LETTER TO JMG

Phenotypic diversity of Menkes disease in mottled mice is associated with defects in localisation and trafficking of the ATP7A protein

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Owing to mutations in the copper-transporting P-type ATPase, ATP7A (or MNK), patients with Menkes disease (MD) have an inadequate supply of copper to various copper-dependent enzymes. The ATP7A protein is located in the trans-Golgi network, where it transports copper via secretory compartments to copper-dependent enzymes. Raised copper concentrations result in the trafficking of ATP7A to the plasma membrane, where it functions in copper export. An important model of MD is the Mottled mouse, which possesses mutations in Atp7A. The Mottled mouse displays three distinct phenotypic severities: embryonic lethal, perinatal lethal and a longer-lived viable phenotype. However, the effects of mutations from these phenotypic classes on the ATP7A protein are unknown. In this study, we found that these classes of mutation differentially affect the copper transport and trafficking functions of the ATP7A protein. The embryonic lethal mutation, Atp7a^{mo11H} (11H), caused mislocalisation of the protein to the endoplasmic reticulum, impaired glycosylation, and abolished copper delivery to the secretory pathway. In contrast, the perinatal lethal and viable mutations, Atp7a^{moMac} (Macular) and Atp7a^{moVbr} (Viable brindle) both resulted in a reduction in copper delivery to the secretory pathway and constitutive trafficking of the ATP7A protein to the plasma membrane in the absence of additional copper. In the case of Viable brindle, this hypertrafficking response was dependent on the catalytic phosphorylation site of ATP7A, whereas no such requirement was found for the Macular mutation. These findings provide evidence that the degree of MD severity in mice is associated with both copper transport and trafficking defects in the ATP7A protein.

Copper is critical for the function of enzymes involved in connective tissue formation, oxidative phosphorylation, antioxidant defence, catecholamine production and pigmentation.[1] This essential role of copper in humans is underlined by the X-chromosome linked disorder of copper homeostasis, Menkes disease (MD), which is caused by mutations in the copper-transporting P-type ATPase, ATP7A (or MNK).[2] This disorder is characterised by neurodegeneration, failure to thrive, developmental delay, and skeletal and connective tissue abnormalities. The ATP7A protein is a key regulator of cellular copper homeostasis, and performs two critical functions. The first involves the supply of copper to copper-dependent enzymes within the trans-Golgi network (TGN), where the ATP7A protein is normally localised.[3 4] The second is copper export, which occurs when raised copper concentrations stimulate the trafficking of the ATP7A protein from the TGN to vesicles and the plasma membrane.[3] The copper deficiency symptoms in MD arise due to defects in ATP7A-dependent copper export from enterocytes into the blood. In enterocytes, copper stimulates the trafficking of ATP7A to the basolateral membrane, which is probably required for transporting dietary copper into the bloodstream.[5] This defect in copper export is not restricted to enterocytes, as it is observed in a variety of cultured cells from patients with MD.[6 7] In addition, there are defects in the transport of copper to enzymes migrating through the TGN.[8–12] Given the bifunctional nature of the ATP7A protein, MD can conceivably arise from ATP7A mutations that affect copper transport into the TGN, copper export from cells, or the trafficking of the protein that regulates the interplay between these two functions. In agreement with this hypothesis, we and others have identified certain mutations that abolish copper-induced trafficking of the protein, with no effect on copper transport function,[13 14] whereas other mutations impair both functions.[15]

Despite progress in understanding the function and regulation of ATP7A, it remains unclear how defects in the ATP7A protein correlate with the severity of MD. An important experimental model that exhibits clinically relevant forms of MD is a collection of mutant mice, named the Mottled mice because female heterozygotes display varying degrees of hypopigmentation of the fur. Mottled mice carry mutations on the X-linked *Atp7a* gene, which shares >90% identity at the amino acid level with the human ATP7A protein.[16] Affected male hemizygotes belong to one of three classes of phenotypic severity: (1) embryonic lethality; (2) postnatal lethality within a few weeks; and (3) postnatal lethality within a few months. The phenotypic diversity of these alleles provides a valuable resource for understanding the molecular basis of MD in humans. However, despite the identification of missense mutations in mice belonging to each phenotypic class, the consequences of these mutations on the transport and trafficking functions of the ATP7A protein are unknown. In this study, we investigated the consequences on ATP7A trafficking and function of the Mottled mutations 11H (embryonic lethal),[17] Macular (perinatal lethal),[18] and Viable brindle (viable)[17] mutations. The 11H mutation resulted in the mislocalisation of the ATP7A mutation to the endoplasmic reticulum, impairment of glycosylation and prevention of copper delivery to the secretory pathway. In contrast, the Macular and Viable brindle mutants impaired, but did not eliminate, copper delivery to the secretory pathway. Surprisingly, however, both these mutations also resulted in constitutive trafficking of the ATP7A protein to the plasma membrane, although via different mechanisms. These findings demonstrate that the severity of MD in mice is correlated with

