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REVIEW ARTICLE

The ABCG2/BCRP transporter and its variants – from structure to pathology

Balázs Sarkadi[1,2], László Homolya[1] and Tamás Hegedűs[2]

1 Institute of Enzymology, Research Centre for Natural Sciences, Budapest, Hungary
2 Department of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary

Correspondence
B. Sarkadi, Institute of Enzymology, Research Centre for Natural Sciences, 1117 Budapest, Magyar tudosok krt 2., Hungary
Tel: +36-1-382-6684
E-mail: sarkadi@biomembrane.hu

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The ABCG2 protein has a key role in the transport of a wide range of structurally dissimilar endo- and xenobiotics in the human body, especially in the tissue barriers and the metabolizing or secreting organs. The human *ABCG2* gene harbors a high number of polymorphisms and mutations, which may significantly modulate its expression and function. Recent high-resolution structural data, complemented with molecular dynamic simulations, may significantly help to understand intramolecular movements and substrate handling, as well as the effects of mutations on the membrane transporter function of ABCG2. As reviewed here, structural alterations may result not only in direct alterations in drug binding and transporter activity, but also in improper folding or problems in the carefully regulated process of trafficking, including vesicular transport, endocytosis, recycling, and degradation. Here, we also review the clinical importance of altered ABCG2 expression and function in general drug metabolism, cancer multidrug resistance, and impaired uric acid excretion, leading to gout.

Keywords: ABCG2; drug metabolism; drug resistance; gout; molecular dynamics; trafficking; variants

The ABCG2 (BCRP, MXR) protein was discovered as a transporter upregulated and causing multidrug resistance in tumor cells, and at about the same time, it was also cloned from normal placenta [1–3]. As demonstrated in the following years, the key physiological function of this transporter is to extrude endo- and xenobiotics, especially in the physiological tissue barriers, including the brain capillary endothelial cells forming the blood–brain barrier (BBB), the intestinal and kidney tubular epithelial cells, and the chorion villi of the placenta. In addition, ABCG2 is physiologically expressed in the liver canalicular membranes, in the mammary glands, and in various progenitor and stem cells, including pluripotent stem cells [4–8]. A medically important site of expression of ABCG2 is in drug-resistant tumors, especially the so-called tumor stem cells or drug-tolerant persister (DTP) cells [5,9–12].

The wide range of the transported substrates of ABCG2 includes uric acid and numerous conjugated endogenous metabolites, as well as hydrophobic and amphipathic drugs and drug conjugates [4,13,14]. This promiscuous drug recognition and transport made this protein an important player in general ADME-Tox

Abbreviations
4-PBA, 4-phenylbutyrate; BBB, blood–brain barrier; COPII, covered with coatomer II; DPT, drug-tolerant persister; endo-H, endoglycosidase H; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERQC, ER quality control; GWA, genome-wide association; HDI, histone deacetylase inhibitor; MD, molecular dynamics; NBD, nucleotide-binding domain; RI, regulatory insertion; SRP, signal recognition particle; THs, transmembrane helices.

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studies, by now with a requirement to study drug–ABCG2 interactions in preclinical drug development [15,16]; http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm). The presence of ABCG2 in a tumor tissue correlates with an unfavorable prognosis in chemotherapy, as substrates of the transporter include anticancer agents, for example, mitoxantrone, doxorubicin, the active metabolite of irinotecan, topotecan, or methotrexate. Even specific, targeted cellular therapies are often counteracted by this nonselective transporter, since targeted agents such as imatinib, sunitinib, and nilotinib are also ABCG2 substrates [5,17–25]. Variants of ABCG2 with impaired expression and/or transport function have been found as major determinants of gout development, as this transporter is a key player in the intestinal uric acid removal pathway [26–29]. In addition, genetic or epigenetic regulation of the ABCG2 expression may significantly affect drug and uric acid metabolism, as well as tumor therapy response [30].

In the following sections of this review, we present the current state of art regarding the high-resolution structure and dynamics of the ABCG2 protein with a special attention to functional regions, and those affected by mutations and polymorphisms. Polymorphic and mutant ABCG2 variants causing reduced expression and function, or even resulting in a knockout phenotype, have been first recognized in focused investigations of drug metabolism or in genome-wide association (GWA) studies, exploring potential genetic associations with multifactorial diseases. Some of the genetic variants strongly affect cellular processing and trafficking pathways required for the appropriate surface localizations and function of the transporter, as well as its modulation by drug interactions. In the last section, we attempt to summarize the medical aspects and importance of the genetic variants/mutations and the regulation of ABCG2 expression.

Recent structural and molecular dynamic studies on ABCG2

Structure and dynamics of the NBDs and transmembrane regions

The ABCG2 protein is a so-called half ABC transporter that homodimerizes to obtain a functional form in the plasma membrane. The nucleotide-binding domain (NBD) is located at the N terminus of the protein, followed by six transmembrane helices (THs). Topology models indicated that the extracellular loops are relatively short, except EL3, the loop between TH5 and TH6 [31]. In EL3, an N-glycosylation site, N596, is present, and the protomers in the homodimer are covalently bound through cysteines at position 603 [32,33].

