Chapter 8

Summary and Perspectives

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Summary

The ability of cells to form two separate membrane domains (ie. apical and basolateral), and to specifically target certain proteins to a particular domain, is required for the proper functioning of many tissues. Disruption of the processes that enable this essential feature can lead to a wide variety of different diseases. The focus of this thesis are two such diseases, namely microvillus inclusion disease (MVID) and Wilson disease (WD), the symptoms of which are (primarily) caused by a failure in the apical targeting of proteins. In the case of microvillus inclusion disease, mutation of the myosin Vb motor protein disrupts the protein trafficking machinery, leading to compromised delivery of multiple trans-membrane proteins. In a large subset of Wilson’s disease patients, mutations in the gene that encodes the ATP7B copper transporter, result in the impaired transport of this protein from the ER to the trans-Golgi, (Huster et al., 2003; Payne et al., 1998) and thereby also to the apical membrane. The main goal of this thesis was to increase our understanding of how protein trafficking is disrupted in these two diseases, and how this gives rise to disease symptoms.

MVID patients show variability in the type and severity of the symptoms they develop (chapter 3). These include the onset time of the disease, severity of villus- and microvillus atrophy, and development of extra-intestinal symptoms such as intrahepatic cholestasis. An outstanding question on the disease mechanism of MVID is to what extent certain MYO5B mutations affect the disease outcome. That is to say, are particular types of mutations responsible for the development or severity of certain symptoms? Properly addressing questions on patient variability requires a baseline understanding of the effect of general myosin Vb deficiency. To establish this, we generated new MVID mouse model by knockout of the Myo5b gene (chapter 4).

Newborn Myo5b knockout mice showed signs of diarrhea and died during the first 12 hours of life. They appeared to suffer from dehydration and had low glucose levels, which are consistent with the absorption defects observed in MVID patients. Examination of the intestines of Myo5b knockout mice revealed ectopic localization of apical plasma membrane markers, microvillus atrophy and formation of microvillus inclusion bodies. These findings confirm that it is a lack
of functional myosin Vb that is the cause of microvillus inclusion disease. In addition, they show that it is not the presence of mutant Myosin Vb protein that is responsible for the hallmark intestinal symptoms seen in MVID patients. Notably, the intestinal defects could be observed in embryos as well, demonstrating that these defects are tissue-autonomous. Thus we can exclude the possibility that, in MVID patients, these same defects are caused by secondary effects associated with MVID (e.g. prolonged lack of nutrients in the intestine, microbiota disruption or iatrogenic effects). The converse is true for defects that are present in patients, but that were not observed in knockout mouse. Abnormalities in the tissue architecture such as villus atrophy were not present in knockout mice, suggesting this is a complication caused by secondary disease effects, or by the presence of particular myosin Vb mutants. Later in chapter 6, we analysed the liver of these mice, and found that they lacked the hepatic defects that were reported in patient samples. This suggests that, like villus atrophy, defects in the liver of MVID patients which are associated with cholestasis, are not likely to be caused by a general myosin Vb deficiency.

Investigating whether certain Myosin Vb mutations cause or exacerbate symptoms, requires genotype-phenotype correlations. For this purpose, proper documentation of known patient mutations is required, preferably with information on the manifesting symptoms. In chapter 5 we document and discuss all newly reported mutations since 2013 (Velde et al., 2013), that are associated with MVID. An interesting recent development is the discovery of unconventional forms of MVID. The intestine of patients with mutations in the genes encoding syntaxin-3 (STX3) or munc18-2 (STXBP2), displays most of the major MVID hallmarks, including microvillus atrophy, intracellular retention of brush border proteins and microvillus inclusion bodies. Both these proteins are involved in facilitating membrane fusion of vesicles at the apical membrane, and thus, like myosin Vb, are thought to be important for transporting proteins to the apical domain. The overlap between these three proteins in terms of their functional pathway and their resulting phenotypes in case of mutation, has led to the suggestion that a common disease mechanism unites the three genes and their encoded proteins. So far this notion has been investigated in a cellular and a murine model (Knowles et al., 2015; Weis et al., 2016), but not in patients. In this work we show that in two MVID patients, munc-18-2 and syntaxin-3 were ectopically localized as intracellular puncta in enterocytes,
rather than at the apical domain. Similarly, syntaxin-3 was localized as intracellular puncta in enterocytes of patients with mutated munc18-2 (FHL5 patients). Taken together with previous in vitro studies, our results confirm the functional link between myosin Vb, syntaxin-3 and munc18-2 in enterocytes.