Abbreviations: ER, endoplasmic reticulum; MD, Menkes disease; MEM, minimal essential medium; PDI, protein disulphide isomerase; TGN, trans-Golgi network

the extent of defects in both transport and trafficking of the ATP7A protein.

METHODS

Reagents and cell culture

All reagents and chemicals were from Sigma Chemical Co. (St Louis, Missouri, USA) unless otherwise stated. Cells were cultured in minimal essential medium (MEM; Gibco BRL) supplemented with 10% fetal bovine serum, penicillin and streptomycin in 5% CO_2, at 37°C.

Plasmids and transfections

The Mottled mutations were generated by PCR, verified by DNA sequencing and reconstituted into the full-length *ATP7A* construct, pCMB344, as described previously.[15] Plasmid constructs bearing the wild type or Mottled mutant cDNAs were transiently transfected into Me32a cells (LipofectAMINE 2000; Gibco BRL), according to the manufacturer's instructions. Plasmid constructs expressing the mutation D1044E, 875TGE-AAA have been described previously.[15] Transfection efficiencies were typically 20% of Me32a cells, and images shown are representative of the population of transfected cells.

Tyrosinase activity assay

Tyrosinase activity was determined using a colorimetric assay based on the enzyme's dopamine oxidase activity, as previously described.[8] Me32a cells were pre-grown overnight in 25 cm^2 flasks, and LipofectAMINE 2000 was used to transiently transfect these cells with 2 μg of the tyrosinase plasmid, pcTYR, together with 2 μg of the various ATP7A expression plasmids (indicated in the relevant figure legends). After culturing for 48 h, cells were harvested by scraping in phosphate-buffered saline, pelleted and sonicated in a buffer containing 2% sodium dodecyl sulphate, 62.5 mmol/l Tris-HCl (pH 6.8), protease inhibitor cocktail (Roche), 1 mmol/l ascorbate and 1 mmol/l bathocuproine disulphonate to chelate free copper. Cell lysates (20 μg) were fractionated by non-reducing 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and tyrosinase activity was colorimetrically determined by incubating gels for 15 min at 37°C in 10 mmol/l phosphate buffer (pH 6.8) containing 1.5 mmol/l L-3,4-dihydroxyphenylalanine and 4 mmol/l 3-methyl-2-benzothiazolinone hydrazone (Sigma). Immunoblotting experiments were performed using either sheep anti-ATP7A antibodies, or goat anti-tyrosinase antibodies (Santa Cruz Biotech, Santa Cruz, California, USA), using an enhanced chemiluminescence detection kit (Roche, Nutley, New Jersey, USA). Relative tyrosinase activity was determined by measuring band intensities using NIH Image J software. Tyrosinase experiments were performed four times with similar results to the gel shown.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described using affinity-purified ATP7A antibodies,[15] and detected using Alexa488 anti-rabbit secondary antibodies (Molecular Probes, Carlsbad, California, USA). Anti-protein disulphide (PDI) antibodies were from Stressgen Biotechnologies.

RESULTS

Localisation of the Mottled mutants

We began this study by engineering each missense Mottled mouse mutation from the three different phenotypic classes into the human ATP7A cDNA expression plasmid (figure 1). Because the human and murine ATP7A proteins share >90% identity,[16] and the amino acid affected by each Mottled mutation is fully conserved in the human protein, we used the human ATP7A protein to characterise the effect of each murine mutation. A fibroblast cell line, Me32a, derived from a patient with MD lacking endogenous ATP7A,[19] was transiently transfected with each Mottled mutant construct to determine the effect of these mutations on the localisation of ATP7A. Using immunofluorescence microscopy, the intracellular localisation of the wild type ATP7A protein (ATP7A-Wt) was found to overlap closely with that of the TGN marker protein, p230 (figure 2). In contrast, the location of ATP7A harbouring the Macular mouse mutation, ATP7A-Mac, was only partially retained in the TGN and this protein was also distributed to post-Golgi compartments and the plasma membrane. Remarkably, a similar shift to post-Golgi compartments was also observed for the Viable brindle mutant, ATP7A-Vbr (figure 2), which only partially overlapped with p230 in TGN. The embryonic lethal mutation, ATP-11H, resulted in a pronounced mislocalisation to a diffuse reticular distribution around the nucleus and throughout the cytoplasm. This distribution was suggestive of the endoplasmic reticulum (ER). These findings suggest that the Macular, Viable brindle and 11H Mottled mutations each result in defective localisation of the ATP7A protein.