The amino acid sequences of the transporters in the ABCG subfamily, outside the conserved NBDs, show no similarity to other human ABC proteins. Because of the lack of sequence homology of TMDs to any ABC protein with a determined structure, no relevant homology models could be built during the 10 years after the first full-length ABC transporter structures were published [34,35]. The length of the sequences corresponding to the ABCG2 transmembrane region suggested a structure similar to bacterial importers with short TM helices, resulting in a close proximity of the NBDs to the inner surface of the plasma membrane. Still, the first reliable ABCG2 structural models [36–38] could only be generated when the ABCG5/ABCG8 heterodimer X-ray structure was determined [39]. Later, several cryo-EM ABCG2 structures confirmed the high structural similarity between the heterodimeric sterol transporter and the homodimeric ABCG2 multidrug transporter [40–43]. These cryo-EM studies revealed various ABCG2 structures in the absence and presence of ATP, substrates, and inhibitors. The first large set of ABCG2 structures was acquired in the presence of anti-ABCG2 antibodies, which helped in structure determination by decreasing the flexibility of the protein [41–43].

The main structural features of ABCG2 are summarized in Fig. 1, and some of them are discussed here in detail. The TM helices of ABCG2 are short and straight, and only TM3 exhibits a significant kink around a Pro residue (P480). In the functional homodimer, TM2, TM5, TM2', and TM5' are located in the center of the TMDs, forming the interface between the two protomers. TM1 interacts with TM2 of the same protomer and with TM5 of the opposite protomer, while TM3, TM4, and TM6 cover the central four helices. The intracellular regions of TMDs in ABCG2 possess important structural properties distinct from so-called 'full' ABC transporters, such as ABCBs or ABCCs. In these full transporters, some of the TM helices cross over to the opposite half, and the intracellular ends of these TM helices are connected with short, so-called coupling helices to the NBDs [35]. In the ABCG2 half transporter, no domain swapping-like conformation is observed, and a coupling helix exists only between TH2 and TH3. The intracellular loop between TH4 and TH5 is too short to interact with the NBD and likely does not leave the membrane bilayer. Interestingly, the so-called connecting helix, which is situated parallel to the bilayer and connected only to TH1, serves a similar function as a coupling helix [39]. This amphipathic connecting helix,

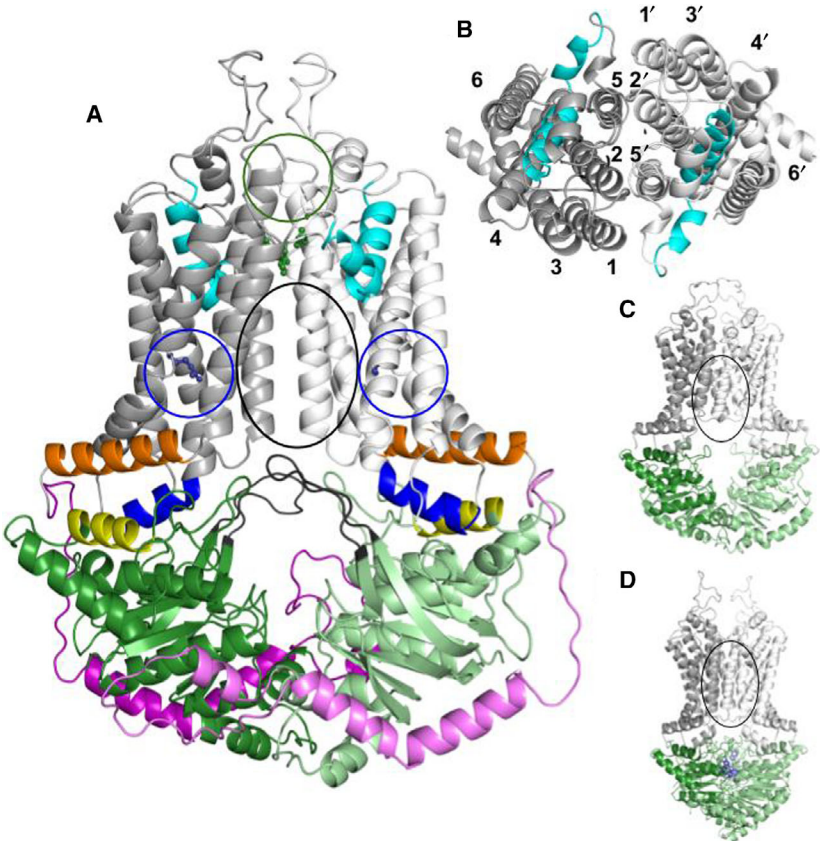

Fig. 1. Conformations of ABCG2 observed by cryo-electron microscopy—potential transport pathway. (A) Side and (B) top views of the inward-facing ABCG2 structure determined in the presence of anti-ABCG2 antibody and in the absence of ATP are shown (PDBID: 6HIJ). Gray: TMD; light gray: TMD′; green: NBD; light green: NBD′; cyan: re-entry G-loop; blue: coupling helix; orange: connecting helix; yellow: Q141-helix; dark gray: RI; violet and raspberry: linker regions; numbers: numbering of TM helices; blue sticks and balls: R482; blue circles: R482 pocket; green sticks and balls: leucine valve; green circle: cavity 2; blue circle: cavity 1 (the central drug-binding pocket). (C) and (D) show ABCG2 structures in the absence of an antibody and ATP (PDBID: 6VXF) and in the presence of ATP (PDBID: 6HZM), respectively. Black circles indicate the lack of cavity 1 in these conformations.

structurally somewhat analogous to the elbow helix of MDR1-like transporters, interfaces with the NBD and provides a second interaction site in addition to the coupling helix (Fig. 1).