While these proteins may be functionally linked in the intestine, this link cannot be generalized to other organs. Extra-intestinal symptoms vary between the three MVID variants. The intrahepatic cholestasis that can develop in MVID patients with MYO5B mutations, has not been observed in the other two variants thus far. To explain this particular symptom, a disease mechanism that is different from the one occurring in enterocytes needs to be considered. The recent discovery of a group of patients with MYO5B mutations suffering from congenital intrahepatic cholestasis, but without conventional intestinal MVID symptoms, can provide useful insights. A total of 26 different MYO5B mutations were described in 17 PFIC patients. MYO5B mutations in patients with isolated cholestasis are more often heterozygous than in MVID patients (though not significantly so). These heterozygous patients with isolated cholestasis and MYO5B mutations often carry at least in one allele, a missense mutation in the motor domain of the protein. Biallelic mutations that lead to a total lack of myosin V protein (e.g. through frameshifts or aberrant intronic splicing), are notably absent in any of the isolated cholestasis patients.

Our work in chapter 6 aimed to elucidate the mechanism through which MYO5B mutations cause intrahepatic cholestasis. It was reported that in MVID patients, the apical transporter protein ABCC2 is mislocalized, and hepatocyte polarity is disrupted (Girard et al., 2014; Gonzales et al., 2017; Schlegel et al., 2018). As mentioned above, we did not observe such hepatic defects in Myosin5b KO mice. ABCC2/MRP2 and structural apical marker radixin were localized in bile canaliculi, indistinguishable from wildtype mice. Similarly, knock-out of MYO5B in hepatic cancer cell line HepG2 or in pluripotent stemcell-derived hepatocytes (hi-Heps), did not induce ectopic localization of ABCC2/MRP2. From these results we can conclude that in hepatocytes, myosin Vb is dispensable for the localization of ABCC2/MRP2 at the bile canaliculi.

To more accurately model the pathophysiological mechanism in patients, we then overexpressed myosin Vb-P660L mutated patient protein in MYO5B knock-out
Chapter 8

HepG2. Strikingly, overexpression of myosin Vb-P660L resulted in the increased intracellular accumulation of ABCC/MRP2 (co-localized with the mutant), when compared to overexpressed wildtype myosin Vb. Our results provide evidence that the P660L mutation is causally linked to the hepatic canalicular defects that are observed in liver biopsies of patients. Taken together with the results on MYO5B knock-out, our findings show that it is not deficiency in myosin Vb that is responsible for hepatic defects, but the presence of a mutated myosin Vb protein. We hypothesized that this effect is somehow caused by the presence of a myosin Vb protein with a non-functional motor domain, but with an intact cargo-binding tail domain. To test this, we generated myosin Vb mutants which lacked the entire motor domain and most of the coiled-coil region, and expressed these in HepG2 cells. This mutant proved even more capable than myosin Vb-P660L in inducing the intracellular accumulation of bile canalicular proteins. These accumulation are collections of vesicles of mostly endosomal identity, containing the endosomal markers rab11, RIP11 and rab8. Interestingly, some trans-Golgi markers were observed in these structures as well, suggesting that these myosin Vb mutants disrupt the transition of vesicles from the trans-Golgi to the endosomal trafficking system.

Further mutagenesis experiments showed that the terminal globular tail domain of myosin Vb is sufficient to induce BC protein accumulation. This inducement is dependent on the binding of this domain to active rab11. Rendering rab11 inactive through overexpression of a dominant negative rab11 mutant, completely abrogated the accumulation phenotype caused by myosin Vb tail domain overexpression. Thus, myosin Vb motor domain mutants cause the intracellular mislocalization of canalicular proteins, through a mechanism that appears to be dependent on an unexpected toxic rab11 gain-of-function. How exactly the binding of mutant myosin Vb tail domain to rab11 leads to the observed phenotype remains unclear. It was recently shown in vitro that the tail domain of myosin Vb induced the homotypic interaction between rab11 proteins located on liposomes, thereby promoting liposome tethering (Inoshita and Mima, 2017). If this process occurs similarly with vesicles in cells, it would provide a compelling hypothetical mechanism for how myosin Vb tail-rab11 interactions could interfere with vesicle trafficking.