The exposure of Me32a cells to raised copper concentrations resulted in the expected trafficking of wild-type ATP7A from the TGN to the plasma membrane (figure 3). The same post-Golgi distribution of ATP7A-Vbr and ATP7A-Mac proteins in basal medium was also observed in copper-supplemented medium. Furthermore, there was no change to this distribution of the ATP7A-Vbr or ATP7A-Mac proteins when copper availability in the basal medium was lowered using the copper chelator, bathocuproine disulphonate (data not shown). As shown in figure 3, the ER-like distribution of the ATP7A-11H mutant protein was not altered by 100 μmol/l copper (figure 3), or a range of higher copper concentrations up to 500 μmol/l (data not shown).

The 11H mutation causes retention of ATP7A in the endoplasmic reticulum

Because the basal localisation of the ATP7A-11H mutant protein was reminiscent of the ER, we sought to verify this by colocalisation experiments with the ER marker protein, PDI. As shown in figure 4A, the localisation of the ATP7A-11H mutant protein in transfected Me32a cells overlapped with the PDI protein, confirming that the 11H mutation resulted in mislocalisation to the ER. Western blot analysis revealed that the ATP7A-11H mutant protein migrated faster than the wild type ATP7A protein, suggesting a defect in glycosylation (figure 4B). This was confirmed upon treatment of cell lysates with the endoglycosidase, N-glycanase (PNGase), which had no effect on the apparent molecular weight of the ATP7A-11H protein, whereas the apparent molecular weight of the ATP7A-Wt protein was reduced to that of the ATP7A-11H protein (figure 4B). Taken together, these findings suggest that the 11H mutation results in mislocalisation of the ATP7A protein in the ER and prevents its glycosylation.

Mislocalisation of Viable brindle, but not Macular mutant to post-Golgi compartments requires the ATP7A phosphorylation site

The effect of both Macular and Viable brindle mutations on the constitutive post-Golgi localisation of ATP7A was reminiscent of the effect of mutations that have been shown to result in hyperphosphorylation of ATP7A.[15] Like all P-type ATPases, the ATP7A protein is transiently autophosphorylated at a specific aspartic acid (D1044) during the catalytic cycle[20] and autophosphatase activity is required to complete the catalytic cycle of ATP7A. We have recently shown that mutations within the phosphatase domain, 875TGE-AAA, which prevent

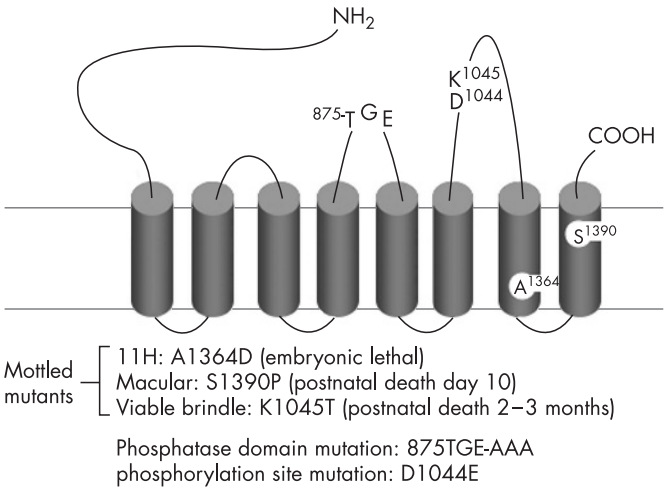

Figure 1 Schematic illustration of the ATP7A protein with the locations of the Mottled and catalytic domain mutations investigated in this study.