The NBD are strongly conserved both at the sequence and structural levels. In ABCG2, the β-subdomain includes a special feature in the form of a longer flexible loop between the first and second β-strands (Fig. 1), at an analogous position to the regulatory insertion (RI) in CFTR NBD1 (discussed in the next section) [44]. The α-helical subdomain is also conserved, and most of the structural deviations among ABCG2 NBD structures arise from the flexibility of the loops. Intriguingly, F142, analogous to F508 in CFTR, interacts with the connecting helix (with amino acids K382 and R383), providing a crucial interface between the TMD and the NBD [45,46]. While CFTR F508 is located at the end of a helix and interacts with a short coupling helix, F142 in ABCG2 is part of a long helix (Fig. 2), which is likely evolved for interacting with the connecting helix, much longer than a coupling helix. Generally, the ATP binding observed in the ABCG2 structure (PDBID: 6HCO) exhibits canonical elements (e.g., the γ-phosphate is coordinated by Q211, corresponding to the catalytic glutamate in the wild-type, the 'switch' histidine H243, and the Q126 located in the Q-loop), but it lacks an A-loop with an aromatic side chain, stacking against the adenine ring of ATP [41,47]. Instead, one side of the adenine moiety interacts with residues V46, I63, and G185 from one NBD, and its other side is oriented toward R184 from the opposite NBD [41]. Interestingly, the residue corresponding to R184 in the bacterial B12 transporter BtuCDF structure [48] also forms a contact with the γ-phosphate. In addition, the hole at the interface of the two NBDs and TMDs is an additional intriguing structural similarity between the distantly related ABCG2 and the BtuCDF proteins [41].

In most of the structures determined in the absence of ATP, that is in an inward-facing conformation, the intracellular ends of the TM helices are not close to each other; thus, the central four helices (TH2, TH5, TH2', and TH5') form the main drug-binding pocket (cavity 1) (PDBIDs: 5NJ3, 6HIJ, 6HCO, 6FEQ, and 6ETI) [41–43]. This pocket is not accessible in the ATP-bound inward-closed structure (PDBID: 6HZM) [41] (Fig. 1). The conformation of TMDs and NBDs is similar in both conformations, and the binding of

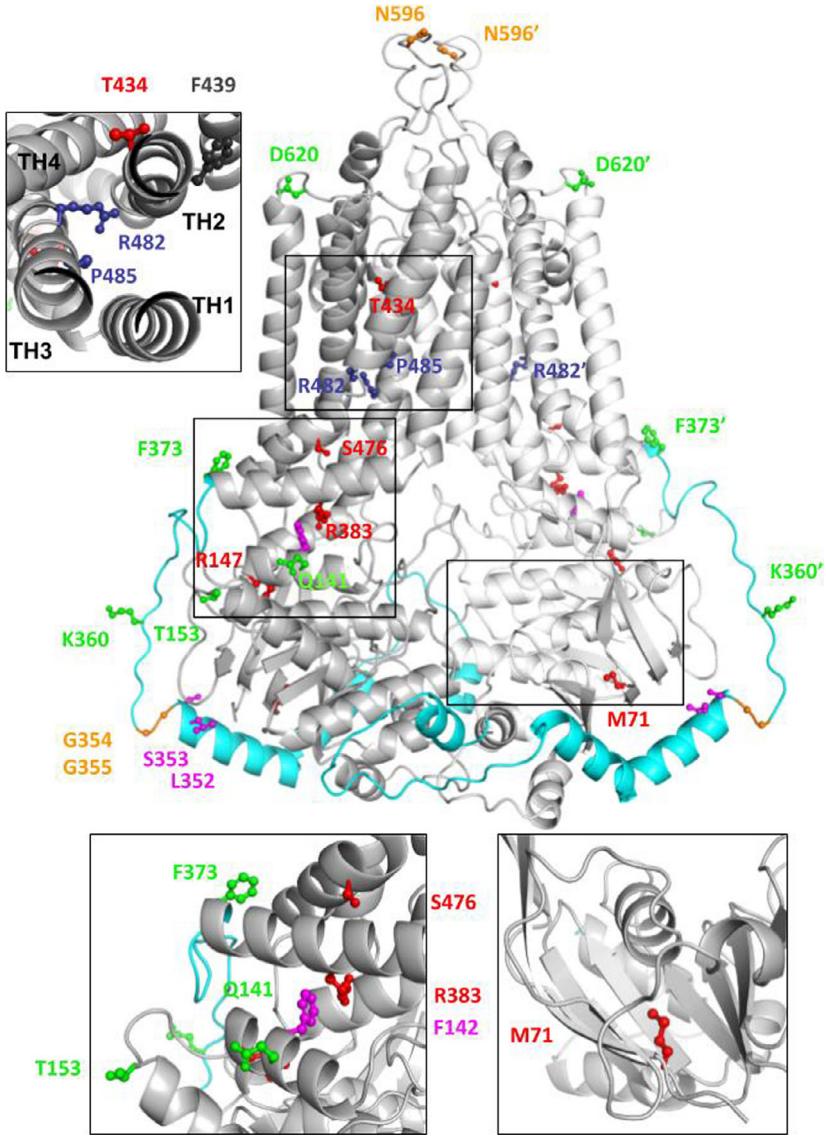

Fig. 2. Localization of functionally important polymorphic variations or mutations in the ABCG2 structure. Location of residues with important amino acid changes is highlighted with stick and ball representation in the context of the inward-facing structure, PDBID: 6HIJ, supplemented with unresolved loops built using Modeller. Gray and light gray: the two protomers; cyan: linker region. Boxed regions are rotated and magnified for visualization purposes and include volumes corresponding to the R482 site (top left), to the TMD/NBD interface (bottom left), and to the NBD β-subdomain (bottom right). Green and red residues label naturally occurring variations with mild and serious effects, respectively. Mild and severe mutations generated for structure–function studies are shown in orange and magenta, respectively. Blue indicates residues which exhibit changes in substrate specificity upon mutations.