In the final experimental chapter of the thesis, the focus is shifted from MVID to
Wilson disease. As previously mentioned, many ATP7B mutations that are carried by WD patients result in the mislocalization of this protein in the ER or other unknown compartments (Huster et al., 2003; Payne et al., 1998). These mutants are not necessarily non-functional however, as many of them have been shown to exhibit significant copper transport activity (Morgan et al., 2004; Rodriguez-Granillo et al., 2008). Thus, it is thought that for many patients, it is the failure of mutant ATP7B to reach the apical plasma membrane, not a lack of protein function, that lies at the heart of WD pathogenesis. Increasing our understanding of how ATP7B trafficking goes awry in WD patients, and finding ways to circumvent this, are promising research directions for finding new therapeutic options. For this purpose, we aimed to develop a new WD disease model, based on the differentiation of patient-derived pluripotent stem cells towards hepatocytes in vitro. However, such a model would only be suitable for studying ATP7B trafficking, if these human induced hepatocytes (hiHeps) form bile canaliculi. In the field of hepatocyte differentiation, it has not been established if hiHeps recapitulate this fundamental property of in vivo hepatocytes. Therefore we first investigated whether hiHeps polarize and form bile canaliculi, using the HUES9 embryonic pluripotent stem cell line.

We generated hiHeps from HUES9 cells through directed differentiation over a 19-day period, which involved the sequential treatment of undifferentiated HUES9 with various growth factors that are known to promote differentiation to the hepatic lineage. The resulting hiHeps showed expression of key hepatic markers HNF4α, AFP, Albumin and alpha-1-antitrypsin, as determined by immunofluorescence and western blot analysis. Additional hepatic hall markers included, the uptake of fluorescent LDL and the presence of large amounts of glycogen visible by electron microscopy. Importantly, hiHeps also formed branching bile canaliculi, which could be visualized by immunofluorescent staining of the hepatic apical markers ABC2/MRP2, BSEP and radixin. These narrow BC lumens could be observed by electron microscopy, were enclosed by tight junctions, and showed formation of microvilli at the apical domain. We then characterized ATP7B trafficking in HUES9-derived hiHeps by exposing the cells to increased copper levels, and determined the localization of endogenous ATP7B. Consistent with previous reports, ATP7B localized in the trans-Golgi at low copper concentration, and was trafficked to the bile canaliculi when copper levels were increased. Thus, we have
Chapter 8

established hiHeps as a novel model for ATP7B trafficking and the study of polarized trafficking in hepatocytes in general.

Next, we generated an iPS line from a Wilson disease patient harboring a H1069Q mutation of ATP7B on one allele, and a non-expressed W779X truncation on the other. This was achieved through Yamanaka factor-based reprogramming of somatic cells derived from urine of the patient. HiHeps derived from this iPS line achieved similar levels of hepatic maturity and BC-formation, when compared to Hues9 or healthy control iPS. In low concentrations of copper, mutant ATP7B was found in the trans-Golgi of patient hiHeps, similar to our results in HUES9 hiHeps. This is striking, since the H1069Q mutation was previously reported to lead to the retention of the mutant in the ER, and not the Golgi. When patient hiHeps were exposed to copper, H1069Q-ATP7B remained in the trans-Golgi and did not translocate to the apical domain. Previous studies had found that treatment with the chemical chaperones phenylbutyrate and curcumin were capable of restoring translocation of H1069Q-APT7B to the plasma membrane. In contrast, we found that addition of these chemical chaperones to patient hiHeps, had no effect on the capability of H1069Q-ATP7B to translocate upon copper exposure.

Finally, we investigated the copper induced translocation of ATP7B in hiHeps derived from MEDNIK syndrome patient iPS. MEDNIK syndrome is a disease caused by mutations in the traffic regulatory gene AP1S1, which shows symptoms of copper metabolism deficiency similar to WD (Martinelli et al., 2013). It has been suggested that AP1S1 is involved in the trafficking of ATP7B, and that the symptoms related to copper metabolism are the result of disrupted ATP7B transport. However, we found that in MEDNIK patient hiHeps, ATP7B behaved indistinguishable from ATP7B in HUES9 hiHeps. In MENDIK hiHeps ATP7B was localized at the trans-Golgi when copper levels were low, and showed ample staining at the canalicular membrane when exposed to copper. Thus, ATP7B translocation to the canalicular membrane is not impaired in MEDNIK patients, and other mechanisms will need to be considered.