dephosphorylation of the ATP7A protein, also result in constitutive trafficking of the ATP7A protein to the plasma membrane.[15] This constitutive trafficking effect of the 875TGE-AAA mutation was confirmed by immunofluorescence (figure 5). We reasoned that the post-Golgi trafficking phenotype of the Vbr and Mac mutations might be due to hyperphosphorylation of the ATP7A protein, similar to the effect of the 875TGE-AAA mutation. Because of limitations in ATP7A transfection efficiencies in the Me32a fibroblasts, direct analysis of Vbr and Mac phosphorylation status was not possible. However, to circumvent this problem, we undertook genetic analysis to examine the role of phosphorylation in the post-Golgi localisation phenotype of these mutations. This was achieved by assessing whether mutation of the aspartic acid phosphorylation site of ATP7A could suppress the post-Golgi localisation of the Vbr and Mac phenotypese. As shown previously, mutation of the phosphorylation site (D1044E) does not affect the normal localisation of ATP7A in the TGN; however, combining the D1044E mutation with the 875TGE-AAA mutant resulted in suppression of the post-Golgi localisation of this double mutant protein (figure 5). Significantly, the introduction of the D1044E mutation into the ATP-Vbr protein also suppressed the post-Golgi mislocalisation (figure 5), confirming the importance of this phosphorylation site for post-Golgi localisation of the Viable brindle mutant. This result was in marked contrast to the Macular mutation, for which the D1044E mutation failed to prevent the post-Golgi mislocalisation of the ATP7A-Mac protein (figure 5). These findings suggest that the post-Golgi localisation of the Viable brindle mutation requires the catalytic phosphorylation site, D1044, whereas the localisation of the Macular mutation does not.

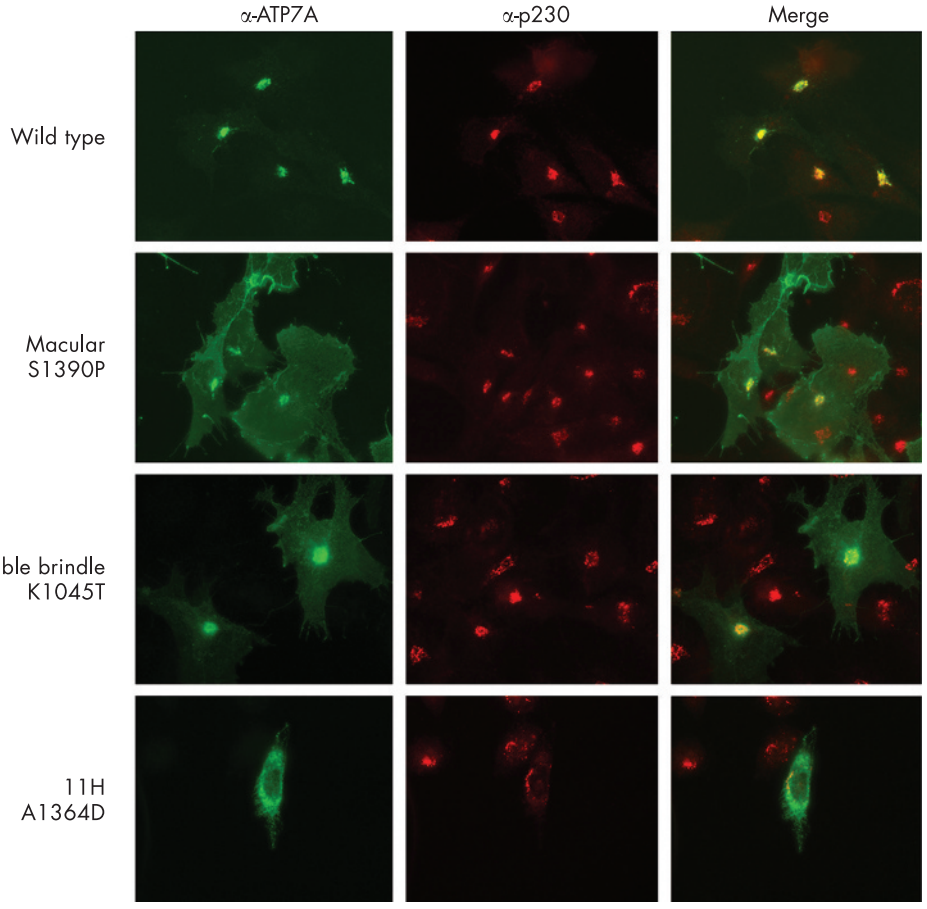

Figure 2 Immunocytochemical localisation of the Mottled mutant proteins using immunofluorescence microscopy. Me32a cells were transiently transfected with plasmids harbouring each mutation within the *ATP7A* cDNA as indicated. Cells were fixed 24 h post-transfection, permeabilised and probed with anti-ATP7A antibodies followed by Alexa-488 anti-rabbit antibodies (green). The location of the trans-Golgi network marker protein, p230, was determined using anti-p230 antibodies followed by anti-mouse Alexa 594 antibodies (red). Regions of co-localisation between ATP7A and p230 are shown in merged panels as yellow labelling.