ATP seems to cause mostly rigid body motions. Importantly, in contrast to the inward-facing structures of ABCB-like proteins [49,50], the ABCG2 structure acquired in the absence of ATP is not open and the NBDs are in contact. Moreover, the extracellular region of the ATP-bound, inward-closed conformation is not similarly open toward the extracellular region as in the full transporters. Since the contacted NBDs in the absence of ATP and the less opened extracellular region with bound ATP can be observed also in the case of the ABCG5/ABCG8 structure (PDBID: 5DO7) [39], these properties are likely characteristic for the ABCG-like transporters.

A recently published ABCG2 structure, which was determined in the absence of ATP and without the conformation-specific antibody segments, revealed a novel conformation (PDBID: 6VXF) [40]. While the NBDs are open and resemble the inward-facing conformation of the apo-ABCG2 structures determined earlier, the intracellular ends of TM helices are closed, resulting in a lack of cavity 1. Beside the rotation of TH5, the middle of TH2 unwinds, and the incorporated F439, which was reported as a crucial residue for substrate recognition and is part of cavity 1 [41,51], points to the opposite direction from the central axis, toward the R482 site. Interestingly, structures determined in the same study in the presence of substrates and inhibitors under similar experimental conditions (i.e., the absence of a conformation-specific antibody segment) exhibit inward-facing TMDs with

the central binding pocket occupied by a small-molecule (PDBIDs: 6VXH, 6VXI, and 6VXJ).

As to the molecular dynamics (MD) of ABCG2, the first simulations were performed with homology models based on ABCG5/ABCG8. Simulations by Laszlo *et al.* [38] indicated that Lys substitution in the Q141K variant likely results in its electrostatic repulsion from K382 located in the connecting helix, and leading to sporadic decoupling of the TMD/NBD interface. This study also showed that R482 is involved in a potential drug-binding pocket and its mutation to Gly alters the dynamics of TH3 and neighboring helices. Ferreira *et al.* [36] investigated the effects of various mutations and location of cholesterol-binding sites. One of the main messages of their MD simulations was that the linkers are important parts of an allosteric pathway, transmitting the effect of conformational changes between the two NBDs. In addition, in the presence of ATP, the helices of the linkers also propagated allosteric signals toward the TMDs. The authors suggested that the ABCG2 linker region may replace the linker region in the full transporters between the two halves. One may also speculate that the ABCG2 linker provides an allosteric coupling between the two protomers, which in full transporters is provided by the crossed-over TM helices.

Khunweeraphong *et al.* [37] interpreted their experiments also in the context of a homology model. They found that charge-reversal mutations of Lys and Arg in and around the coupling helix (a.a. 451–463) exhibited only a mild effect on mitoxantrone transport, while mutating negatively charged residues in this region abolished that transport function. They demonstrated the importance of salt bridges E451/K473 and R383/E458, thus added the distal part of the NBD/TMD interface [37] to the proximal part, formed by F142 clamped by K382 and R383 [38] (Fig. 2, bottom left inset). Khunweeraphong *et al.* [52], using molecular dynamic simulations with an inward-facing cryo-EM structure (PDBID: 6ETI), also demonstrated experimentally that L555 is indispensable for transport function and mutations of the di-leucine valve (L554/L555) disrupt the seal between cavity 1 (the central large binding pocket) and cavity 2 (the upper cavity). While the increased basal ATPase activity, the increased EC_{50} of E1S-induced ATPase stimulation, and the increased E1S transport activity of the L554A mutant had already suggested the 'leucine plug' as a checkpoint during substrate transport [41], these simulations [52] potentially explain the observed altered transport function and the uncoupling of the ATPase and transport activities at the atomistic level.

Hidden, potentially important regions not visualized in atomic-level structures

Flexible regions in proteins are usually not resolved in either the X-ray or the cryo-EM structures, and such 'invisible' regions also exist within the ABCG2 structure. The first such segment is the N-terminal tail of 32 amino acids, preceding the NBDs. This region is likely intrinsically disordered, based on secondary structure and IUPRED predictions [53]. Two prolines at positions 11 and 21 are present, and since several hydrophobic amino acids are surrounding P11, this region probably interacts with other parts of ABCG2 or the membrane bilayer.

A short segment between the first and second β-strands of the NBD (a.a. 47–60) is also not resolved in any of the structures. Since this location is analogous to the CFTR NBD1 RI [44], and is unlikely to interact with the adenine ring of ATP, in ABCG2 we suggest to call this loop also RI, instead of A-loop, as suggested in Ref. [47]. Our knowledge on the function of RI in CFTR is limited. Albeit the CFTR RI possesses a phosphorylation site (S422), it does not seem to significantly contribute to the phosphorylation-dependent regulation of CFTR [44]. While RI deletion in CFTR results in stabilization of both the isolated NBD1 and the full-length protein, the effects of this deletion on CFTR processing and rescue by various drugs are complex [54,55]. A potential structural analog, the so-called gating helix, was observed in bacterial transporters, such as the O-antigen polysaccharide ABC transporter (PDB ID: 6OIH) [56]. In this protein, the gating helix forms a contact with the lipid bilayer and has an important role in the first steps of substrate transport.

