reverse primer had a sequence encoding an epitope of the haemagglutinin influenza virus (HA tag) located in frame with AP1S1 sequence. After confirming sequence fidelity, the complementary DNA was subcloned in the eukaryotic expression vector pcDNA3.1 (Life Technologies). The construct was used to transfect patient’s fibroblasts using Lipofectamine® 2000 (Life Technologies) according to the manufacturer’s instructions. ATP7A distribution and compartmentalization were analysed under basal conditions and after increasing copper concentration in culture medium (200 μM of CuCl₂ for 3 h). Multiple stainings were performed overnight using a rabbit polyclonal anti-ATP7A (dilution 1:1000, Abcam), a mouse monoclonal anti-p230 antibody directed against the trans-Golgi network (1:500; BD Transduction Laboratories), a rat monoclonal anti-HA antibody (1:300; clone 3F10, Roche-Applied-Science), all diluted in 1% bovine serum albumin/PBS. The secondary antibodies were linked to Alexa Fluor® 405, 488 and 555 dyes (Molecular Probes). Nuclei were labelled with 1 μg/ml Hoechst 33342 (Molecular Probes) or DRAQ5 (Biostatus). Images of immunostained objective.

Results

Case series

The index patient (Patient 1 in Tables 1 and 2) was the third born of healthy consanguineous Sephardic Jewish parents. An elder sister died at 50 days of age from intestinal pseudo-obstruction without showing skin and hair changes. The index patient was born at term, neonatal growth parameters were normal. From birth she manifested alternating diarrhoea and constipation, recurrent infections, migrating erythematous skin patches, recurrent venous thromboses, hypotonia, progressive growth and psychomotor delay. Sensorineural deafness, hypopigmented and lax skin, ichthyosis, sparse woolly greyish hair and hepatomegaly were later recorded. Routine laboratory and extensive metabolic investigations displayed intermittent elevation of transaminases (up to six times), raised total bile acids (65 μmol/l; normal values 0–6), severe and persistent reduced concentrations of serum copper (range 2.4–2.8 μmol/l; normal values 12.7–22.0) and ceruloplasmin (range 0.13–0.2 μmol/l; normal values 2.0–4.3), with increase of calculated free copper level (1.9 μmol/l; normal value <1.6) and increased urinary copper excretion (2.55 μmol/l/24 h; normal values 0.07–0.80). Zinc level was in the high/normal range. Additional abnormalities included elevation of lactate in blood and urine, increase of plasma alanine, borderline elevation of plasma very long chain fatty acids (C26:0 = 1.0–1.3 μmol/l; normal value <0.9), with normal levels of pristanic acid, phytic acid and pipecolic acid. Increased copper content (0.58 μg/mg; normal values 0.02–0.06) was detected in a liver biopsy performed at the age of 2 years, which showed normal morphology. Extensive genetic studies, including karyotype, array-comparative genomic hybridization, sequencing of ATP7A and ATP7B and other copper-related genes (ATOK, SLC31A1, CCS, SLC11A2, MT3, SCO1, SCO2, MURR1, COX11, COX17, COX19, COX23 and CP) were normal. Large ATP7A and ATP7B deletions/duplications were excluded by quantitative real-time PCR. At 7 years of age she showed, together with neurocutaneous signs, microcephaly, severe mental retardation (Major Depression Inventory score <45 at Bayley Mental Developmental scale), autistic–like features (social and communication impairments, stereotypical responses, behavioural inflexibility), growth retardation, intractable itching and hepatomegaly. Dermatological findings are shown in Fig. 1A and B. Patient’s hair study by light microscopy documented trichorrexis nodosa.

Eye examination, including slit-lamp study of anterior chamber, excluded Kayser–Fleischer ring. Ultrasound disclosed increased liver echogenicity. X-ray displayed severe osteoporosis, and no evidence of occipital horn. Nerve conduction velocities were unremarkable. MRI showed brain atrophy and bilateral symmetrical hyointensities in caudate nuclei and putamina at T2-weighted images, a pattern similar but milder than in Wilson’s disease (Fig. 1C), and absence of vascular abnormalities at angio-MRI. A second liver biopsy displayed intrahepatic cholestasis and confirmed hepatic copper accumulation (0.74 μg/mg). Given the similarities to Wilson’s disease, we attempted oral zinc acetate therapy (50 mg/day) to reduce liver copper overload. On this therapy, we observed a marked improvement of behavioural disturbances and clear relief from itching. Cognitive function also improved, showing after 1 year of treatment a Major Depression Inventory score of 60 at Bailey scales, compatible with moderate mental retardation. Biochemically, serum transaminases, plasma ceruloplasmin (20.0 μmol/l), serum free copper level (<1.6 μmol/l) and urinary copper excretion (0.06 μmol/l/24 h) normalized, along with striking reduction of plasma total bile acids levels (10.2 μmol/l) and liver copper overload (0.42 μg/mg); blood and urine lactate were constantly normal (Table 1).