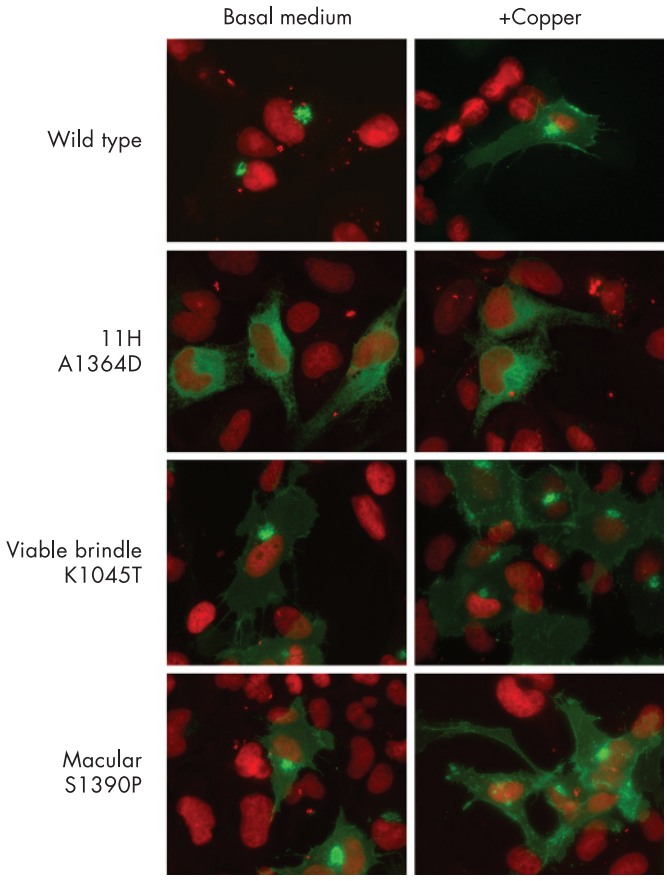

Figure 3 Effect of copper on the localisation of the Mottled mutant proteins. Immunofluorescence microscopy was used to assess the localisation of each Mottled mutant protein transiently expressed in Me32a cells in the presence or absence of 100 μmol/l $CuCl_2$ for 6 h. Cells were fixed 24 h post-transfection, permeabilised and probed with anti-ATP7A antibodies followed by Alexa-488 anti-rabbit antibodies. Nuclei were stained red using propidium iodide.

Complementation analysis by Mottled mutants of fibroblasts from patients with MD

We further characterised the Mottled mutants by assessing their ability to transport copper into the trans-Golgi network to the copper-dependent enzyme tyrosinase. We have previously shown that copper delivery to tyrosinase is dependent on ATP7A protein activity.[8] This is shown in figure 6A, where transfection of the tyrosinase expression plasmid pcTyr into the Me32a cells failed to restore tyrosinase activity, whereas coexpression of both ATP7A-Wt and tyrosinase in these cells resulted in tyrosinase activation. However, the ATP7A-Mac and ATP7A-Vbr proteins were only partially able to restore copper delivery to tyrosinase relative to ATP-Wt (figure 6A). This finding was in marked contrast to the ATP7A-11H protein, for which tyrosinase activation was absent; however, the relatively low expression of the ATP7A-11H protein in Me32a cells added a confounding variable that made this result inconclusive (figure 6A). To control for this variable, we assessed tyrosinase activity in cells expressing increased levels of the ATP7A-11H protein relative to wild-type ATP7A. This was achieved by altering the amount of the individual expression plasmids used in transfection experiments. As shown in figure 6B, the ATP7A-11H mutant protein was unable to activate tyrosinase even when expressed at higher levels than ATP7A-Wt. Taken together with earlier results, these findings suggest that the Mottled 11H mutation causes mislocalisation to the ER and prevents copper delivery to the secretory pathway.

DISCUSSION

In this study, we investigated how Mottled mutations of varying phenotypic severity affect the copper transport, localisation and trafficking functions of the ATP7A protein. Mutations were investigated from three phenotypic classes: the embryonic lethal 11H mutation, the perinatal lethal Macular mutant and the longer-lived viable mutant, Viable brindle.