Based on these data, two potential functional roles can be proposed for the ABCG2 RI. First, it may collect cholesterol molecules and participate in cholesterol-dependent regulation of ABCG2 function [57]. Second, RI can cover the lateral side of the substrate entry point, thus regulate the entry of substrate molecules. This potential role of RI in substrate specificity is underlined by the effects of several variants. The R56Q variant (allele frequency: 4.96E-05, dbSNP: rs543249891 gnomAD ID: 4-89060981-C-T) likely has a decreased doxorubicin transport, since R56Q was associated with an increased survival of cancer patients with administered doxorubicin [58]. Another variation in this region, G51C, was reported to exhibit a decreased transport of porphyrin and methotrexate [59,60], although a C55S mutation in this region had an SN-38 transport similar to that of the wild-type [61]. While the effects of CFTR RI on CFTR

processing and interaction with drugs have already been studied [55], the role of ABCG2 RI in trafficking has not been investigated in detail.

In ABCG2, the linker region (a.a. 300–372) between NBD and TMD includes a V-shaped α-helical region (a.a. 326–353) surrounded by unresolved N- and C-terminal loops (a.a. 311–325 and a.a. 354–367). The flexibility of the loop likely allows protein–protein interactions, similarly to several eukaryotic linear motifs, containing sorting and degradation patterns [62,63]. The N-terminal linker loop (likely more flexible than the C-terminal one) can also exhibit conformations which partially cover the substrate entry site, thus influencing substrate entry. The C-terminal linker loop has to avoid clashes with NBD while reaching the connecting helix, and sequence features indicate its restriction at the bilayer (e.g., hydrophobic residues F364 and Y369; a stretch of four lysines between 357 and K360) [63]. As discussed above, the full linker may play an important role in communicating allosteric signals [36].

A suggested pathway and dynamics for substrate transport in ABCG2

The recent paradigm for the substrate binding and translocation of ABCG2 includes two main steps. First, the substrate binds to the central cavity, made possible by the separation of NBDs and the opening of the intracellular ends of TM helices. Second, the substrate passes from cavity 1 (the central binding pocket) to cavity 2 toward the extracellular space, through the di-leucine valve (L554/L555) [43,52,64]. However, this type of a description for substrate binding is likely oversimplified and limits our efforts for understanding substrate recognition and transport.

Importantly, a recent ABCG2 structure in the absence of ATP was determined without a stabilizing antibody, and this structure exhibits closed intracellular ends of TM helices [40]. This structure (PDBID: 6VXF) and the substrate-bound inward-facing conformations (PDBIDs: 6VXH, 6VXI, and 6VXJ) allow to suggest two possible mechanisms of substrate entry to the central binding pocket, cavity 1: (a) There is an equilibrium between the inward-facing and inward-closed TMD conformations and the substrate can enter to cavity 1 if the TM helices are open and this central pocket exists and (b) the substrate is recognized by the amino acids around the R482 site, including F439, pointing toward this site in the 6VXF structure, and facilitates the refolding of the central part of TH2, thus the reorganization of the TH to form cavity 1.

In this latter case, the constant contact between the substrate and F439 is a rational scenario, and F439 may pull and push the small substrate molecule toward to central axis into the *in situ* forming binding pocket. This mechanism may be similar to the induced fit often observed in the case of ligands and receptors, although in a more active manner. Altogether, the observed ABCG2 conformations support the hypothesis that ABCG2-like transporters do not function *via* an alternating access mechanism but perform peristaltic movements to expel the substrate from the intracellular to the extracellular space [50,52].

As to the components of the central binding pocket (cavity 1), currently there are no experimental data demonstrating direct binding of substrates to either RI or R482G, while several *in silico* studies support the assumption that substrates directly interact with these regions and the observed effects are not the results of allosteric effects. The strongest support for nonallosteric effects of mutations in RI is provided by data on the analogous structural element, the gating helix in bacterial transporters. In those cases, the RI analogs were demonstrated to interact directly with transported molecules and participate in the engagement of the substrate with the translocation pathway [56]. Computational studies, including our *in silico* docking [38] and equilibrium molecular dynamic simulations (submitted, http://abcg.hegelab.org), indicate that a physiological transported substrate, uric acid, visits the R482 pocket several times. These observations strongly indicate that the R482 region is an important site along the substrate translocation pathway. In addition, the side chain of N436, which was shown to be essential for estrone 3-sulfate transport [41], is located at the top of the R482 pocket, forming the boundary between the R482 site and the central pocket (cavity 1). Independently from the transport mechanism, the substrate access in the case of the bottom-open conformation is more limited than expected on the basis of the available structures, as the RI region and the N-terminal flexible loop in the linker region cover—at least partially—the opening toward cavity 1 (Fig. 1). In addition, data on several ABCG2 variants and mutants, exhibiting altered substrate specificity, indicate that regions other than the central binding sites have a marked influence on substrate specificity.