We have shown in the final chapter that stem cells can be differentiated to hepatocyte-like cells (hiHeps), which develop functional, branching bile canaliculi. These hiHeps can be used to study polarized trafficking processes, as we exemplify by studying the trafficking of ATP7B in response to copper, in healthy and diseased
cells. This has yielded unexpected new insights in the trafficking mechanism of the common H1069Q mutant, and its responsiveness to treatment with chemical chaperones, which are at odds with previous studies using overexpressed ATP7B and/or immortalized cell lines. Our results highlight the importance of studying endogenously expressed mutant proteins in patient-own cells, with regard to developing and testing new therapeutic strategies.

**Perspectives**

*Microvillus Inclusion Disease*

The work presented in this thesis aimed to increase our understanding of the pathogenesis of microvillus inclusion disease. Specifically on why certain symptoms variably manifest in different patients. The generation of a new knock-out myosin vb mouse model has provided new insights on how myosin Vb deficiency affects mammalian organ systems, without interference from mutant protein or secondary disease complications. We observed no villus atrophy or liver defects in these mice, suggesting that in patients, these symptoms are not primarily the result of loss of myosin Vb. In chapter 8, we investigate the subject of MVID associated liver symptoms, and show that is the presence of mutant myosin vb, and not myosin vb deficiency, that is responsible for defective trafficking of canalicular proteins in hepatocytes. Specifically the presence of a myosin Vb mutant with a defective motor domain, and a functional rab11-binding tail domain. We can speculate that that cholestasis is more likely to occur in patients with a missense mutation in the motor domain.

Should we then advice against a bowel transplant in these patients, in favor of a bowel and liver co-transplant to prevent the development of cholestasis? At this stage, such treatment advice is still premature, and further confirmation of our hypothetical model will be required. The effect of additional motor domain myosin Vb mutants on canalicular proteins will have to be investigated. Preferably using endogenously mutated myosin Vb (by CRISPR-CAS9 technology), or by using hi-Heps generated from patient iPS lines. These methods will ensure that any observed phenotypes are not due to artificially high levels of overexpressed mutant.
While we have shown that mutant myosin Vb protein can cause the intracellular accumulation of canalicular proteins, it is not yet certain if this would ultimately lead to cholestasis. One of these mislocalized canalicular proteins is ABCC2/MRP2. This transporter has a broad substrate specificity and exports various anionic compounds, including divalent bile salts with two negative charges such as sulfated tauro- or glycolithocholate (Trauner and Boyer, 2003). Congenital loss of ABCC2/MRP2 leads to Dubin-Johnson syndrome, which is a relatively benign disorder characterized by jaundice due to failure to export conjugated bilirubin into the bile. This is a different type of cholestasis as seen in MVID patients, which is characterized by build-up of bile salts, resulting in pruritus. Still, Dubin-Johnson patients have increased levels of serum bile salts (Douglas et al., 1980; Kimura et al., 1991; Kimura et al., 1995), and show a lower clearance rate of bile salts from the blood when challenged by oral administration of the secondary bile salt ursodesoxycholate (UDCA) (Hironaka et al., 1981). Similarly, Abcc2-deficient rats show increased bile salt levels in blood serum (Jansen et al., 1985; Paulusma et al., 1996). Thus, while ABCC2 deficiency in itself is not likely to increase bile salt concentrations to pruritus-inducing levels, it could be a contributing factor to this symptom when bile salt circulation is challenged.