Unlike the embryonic lethal 11H mouse, Macular and Viable brindle mice survive gestation and die within a few weeks or months of birth, respectively. It was therefore considered likely that these mutations result in less severe consequences on the function of the ATP7A protein. Consistent with this hypothesis, the Macular and Viable brindle mutations failed to abolish copper transport to tyrosinase. This finding reveals a positive correlation between the impairment of ATP7A copper transport activity and the phenotypic severity of MD. An unexpected finding of our studies was that all three Mottled mutations affected the intracellular distribution of the ATP7A protein. Again, there was a direct correlation between the extent of ATP7A mislocalisation and disease severity in the Mottled mice. The embryonic lethal 11H mutation resulted in retention of ATP7A in the ER and impaired its glycosylation. The 11H mutation replaces alanine with a positively charged aspartic acid residue in the seventh transmembrane domain.[17] Such a non-conservative substitution was predicted to alter the conformation of this domain and probably accounts for its mislocalisation in the ER. Neither the addition of copper to cells, nor the lowering of the cell-culturing temperature (data not shown), corrected the mislocalisation of the 11H mutant protein. Because copper is essential for embryogenesis, our findings suggest that the embryonic lethal phenotype of $Atp7a^{mo11H}$ hemizygous mice is probably a consequence of

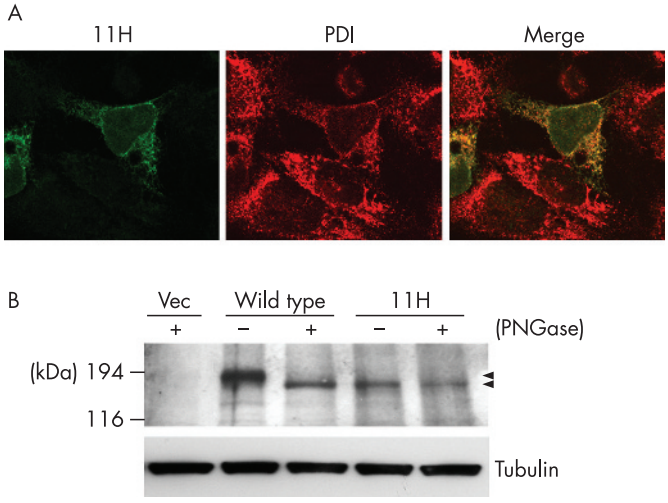

Figure 4 Analysis of the 11H mottled mutation on the ATP7A protein. (A) Co-localisation of the 11H Mottled mutant protein with endoplasmic reticulum marker, protein disulphide isomerase (PDI). Me32a cells were transiently transfected the 11H mutant expression plasmid and then fixed and permeabilised 24 h post-transfection. Cells were probed with anti-ATP7A antibodies followed by Alexa-488 anti-rabbit antibodies (green) and antibodies against PDI, and visualised with anti-mouse Alexa 594 antibodies (red). Regions of co-localisation between ATP7A and PDI are shown in the merged panel (yellow). (B) Impaired glycosylation of the Mottled 11H mutant protein. Immunoblot analysis was used to demonstrate the lower apparent molecular weight of the 11H-ATP7A protein in transiently transfected Me32a cells. Cell lysates were also treated for 1 h at 37°C with 0.2 U of the endoglycosidase PNGase F, which cleaves N-linked glycosyl moieties. This treatment increased the mobility of the wild-type ATP7A, in contrast to the 11H protein whose mobility remained unchanged. Tubulin protein levels were detected in parallel to indicate protein loading (lower panel).