We suggest that investigating the role of not only cavity 1 but also the full substrate translocation pathway should lead to a better understanding of substrate recognition and thus predictions of drug–drug interactions. The dynamics of protein regions around the substrate entry site probably determines the affinity of a molecule for the RI and the linker region, which is

required for substrate entry and transport. When engaged, the substrate may visit several pockets (e.g., the R482 site) along the translocation pathway that may be slightly different for various substrates. For example, those molecules, which exhibit no altered transport by an R482G mutant, likely do not visit the R482 site. Even if different molecules traverse through the same physical region of the protein, they may interact with different patterns of amino acids. Interaction of drugs with other sites than cavity 1 formed by the four central helices (TM2, TM2', TM5, and TM5') are supported by ABCG2 structures with inhibitors. For example, the inhibitory properties of the tariquidar-derived MB136 can be explained by the increased binding affinity caused by its interactions with additional regions in TM1 [41], which is likely accessible also for substrates along the transport pathway.

The above notion of patterning is supported by the variable importance of amino acids within cavity 1 in the ABCG2-dependent drug transport. Manolaridis *et al.* [41] identified N436 to be crucial for estrone 3-sulfate transport. In contrast, Gose *et al.* [51] found F439 as the single residue indispensable for drug transport, while mutations of N436 did not affect the translocation of the substrates used in their study. A potential explanation for this discrepancy is that different substrate molecules all have to visit the central binding pocket, while interacting with different sets of amino acids in this region. Accordingly, the proposed multiple ABCG2 drug-binding pockets [65] may not represent different pockets in a classical sense, but alternative interaction surfaces along the translocation pathway with different affinities for various substrates.

Localization of functionally important polymorphic variations or mutations in the ABCG2 structure—role in folding or transport function

Mutations and polymorphic variations are spread all over the ABCG2 protein sequence (see Section 1), and the recently determined structures supplemented with 3D bioinformatics may help to understand their effects. Some of the well-characterized mutations are discussed below and presented in Fig. 2.

V12M [66,67] positioned in the flexible N-tail, and D620N [68] located at the end of EL3, does not have an effect on functional expression of ABCG2. The glycosylation site N596 also resides in this latter extracellular loop. Mutations at this site (N596Q and N596A) do not seriously affect protein folding and trafficking when ABCG2 is overexpressed [33]. However, it has also been shown that N596Q increases the ubiquitin-mediated proteasomal degradation of ABCG2 in a single-copy Flp-In-293 system, and thus, a stabilizing role of the N-glycan was proposed [69].

Mutations at this site (N596Q and N596A) were first reported not to affect protein folding and trafficking [33], while in a more sensitive assay an increased ubiquitin-mediated proteasomal degradation was observed [69]. The effect of K360del, as this deletion is located in the unresolved C-terminal part of the linker region, is also mild. Similarly, amino acids 354 and 355 at the C terminus of the linker helix can be replaced by alanine with no major alterations in the ABCG2 expression or function [70]. In contrast, mutations of L352 and S353, located in a well-defined helical structure, are crucial for the proper functional expression of ABCG2 [70].

Mutations in regions with well-defined secondary structures but at residues positioning their side chains to the solvent are also expected to exhibit relatively mild effects. Examples for this type of alterations include T153M [71,72] and Q141K [66], both located in the NBD. Still, this naturally occurring latter variant has both trafficking and functional impairments. Similarly, the side chain of M71 is buried inside the β-subdomain of the NBD, and the M71V mutation has a negative effect on ABCG2 expression and trafficking. Valine at this position was also found to alter residue contacts as monitored by molecular dynamic simulations. This resulted in a disturbed allosteric communication network, in which residues within a secondary structural unit exhibit less noncorrelated motions when compared to the wild-type protein [73]. The imbalanced dynamics of NBD likely caused an altered NBD folding and diminished domain-domain assembly, similar to that observed in the case of CFTR NBD1 mutants [74]

Amino acid changes in functionally important complex structural regions are frequently detrimental. Mutation R147W [75] disrupts the electrostatic interaction with E199. This likely destabilizes the helix incorporating R147 and interacting with the connecting helix, which is part of the structurally and functionally important NBD/TMD interface. F142 (homologous to CFTR F508) is also located in this helix and is in contact with R383 in the connecting helix [76]. Mutations of these residues (F142del, R383C) and other amino acids in the connecting helix (F373C [75]) most likely destabilize the interface and have major effects on protein folding and function [45,46].

There are two naturally occurring variants, T434M and S476P, which are located in TM helices. Both of these variants abolish transport function [75]. The deleterious effect of S476P is likely related to the effect of this proline residue on the structure and dynamics

of helix backbone. However, this helical region contains several Pro residues (P480 and P485); thus, the structure and dynamics of this helix, associated with function and substrate recognition, are delicately balanced. It has been shown that the P485A variant exhibits a decreased efflux activity for BODIPY-prazosin, but not for mitoxantrone and Hoechst 33342 [77]. Other studies also demonstrated a major role of this residue in transport function [78]. Importantly, R482, which also affects ABCG2 substrate specificity if mutated [79,80], also resides in this region. All these data and *in silico* docking [38] suggest that there is a transiently used binding pocket around R482 along the substrate translocation pathway. Nevertheless, changes in substrate specificity may be caused not only by altered side chains, but also by the altered conformation and dynamics of TM helices upon mutation [38]. These observations suggest that modified substrate recognition may be complex and involve both direct side-chain interactions with the substrates and allosteric mechanisms.