Regardless of the copper-related metabolic abnormalities, the clinical picture prompted us to suspect MEDNIK syndrome and investigate AP1S1 as a candidate gene.
Table 1 Relevant clinical and biochemical changes in Patient 1 before and on zinc acetate therapy

<table>
<thead>
<tr>
<th></th>
<th>Before therapy</th>
<th>On therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ</td>
<td>&lt;45</td>
<td>60</td>
</tr>
<tr>
<td>Liver copper</td>
<td>0.74</td>
<td>0.42</td>
</tr>
<tr>
<td>µg/mg (0.02–0.06)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Serum free copper level</td>
<td>1.9</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>µmol/l (&lt;1.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary copper/24 h</td>
<td>2.55</td>
<td>0.06</td>
</tr>
<tr>
<td>µmol/l/24 h (0.07–0.80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST/ALT</td>
<td>266/111</td>
<td>67/39</td>
</tr>
<tr>
<td>UI/I (&lt;40)</td>
<td></td>
<td></td>
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<tr>
<td>Blood lactate</td>
<td>3.6</td>
<td>1.4</td>
</tr>
<tr>
<td>µmol/l (&lt;2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bile acids</td>
<td>65</td>
<td>10.2</td>
</tr>
<tr>
<td>µmol/l (0–6)</td>
<td></td>
<td></td>
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</tbody>
</table>

AST/ALY = aspartate aminotransferase/alanine aminotransferase. Normal values are given in brackets.

Table 2 Clinical, biochemical and neuroradiological features of patients with AP1S1 mutations

<table>
<thead>
<tr>
<th>Family</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Code pedigree</td>
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<td>II:2</td>
<td>II:1</td>
<td>II:2</td>
<td>II:2</td>
</tr>
<tr>
<td>Gender</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Age</td>
<td>8 y</td>
<td>27 y†</td>
<td>28 y</td>
<td>19 y</td>
<td>1.5 y‡</td>
</tr>
</tbody>
</table>

**Clinical features**
- Mental retardation
- Enteroopathy
- Deafness
- Neuropathy
- Ichthyosis
- Hyperkeratosis
- Erythrodermia
- Hepatopathy
- Liver fibrosis

**Laboratory abnormalities**
- AST/ALT
  - UI/I (<40) 266/111
  - Gamma-glytamyl transpeptidase
  - UI/I (<40) 38
  - ALP
  - UI/I (31–125) 1036
  - Total bile acids
  - µmol/l (0–6) 65
- Copper µmol/l (12.7–22) 2.4–2.8
- Ceruloplasmin µmol/l (2.0–4.3) 0.1–0.2
- Serum free copper level µmol/l (<1.6) 1.9
- VLCFA
  - ↑
  - ↑
  - ↑
  - ↑
  - ↑

**Brain MRI**
- Cerebral atrophy
- Basal ganglia abnormalities

ALP = alkaline phosphatase; AST/ALY = aspartate aminotransferase/alanine aminotransferase; N = normal; nd = not determined; VCLFA = very long chain fatty acids. Normal values are given in brackets.

*†died.
*Laboratory values in Patient 1 correspond with pretreatment phase.

AP1S1 mutation and expression

Sequencing of the whole AP1S1 gene coding regions and splice sites identified a homozygous single G insertion in a stretch of eight consecutive Gs between nucleotides 356 and 365 in exon 4 (Fig. 2A). Both parents and the eldest sister were heterozygous for this mutation. Because of the lack of biological samples, genetic analyses were not performed for the sister who died soon after birth. This mutation was predicted to cause a premature stop codon, 17 amino acids downstream of the mutation site (p.Asp322GlyfsX17). As shown in Fig. 2B, this homozygous mutation induced an 80-fold reduction of AP1S1 messenger RNA expression levels in patient’s fibroblasts when compared with wild-type controls. Similarly, the complete absence of AP1S1 protein was detected at immunoblot analysis (Fig. 2C). Both parents and the healthy sister, heterozygous for this mutation, had a 40-fold reduction of AP1S1 messenger RNA expression levels (data not shown).
Confirmation of clinical and biochemical findings in French-Canadian MEDNIK patients

To confirm the pathogenic link between AP1S1 mutation and copper-related abnormalities observed in the index patient, we reviewed the whole French-Canadian series of original MEDNIK patients (Saba et al., 2005; Montpetit et al., 2008). Because only two patients of this cohort were still alive, data from the other patients were retrospectively reviewed by clinical records.