The primary transporter of bile acids across the apical domain of hepatocytes is BSEP (Trauner and Boyer, 2003). Patients harboring mutations in the gene encoding BSEP develop progressive familial intrahepatic cholestasis type 2. In contrast to Dubin-Johnson syndrome, the cholestasis that these patients develop is characterized by pruritus and liver failure, in addition to jaundice, which is more akin to the symptoms seen in MVID-associated cholestasis. Like ABCC2/MRP2, BSEP was found to be mislocalized in the liver tissue of MVID patients (Girard et al., 2014; Schlegel et al., 2018). Considering its importance in bile acid transport, it is seems reasonable to speculate that the mislocalization of BSEP is the major driver of MVID-associated cholestasis. We hypothesize that BSEP trafficking to the apical membrane is impaired by the presence of mutant myosin Vb similarly to what we have shown for ABCC2 in HepG2. The inclusion of BSEP localization will be prudent in any further research. We were unable to address the localization of BSEP in this work due to technical difficulties. HepG2 cells do not express BSEP endogenously, and human BSEP cDNA is toxic to E.Coli bacteria (Noé et al., 2002; Stindt et al., 2011), which severely limits conventional molecular biology techniques.
for generating overexpression cDNA vectors. HepG2 cells expressing GFP-tagged mouse BSEP showed only very faint GFP signal at ectopic locations (not shown), suggesting this construct is unstable and/or degraded. Again, the use of hiHeps generated from patient iPSC might circumvent these issues, since BSEP is expressed endogenously in hiHeps. For any further experiments, functional assays for bile acid transport (e.g. by using isotope-labeled or fluorescently-labeled bile acid analogs) would be useful to confirm the link between transporter mislocalization and failure to export bile acids.

In addition, the molecular mechanism on which our proposed model for MVID-associated cholestasis is based, remains to be elucidated. In particular, how does the presence of myosin Vb tail domain induce the clustering of endosomal vesicles through rab11 binding? As mentioned before, the recent discovery that myosin Vb tail domain induced the tethering between rab11-coated liposomes provides a suitable hypothesis for this phenomenon (Inoshita and Mima, 2017). The authors suggest that binding of myosin Vb tail domain induces a homotypic interaction between rab11 molecules on opposing membranes. However, the authors did not do experiments that exclude the possibility of interaction between the tail domains that are attached to rab11. Further research on the biochemical mechanisms of this process will likely be the first step. At the moment it is not clear how the possible occurrence of rab11 homotypic interactions can be proven in cells. Conventional methods for studying protein-interaction in cells like yeast-two-hybrid or FRET are not applicable to studying the interaction of a protein with another molecule of the same protein on an opposing membrane. Discovering the conformational changes and key amino acid residues that are responsible for homotypic interactions between rab proteins will be important for future elucidation of a possible tethering mechanism. If these can be identified, it will allow the testing of tethering-deficient rab11 mutants in combination with myosin Vb tail domain expression.

The discrepancy we demonstrate between knock-out of myosin Vb and tail domain overexpression, has implications beyond the specific topic of myosin Vb function in the liver. The overexpression of myosin Vb tail domain has been widely used in protein trafficking research as a stand-in for myosin Vb deficiency, under the assumption that it acts dominant negatively, competing with endogenous myosin Vb (Fan et al., 2004; Gupta et al., 2016; Hales et al., 2002; Lapierre et al., 2001; Lisé et
al., 2006, 2006; Millman et al., 2008; Nedvetsky et al., 2007; Swiatecka-Urban et al., 2007; Wakabayashi et al., 2005). However, we have shown that overexpression of myosin Vb tail domain cannot be equated with true myosin Vb deficiency. There is no reason to assume that our findings on this discrepancy are specific to the liver. Indeed, we found that overexpression in the intestinal cancer cell line Caco-2 also induced the accumulation of apical markers such as ANO6, but not in Caco-2 MYO5 KO cells (data not shown). While some studies validate their findings from myosin Vb tail domain overexpression with MYO5B knock-out or knock-down, many do not. The interpretation of the results of these studies may have to re-evaluated. Certain trafficking phenotypes which are attributed to a specific lack of myosin Vb function, may in fact be caused by a general disruption of post-Golgi endosomal trafficking.