Cellular trafficking of the ABCG2 protein

The role of ER in the folding and processing of ABCG2

Biosynthesis, folding, assembly, and initial posttranslational modifications of membrane proteins take place in the endoplasmic reticulum (ER). Cotranslational translocation is performed by the coordinated action of ribosomes docked by a signal recognition particle (SRP) to the SRP receptor in the ER and the heterotrimeric translocon complex Sec61, which facilitates the membrane insertion and proper orientation of the newly synthesized membrane protein. It has recently been reported that the ER membrane protein complex has an essential role in the correct insertion of the first transmembrane domain, which is a critical step of membrane protein topogenesis [81]. As a result of subsequent folding and assembly, assisted by molecular chaperones in the ER lumen such as the binding immunoglobulin protein (BiP or GRP-78) or calnexin/calreticulin, the newly synthesized membrane proteins attain their tertiary and quaternary structures (see Fig. 3).

During this complex process, multiple errors can occur, resulting in misfolded proteins. These not properly folded species (also called 'toxic conformations') tend to aggregate as being detrimental for normal cellular functions; therefore, their removal is essential. The ER quality control (ERQC) network, also known as the central quality control system, continuously scrutinizes and manages the newly synthesized proteins. Correctly folded conformations, when pass the ERQC, are targeted for export to the Golgi apparatus, and ultimately to the plasma membrane, although they are deferred in the ER for a while. For instance, the half-life time of the CFTR in the ER is 1–2 h [82], whereas that of the ABCG2 protein is about 45 min (L. Homolya unpublished data). As a part of the ERQC network, the endoplasmic reticulum-associated degradation (ERAD) recognizes the misfolded proteins and targets them for proteolysis. ERAD involves several different mechanisms, including recognition, deglycosylation, retrotranslocation to the cytosol, polyubiquitination, and ultimately proteasomal degradation of the unwanted conformations. Interestingly, a substantial fraction (40–60%) of the CFTR, even in its wild-type form, gets misfolded and subsequently degraded [83,84]. In contrast, just a minor proteasomal degradation of the wild-type ABCG2 was observed [69,73,76,85]. Improper folding and partial or complete degradation, however, frequently occur with mutant/polymorphic variants, which are discussed later in this section.

Two critical checkpoints governing the central quality control of ABCG2 biosynthesis have been proposed [86]. One of them involves cysteinyl disulfide bonds. Three out of the 12 cysteine residues in the ABCG2 (C592, C603, and C608) are localized to the large extracellular loop between TM5 and TM6. Only these cysteines are exposed to oxidative environment, that is, the ER lumen or the extracellular space, and consequently, only these residues are able to form disulfide bridges. As mentioned earlier, intramolecular disulfide bonds are established between C592 and C608 residues, whereas an intermolecular disulfide bridge, connecting the two halves of the homodimer, is formed by the 603 cysteines. It has been reported that the formation of intermolecular cysteinyl disulfide bond is not essential for proper localization or transport activity [32,61,87], whereas the C592-C608 intramolecular disulfide bond is required for correct folding and protein stability [87]. The variants of ABCG2, mutated at these latter sites, undergo retrotranslocation, polyubiquitination, and proteasomal degradation [85].

The other checkpoint proposed for ABCG2 quality control involves glycosylation. Asparagine 596, localized in the extracellular loop between TM5 and TM6, is subject to N-linked glycosylation. The core oligosaccharide is added to this residue in the ER, whereas a more complex glycan structure is acquired in the Golgi apparatus. Initial studies reported that glycosylation