Table 2 summarizes clinical, genetic and laboratory data of the whole series of known patients with MEDNIK syndrome. Family pedigrees are presented in Supplementary Fig 1. All patients showed peculiar dysmorphic features (high forehead, mongoloid facial appearance, depressed nasal bridge, low set ears, growth retardation) moderate to severe mental retardation, enteropathy with congenital diarrhoea, sensorineural deafness, ichthyosis, hyperkeratosis and erythrodermia. Peripheral neuropathy, a pivotal feature of MEDNIK syndrome, was not recorded in our index patient and in one patient from the French-Canadian series. As these two patients were younger at the time of study, it can be hypothesized that peripheral neuropathy may appear later in the course of the disease. Eye examination, including slit-lamp study of the anterior chamber, excluded Kayser–Fleischer ring. Osteoporosis was detected at skeletal X-ray. Hair examination revealed occasional trichorrhexis nodosa and irregular hair shaft calibre. All investigated patients presented mild elevation of very long chain fatty acids with normal levels of phytanic and pristanic acids. Further studies in blood (e.g. cell count, amino acids, essential fatty acid, zinc, homocysteine, folic acid and vitamin B12) and urine (amino acids, organic acid) were normal.

Besides the known features of MEDNIK phenotype, all presented with brain atrophy. In two patients, symmetrical T2 hyperintensity of basal ganglia, mainly involving caudate and putamen, were detected at MRI. A novel sign recorded in all subjects was the presence of liver disease. Liver disease was characterized by hepatomegaly, increased transaminases with signs of intrahepatic cholestasis as indicated by raised total bile acids, AST/ALT (aspartate aminotransferase/alanine aminotransferase) ratio >1, and normal levels of gamma-glutamyl transpeptidase in most cases. Alkaline phosphatase was generally increased. Ultrasound disclosed hepatomegaly and increased liver echogenicity. In two patients, liver fibrosis and/or cirrhosis were detected at liver biopsy. Remarkably, the three investigated subjects from the original series shared with the index patient a profound reduction of plasma copper and ceruloplasmin and increase of serum free copper level. We could not study urinary copper excretion over 24 h because of the lack of consent by the parents of living patients.
**AP1S1 mutation affects intracellular copper metabolism**

Intracellular copper metabolism was studied in skin fibroblasts from Patient 1. Fibroblast copper incorporation rate (0.21 μCi of incorporated 64 Cu/mg protein; normal control subjects 0.064–0.075; Menkes disease 0.47–1.05) and copper retention (patient 47%; normal control subjects 31–36%, Menkes disease 92–97%) showed intermediate values between normal control subjects and patients with Menkes disease. Intracellular copper concentration was within normal limits (42.5 ng/mg proteins; control subjects 21.2–46.0), and lower than that observed in Menkes disease (295–321 ng/mg proteins).

**AP1S1 mutation affects mitochondrial cuproenzymes and peroxisomal metabolism**

To determine the possible consequence of the lack of copper incorporation in cuproenzymes, we investigated the expression level of different copper-dependent enzymes in fibroblasts from Patients 1 and 4. As shown in Fig. 3A, marked reduction of COX-II and -IV subunits and superoxide dismutase was observed, whereas the copper unrelated proteins SDH-70 and porin were normally expressed. Consistent with these results, the fibroblasts of Patients 1 and 4 showed a severe reduction of COX activity (Fig. 3B). Marked reduction of PMP70 expression, a protein involved in the transport of long- and branched-chain acyl-CoAs in peroxisomal membrane, was detected in patients’ fibroblasts by immunofluorescence analysis (Fig. 3C).