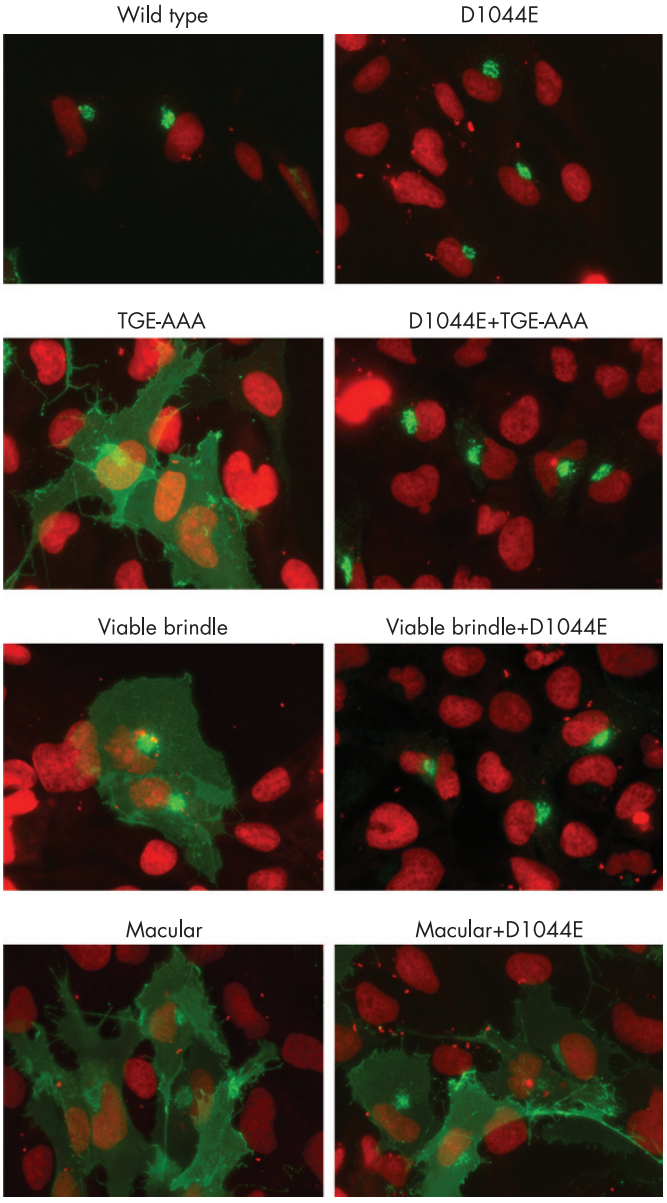

Figure 5 Analysis of the Asp-1044 phosphorylation site for post-Golgi localisation of the Macular and Viable brindle mutant proteins. Immunofluorescence microscopy was used to determine the localisation of ATP7A proteins containing either single or double mutations as indicated. Me32a cells were transiently transfected with plasmid constructs, allowed to recover for 24 h in basal medium, fixed, permeabilised and probed with anti-ATP7A antibodies followed by Alexa-488 anti-rabbit antibodies. Note that the mutation of the aspartic acid 1044 abolishes the post-Golgi staining of the Viable brindle mutant protein, but not the Macular mutant protein. Nuclei were stained red using propidium iodide.