**Wilson disease**

We have demonstrated for the first time the copper-induced polarized trafficking of endogenous ATP7B in hepatocyte-like cells (hiHeps) derived from pluripotent stem cells *in vitro*. This new model system represents a great improvement in terms of accurately reproducing the *in vivo* context of ATP7B transport, when compared to conventional, cancer cell line based models. This is important, as elucidating the trafficking routes of ATP7B in these conventional models has proven to be challenging. This topic has long been a subject of intensive debate, and various subcellular localizations and trafficking routes of ATP7B have been proposed over the years (Harada et al., 2000, 2005; Hung et al., 1997; Lalioti et al., 2016; Nyasae et al., 2014; Polishchuk et al., 2014; Roelofsen et al., 2000; Schaefer et al., 1999). It is hard to say what exactly can explain the discrepancies between some of these studies, but two likely candidates are differences in the use of cell line models, and in the study of endogenous or exogenous ATP7B. Because of the relative similarity between hiHeps and true hepatocytes, insights gained on ATP7B trafficking in a hiHeps model will be more reliable, and could prove decisive in settling contentious issues. Moreover, studying endogenous ATP7B avoids the risks of introducing misleading overexpression artifacts. While trafficking of wildtype endogenous ATP7B can be studied in hepatic cancer cell lines, endogenous expression of patient mutant ATP7B variants requires labor intensive genome editing. Thus, hi-
Heps are particularly appealing to study the trafficking of mutant ATP7B variants in patient-derived cells, and test potential new treatments.

In recent years, two previously disputed issues on ATP7B trafficking have been settled: 1. ATP7B normally localizes to the trans-Golgi of hepatocytes, and 2. It translocates to the apical canalicular domain when copper levels are increased to above a certain threshold level. What remains contentious is the trafficking route that ATP7B uses to reach the apical domain. Polishchuk and colleagues have demonstrated that copper-induced ATP7B trafficking occurs through lysosomal exocytosis (Polishchuk et al., 2014). However, this was contested by two studies (Lalioti et al., 2016; Nyasae et al., 2014), that determined that ATP7B reaches the apical membrane indirectly, by trafficking to the basolateral membrane first, followed by transcytosis to subapical endosomes and the apical membrane. In addition, Gupta and colleagues had previously reported the involvement of subapical recycling endosomes in the trafficking of ATP7B (Gupta et al., 2016). In hiHeps, we saw clear localization of ATP7B in the LAMP1-positive lysosomes in response to copper treatment, which supports a lysosomal-exocytosis model for ATP7B translocation. Still, it would be useful to investigate if transport through any of the other proposed trafficking routes occurs in hiHeps. A particular interesting difference between these studies is the use of varying Cu concentrations to induce ATP7B translocation. We have not done a comprehensive dose-response experiment with Cu in hiHeps in this study. Such an experiment would be useful to determine whether trafficking routes vary at different copper concentrations, and to establish what concentrations of copper are toxic for hiHeps. The latter will also be essential for studying copper-induced toxicity in WD patient-derived hiHeps.

Our study of the common Wilson disease causing mutant H1069Q-ATP7B in hiHeps yielded interesting new insights into its pathogenicity. We confirm that expression of this mutant is greatly reduced, presumably due to rapid ER-associated protein degradation (ERAD). But unlike reported in in previous studies, H1069Q-ATP7B that escapes this fate is not retained in the ER, but is capable of reaching the trans-Golgi. This pool of H1069Q-ATP7B protein is unresponsive to copper-induced translocation however, adding a second bottleneck to the trafficking of this mutant. This is problematic for new treatment strategies that focus on preventing ERAD of the H1069Q mutants (such as chemical chaperones), as this
will not solve the issue of Golgi-retention. Should we abandon this approach to restoring mutant ATP7B function? Before we can answer this, we need to verify that there is truly no translocation of the H1069Q mutant whatsoever. Considering that fluorescence intensity of ATP7B at the bile canaliculi is not particularly high even in wildtype copper-treated cells, and that patient hiHeps have greatly reduced expression of ATP7B, it is conceivable that we would not be able to detect the translocation of a small percentage of the Golgi-residing mutant H1069Q-ATP7B by immunofluorescence. In this case, if ERAD prevention is sufficiently high, it might be capable of increasing mutant ATP7B translocation to the BC. Before definitively abandoning ERAD-based approaches, it would be helpful to determine their effect, if any, by using functional assays to determine copper metabolism and excretion. These could include the visualization of copper flow with fluorescent copper sensing compounds, or determining tolerance to copper-induced toxicity. Developing such functional assays will also be important for testing other novel treatments that are not based on ERAD prevention. Finally, the H1069Q mutant might be unique in its Golgi-retention phenotype, and perhaps other, less common missense mutations that result in misfolding might respond better to ERAD prevention strategies.