**AP1S1 regulates the correct intracellular localization of ATP7A**

The subcellular localization of ATP7A was investigated in fibroblasts from Patients 1 and 4 in comparison with a healthy control subject, both in basal condition and after increasing copper concentration to cell culture medium. As shown in Fig. 4A, under basal copper concentration, ATP7A in control fibroblasts showed an extensive co-localization pattern with the trans-Golgi marker p230 (yellow merge signal). In response to copper cell loading, ATP7A translocated to the plasma membrane (Fig. 4B). Interestingly, in AP1S1 mutant fibroblasts we observed that ATP7A protein was mainly localized to the cell periphery and linked to cytoskeletal filaments both in baseline conditions and after increasing the copper concentration (Fig. 4A and B). ATP7A expression in patients’ fibroblasts was quantitatively normal as detected by western blot analysis (data not shown).

In a rescue experiment, with overexpression of wild-type AP1S1 in mutant fibroblasts, we restored the correct co-localization of ATP7A within the trans-Golgi network (yellow merge signal) under basal copper concentration, as well as the physiological transfer to the cytoskeleton and plasma membrane after copper exposure (Fig. 5).
Discussion

We detected severe perturbations of copper homeostasis in a patient with MEDNIK syndrome, a metabolic abnormality previously unrecognized in this disease (Saba et al., 2005; Montpetit et al., 2008). When re-evaluated, the original French-Canadian patients (Saba et al., 2005; Montpetit et al., 2008) confirmed reduced serum copper and ceruloplasmin, increase of serum free copper level and, as with the index patient, previously unreported brain atrophy and colestathic lepathopathy, adding these two new symptoms to the cardinal phenotypic findings of the disease. Patients with MEDNIK syndrome display a unique puzzling picture, combining clinical and biochemical signs of both Menkes and Wilson diseases. Some of the neurological, cutaneous and skeletal symptoms, along with low plasma copper and ceruloplasmin are similar, but milder than those of Menkes disease. Interestingly, erythroderma has recently been reported as a first manifestation of Menkes disease (Galve et al., 2012). On the other hand, hepaticopathy with liver copper accumulation associated with symmetrical T2 hyperintensities of caudate nuclei and putamina at brain MRI, along with increased urinary copper excretion are reminiscent of Wilson disease.

To date, the pathogenetic mechanism underlying MEDNIK syndrome has not been completely understood. AP1S1 codes for the small-subunit σ1A of the AP-1 complex. Four ubiquitous adaptor protein complexes exist, each composed of two large, one medium, and one small subunit, and mediate intracellular trafficking linking clathrin to receptors in coated vesicles (Boehm and Bonifacino, 2002), selectively sorting cargo between cell membrane, trans-Golgi network and endosomal compartments. AP-1 is located in the trans-Golgi network and interacts with the cytoskeleton, modulating the loading of nascent AP1-coated vesicles onto the appropriate microtubule tracks (Orzech et al., 2001).

Some phenotypic changes reminiscent of MEDNIK syndrome were obtained by knocking down ap1s1 expression in zebrafish larvae, which showed altered skin formation, reduced pigmentation, and severe motility deficits (Montpetit et al., 2008). A similar phenotype was reproduced when zebrafish partially knocked down for ap1s1 were investigated in low-copper environmental conditions, indicating the connection of this gene with copper metabolism (Ishizaki et al., 2010).

As copper-related pathways are highly conserved throughout species, the ap1s1 zebrafish model may provide insights to copper-metabolism changes in patients with MEDNIK syndrome. Intracellular copper trafficking is crucial for several processes, including its transport to cupro enzymes, recycling of copper-ATPasers (Lutsenko et al., 2007), and cellular copper detoxification (Jo et al., 2008). The functions of ATP7A and ATP7B can be described as biosynthetic (i.e. the delivery of copper to the secretory pathway for metallation of cuproenzymes) and homeostatic (i.e. the export of copper excess from the cell). These dual functions are associated with their distinct intracellular targeting, depending on copper concentration. Under basal copper conditions, the localization of copper pumps in the trans-Golgi network reflects their role in the delivery of copper to copper-dependent enzymes (Hung et al., 1997). When the intracellular levels are