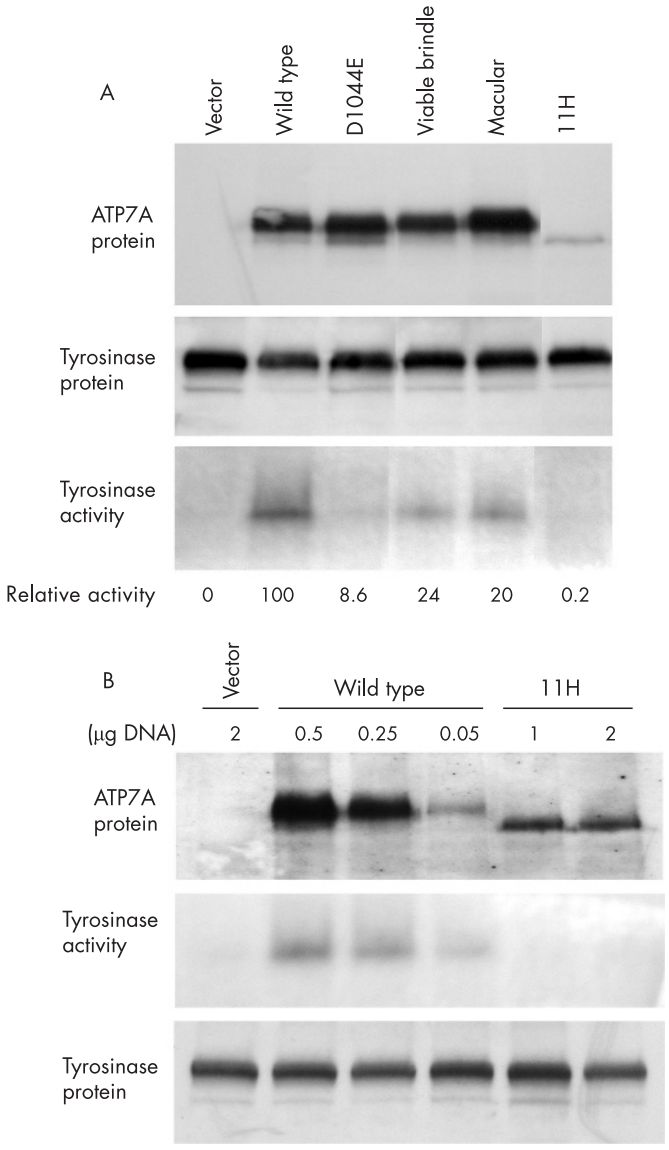

Figure 6 Differential ability of Mottled mutants to complement deficient tyrosinase activity in fibroblasts from patients with MD. (A) The ability of Mottled mutant proteins to activate tyrosinase in fibroblasts from patients with MD. Me32a cells were co-transfected with the tyrosinase expression plasmid, pcTYR and either vector alone (lane 1), or the ATP7A expression plasmids for Wt-ATP7A (lane 2), D1044E (lane 3), Viable brindle (lane 4), Macular (lane 5), or 11H (lane 6). The upper panel is an immunoblot showing the corresponding levels of each ATP7A mutant protein in the transfected cells detected using affinity-purified ATP7A antibodies. The middle panel shows the bands produced by the oxidation of L-dopamine by tyrosinase in transfected cells. The lower panel is an immunoblot showing the corresponding levels of transfected tyrosinase protein in each sample. Percentage tyrosinase activity was determined against ATP-Wt and normalised against ATP7A level and tyrosinase protein levels. (B) Further analysis of the ability of 11H mutant protein to activate tyrosinase protein under varying levels of expression relative to ATP7A-Wt. Tyrosinase activity assays were performed as described in (A); however, the amounts of ATP7A expression plasmid were varied as indicated to allow different levels of ATP7A-11H and ATP7A-Wt proteins to be expressed in Me32a cells.

mislocalisation and failure to transport copper across the placenta and within the developing embryo.

Both the Macular and Viable brindle mutations shifted the steady-state equilibrium of ATP7A towards post-Golgi compartments, which was reminiscent of the effect of hyperphosphorylation mutations, such as those that destroy the phosphatase domain (875TGE).[15] This finding highlighted the possibility that the post-Golgi mislocalisation of Macular or Viable brindle mutants may occur via hyperphosphorylation of ATP7A. This hypothesis was supported by the finding that the post-Golgi mislocalisation of the Viable brindle mutant was dependent on an intact phosphorylation site, whereas the Macular mutation was not. Moreover, the Viable brindle mutation substitutes a lysine for threonine immediately adjacent to the aspartic acid that is specifically phosphorylated during catalysis. Such a change may interfere with the dephosphorylation of ATP7A, thereby extending the duration of phosphorylation of the protein and consequently, the frequency of its exocytic trafficking from the TGN. The absence

Key points

- Mutations in the Mottled mouse result in mislocalisation of the ATP7A protein.
- The phenotypic severity of Mottled mutations is correlated with impairment of ATP7A-dependent copper transport.
- Post-Golgi localisation of ATP7A caused by Viable brindle and Macular mutations occur via distinct mechanisms.

of a requirement for aspartic acid 1044 for the post-Golgi localisation of the Macular mutant protein suggested that this mislocalisation process is independent of catalytic phosphorylation. The Macular mutation occurs in the eighth transmembrane domain (S1390P),[18] which may be critical for its retention in the TGN by preventing secretion of ATP7A via bulk flow to the plasma membrane.

Despite the finding that Viable brindle and Macular mutants have similarly impaired copper transport activity and localisation of the ATP7A protein, the life expectancy of the Atp7a^{moMac} mice is a few weeks of birth,[21] whereas Atp7a^{moVbr} mice survive to a few months of age. In addition to these differences in longevity, Atp7a^{moVbr} mice exhibit predominantly connective tissue abnormalities that are thought to arise from reduced activity of copper-dependent lysyl oxidase.[22] In contrast, Atp7a^{moMac} mice display neurological defects such as seizure and neurodegeneration.[23] The reasons for these differences are not clear. One possibility is that the Macular and Viable brindle mutations differentially affect trafficking to basolateral membranes in polarised cells, especially the endothelia of the blood brain barrier, where ATP7A is required for copper transport into the central nervous system.[24 25] Alternatively, recent studies of hippocampal neurons have indicated a role for ATP7A in providing copper for the nitrosylation of the NMDA receptor in order to suppress neuronal death via excitotoxicity.[26] This process requires post-Golgi vesicular trafficking of the ATP7A protein in a manner that is independent of copper.[27] Such a process may be specifically defective in Macular mice, thus giving rise to neurological disease.

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