Despite the novel insights gained in our study, the behavior of the H1069Q-ATP7B mutant remains enigmatic. Its apparent Golgi-retention raises a new question: Why is the H1069Q mutant incapable of copper-induced translocation to the BC? Binding of copper to the metal binding domains at the N-terminus induces the phosphorylation of certain key amino acid residues (Hasan et al., 2012). These phosphorylated residues then induce conformational changes that promote the exit of ATP7B from the Golgi. It is not immediately clear how the H1069Q mutation would affect this process. The His$^{1069}$ residue contributes to the ATP-binding pocket, and its mutation is thought to decrease the affinity for ATP and prevent the formation of a catalytic phosphointermediate (Dmitriev et al., 2011). In addition, it decreases the stability of the N-domain, which is likely to contribute to ERAD of the mutant, and possibly also its failure to exit the Golgi. However, the exact biochemical mechanism is unclear at this point. It would be useful to observe the copper-induced Golgi exit of ATP7B mutants which harbour mutations in other ATP-binding pocket residues. Preferably these would be mutations that diminish ATP affinity, but do not affect N-domain stability, in order to determine which of
these two factors affect trans-Golgi exit in the case of H1069Q-ATP7B.

Another aspect of ATP7B trafficking that remains unclear is the involvement of AP1S1. We have determined that are no clear defects in AP1S1 deficient MEDNIK patient-derived hiHeps in terms of ATP7B trafficking to the apical domain. If not through perturbing ATP7B trafficking, how does AP1S1 deficiency result in Wilson disease-like symptoms in MEDNIK syndrome? Perhaps loss AP1S1 of affects other aspects of ATP7B trafficking that are not directly apparent in our translocation assays. Examining translocation through immunofluorescence as we did in this study is not very accurate in terms of quantification (i.e. percentage of total ATP7B protein trafficked to the BC), and is more suited for binary assessments (i.e. whether translocation occurs). Thus, we cannot exclude the possibility that MEDNIK hiHeps have reduced levels of ATP7B at the BC. The dileucine motif of ATP7B with which AP1S1 is thought to interact, is versatile in its function. It has been associated with the proper localization of ATP7B in the trans-Golgi and its recycling at the plasma membrane (Jain et al., 2015; Lalioti et al., 2016). Furthermore, this motif has been reported to sort and retain cargo in lysosomes (Braulke and Bonifacino, 2009), although this has not been determined for ATP7B. Disruption of any of these trafficking processes might diminish the amount of ATP7B that is ultimately present on the BC. Still, this reduction would have to be substantially higher than 50%, as heterozygous carriers of a pathogenic ATP7B mutation show no symptoms whatsoever. We have found no indications for a very large reduction of apical ATP7B in our MEDNIK patient-derived hiHeps by immunofluorescence. Therefore, alternative mechanisms beyond disruption of ATP7B trafficking should be considered to explain the defective copper metabolism observed in MEDNIK patients.

Future work on studying WD with hiHeps should focus on expanding the amount of iPS lines of patients, to encompass a bigger variety of ATP7B mutations. It would be useful to join the iPS lines of separate research groups into a central biobank, or at least to register generated lines into a database that is available online. This would be great resource for the testing of new treatments strategies when they are discovered in more basic disease models or by biochemical studies. This kind of approach can help prevent wasting resources on treatments that will most likely fail in patients, and provide additional support to start clinical trials.
in those treatments that prove effective. Beyond Wilson disease, the approaches outlined in this work can be applied for various other hepatic diseases, particularly those that are congenital or involve hepatocyte polarity (see Treyer and Müsch, 2013). Ultimately, the disease modeling approach may be adapted for therapeutic purposes to generate hepatocytes for transplantation. This would require generating iPS with non-integrative DNA transfer methods, and the use of gene editing technology to correct the faulty gene. Moreover, hepatocytes will need to be generated with high efficiency, and even then, some purification method may be necessary to remove non-hepatocytes from the differentiated cell population. Finally, many safety concerns will need to be addressed (e.g. genome stability, gene editing off-target effects) before actual transplantation can occur. Thus, it seems that we are still many years away until hiHep transplantation becomes a viable therapeutic option. However, the initial steps to achieving this are being made animal models (Carpentier et al., 2014; Chen et al., 2015; Ramanathan et al., 2015; Tolosa et al., 2015; Yusa et al., 2011), and it is with great anticipation that we follow the progress in this field.

References


Summary and perspectives


Summary and perspectives


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