Figure 4 AP1S1 mutation induces an altered subcellular localization of ATP7A in dermal fibroblasts. Cells were co-stained with antibodies against ATP7A (red) and p230, the trans-Golgi network marker (green), and with Hoechst nuclear counterstain (blue). (A) Under normal copper concentrations, fibroblasts from control subjects displayed colocalization (yellow, merge signal) of ATP7A and p230, whereas the AP1S1 mutant fibroblasts from Patients 1 and 4 (Pt) showed reduced trans-Golgi localization and more diffuse ATP7A signal (red) throughout the cells (arrows). (B) With increasing copper concentration (200 μM), the cytoskeletal ATP7A distribution was increased in control cells (arrows), and the degree of co-localization between ATP7A and p230 was reduced (green signal, merge). Fibroblasts of patients showed a clear and brilliant ATP7A cytoskeletal distribution that did not overlap with the trans-Golgi network (green signal, merge).
The two copper-ATPases sequester copper into vesicles to export excessive copper out of the cell (Lutsenko et al., 2007; Jo et al., 2008). Both proteins show a C-terminal poly-leucine endocytic motif that has been suggested to drive them into the clathrin-mediated endocytic pathway (Petris et al., 1999; Lane et al., 2004; Lutsenko et al., 2007). As the $\sigma$ subunit of AP-1 is considered to interact, in combination with the $\gamma$ subunit, with cargo proteins’ dileucine-based recognition signals (Janvier et al., 2003; Coleman et al., 2005), all of these findings led us to hypothesize that a defect involving AP1S1 would cause changes in copper metabolism by impairing function and trafficking of copper-ATPases (i.e. less retention in the trans-Golgi and more accumulation at the plasma membrane), resulting in the clinical and biochemical features of MEDNIK syndrome. To assess this possibility, we investigated the effects of AP1S1 mutation on intracellular copper metabolism and its relation with ATP7A, the only copper-ATPase expressed in fibroblasts. Our studies showed that AP1S1 regulates the intracellular trafficking of ATP7A, and that

**Figure 5** AP1S1 transfection restores the correct distribution and compartmentalization of ATP7A in fibroblasts of patients with MEDNIK syndrome. AP1S1 transiently transfected patients’ fibroblasts were immunolabelled with antibodies against anti-HA tag (grey), anti-ATP7A (red) and anti-p230 (green), with (+CuCl2) or without (untreated) copper treatment. In untreated AP1S1-transfected cells (grey), the ATP7A localization was homogeneous in the cytoplasm and significantly concentrated in the trans-Golgi cisternae (arrow, yellow merge signal in the inset). After copper treatment, AP1S1-transfected cells (grey) showed the characteristic peripheral and cytoskeletal distribution of the ATP7A protein (arrowheads), as well as a reduced colocalization pattern with the trans-Golgi network network (arrow, green-yellow signal in the inset). Nuclei were counterstained with DRAQ5 (pseudocoloured in blue).
mutation of AP1S1 causes abnormal intracellular copper trafficking with secondary deficiency of copper-dependent enzymes, demonstrating that the pathogenetic mechanism of MEDNIK syndrome is due to a qualitative abnormality of intracellular copper metabolism. Structural similarities of ATP7B with ATP7A (Lutsenko et al., 2007) suggest that impairment of a clathrin-dependent intracellular trafficking may also affect the function of the Wilson’s disease protein, explaining liver copper accumulation and Wilson’s-like phenotype in MEDNIK syndrome.

This would be consistent with emerging knowledge on the role of AP-1 complex in sequestering cargo proteins in certain compartments of polarized cells. Our results may therefore clarify considerable confusion regarding the mechanism(s) of ATP7A/ATP7B intracellular trafficking, which have been suggested as being both clathrin-mediated and clathrin-independent, caveolin-mediated and caveolin-independent, as well as Rac 1-regulated (La Fontaine and Mercer, 2007). It is possible that other trafficked membrane proteins could also be affected by AP1S1 mutations, contributing to the complex pathogenesis of MEDNIK syndrome. In particular, the increase of plasma very long chain fatty acids, a hallmark of peroxisomal disorders, and decreased PMP70 expression in the fibroblasts of patients with MEDNIK syndrome may suggest a role of AP1S1 in peroxisomal function. However, our studies do not allow us to conclude whether these findings are related to the defect of cellular copper trafficking or are the consequence of a putative effect of AP1S1 on the targeting of PMP70 to peroxisomes (Iwashita et al., 2010).

In one patient, liver copper overload was tentatively treated with zinc acetate, a drug recommended to reduce copper intestinal absorption in Wilson disease (Bruha et al., 2011). This approach was successful in reducing copper overload and cholestasis, with clear improvement of the patient’s clinical and neurological conditions. Although this therapy was given only in one patient, it appeared to be effective in improving clinical signs and biochemical abnormalities related to copper overload.

In conclusion, we have shown the functional relationship of AP1S1 with copper homeostasis, helping to elucidate the pathogenetic mechanism of MEDNIK syndrome, a treatable disease that can now be listed among the inherited disorders of copper metabolism in humans.

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Supplementary material

Supplementary material is available at Brain online.

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