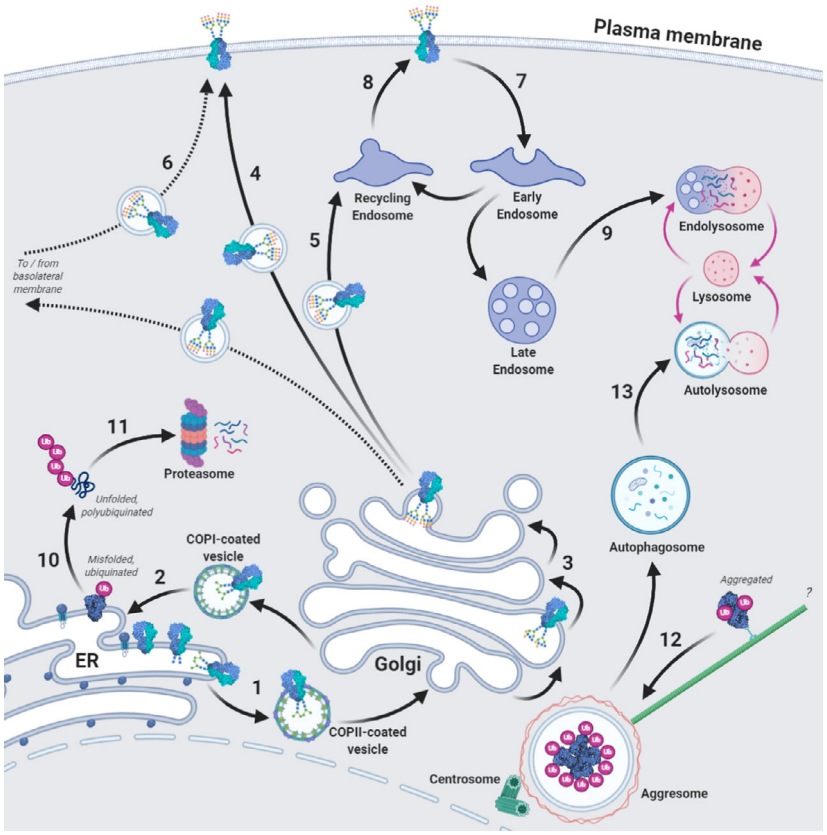

Fig. 3. Cellular routing of the ABCG2 protein. Following biosynthesis, ABCG2 undergoes folding, dimerization, and core glycosylation. If passing the ERQC system, the protein traffics to the Golgi apparatus (1). Quality control mechanism in the Golgi may send the protein back to the ER by retrograde trafficking (2). While traveling through the Golgi stacks (3), ABCG2 becomes fully glycosylated. Following maturation, the protein is delivered to its business end, the plasma membrane. The cellular route taken by ABCG2 to the cell surface still remains to be revealed—these may include direct delivery to the plasma membrane (4), trafficking *via* the endosomal pool (5), and transcytotic pathway *via* the basolateral membrane (6). The transporter in the plasma membrane may become subject of endocytosis (7) and recycling (8). Surplus protein is degraded by lysosomal proteolysis (9). Misfolded proteins in the ER are targeted to cytosol, become polyubiquitinated (10), and degraded in the proteasomes (11). When the capacity of ubiquitin–proteasome system is exceeded, excess proteins tagged with ubiquitin are transferred to aggresomes by retrograde transport along the microtubules (12). Subsequently, these species are subject to autophagic degradation (13).

on N596 is not required for proper expression, delivery to the cell surface, or the transport activity of ABCG2 [33,88]. However, more careful studies demonstrated that disruption of N-linked glycosylation by pharmacological inhibition, or an N596Q mutation, results in destabilization and partial degradation of ABCG2 by the ubiquitin–proteasome system [69]. Derlin-1, a pseudoprotease involved in the retrotranslocation step of ERAD, has been proposed to promote proteasomal degradation of the nonglycosylated form of ABCG2, and facilitate ER retention of its properly folded and glycosylated form [89].

When the capacity of ubiquitin–proteasomal pathway is exceeded or its activity is blocked, misfolded proteins, translocated to the cytosol, form aggregates. These ubiquitin-rich inclusion bodies encapsulated in a vimentin cage, called aggresomes, are localized perinuclearly in the vicinity of the centrosome and facilitate protein degradation by the autophagy pathway [90]. Aggresome formation has first been reported with the most frequent ΔF508 mutant variant of CFTR, which has a folding defect [91]. Also, cellular aggregation of various ABCG2 variants with folding problems has been demonstrated [85,92]. While the expression of wild-type ABCG2 is unaffected, that of the frequent Q141K polymorphic variant increases about threefold when the cells are treated with the autophagosome inhibitor 3-methyladenine [92], suggesting a substantial role for autophagosomal degradation for variants with folding defect.

Trafficking of ABCG2 beyond the ER

Plasma membrane-resident proteins, when pass through the ERQC, are released from the ER at the ribosome-free ER exit sites and transferred to the Golgi apparatus by vesicles covered with coatomer II (COPII). Subsequently, COPII-coated vesicles fuse into vesicle clusters, called ER-Golgi intermediate compartment, before merging into the cis-Golgi network. ABCA1 and CFTR have been shown to traffic from the ER to the Golgi *via* this COPII-coated vesicle-mediated pathway [93,94]. Quality control mechanisms exit beyond the ER, exemplified by the Rer1 and ERp44 proteins. Unwanted protein species in the Golgi are recognized by these proteins and targeted to retrograde trafficking to the ER *via* COPI-coated vesicles.

Glycoproteins, such as ABCG2, while trafficking along the Golgi stacks *via* COPI-coated vesicles, undergo Golgi apparatus-mediated glycoprocessing, through which a complex glycan structure is added to the core oligosaccharides. Most of the carbohydrates (except for a single GlcNAc residue) can be enzymatically removed by N-glycosidase F from the ABCG2, but not by endoglycosidase H (endo-H) [69,92], suggesting a complex structure for the glycan of ABCG2, and allowing distinguishing between ABCG2 species that reached the Golgi apparatus and those that retained in the ER. Interestingly, the majority of the Q141K-ABCG2 variant, subject to increased proteasomal and autophagosomal degradations, is found in a fully mature form, resistant to endo-H deglycosylation [92]. Moreover, the inhibition of lysosomal pathway by bafilomycin increases the expression level of the Q141K and the glycosylation-incompetent N596Q variants approximately twofold [69,92]. These findings suggest that a substantial portion of these ABCG2 variants passes the central quality control mechanisms, which is in sharp contrast to that found with CFTR ΔF508 and various ABCB1 (MDR1 or Pgp) mutants, which fail to mature and are retained in the ER or targeted to proteasomal degradation [95–97].

After maturation, plasma membrane proteins can be transferred from the Golgi to their business end in various ways. The conventional or constitutive pathway involves membrane vesicles, in which the membrane proteins as a cargo are shipped directly to the plasma membrane. Alternatively, vesicles can also enter the endosomal compartments to constitute an intracellular reservoir of membrane proteins, which can later be mobilized to the cell surface. In polarized cells, many apical membrane proteins first traffic to the basolateral domain and then transferred to the apical surface by transcytosis. Various plasma membrane-resident ABC transporters take different routes. For example, ABCC2 (MRP2) has been reported to be delivered directly to the cell surface [98], whereas ABCB11 (BSEP) traffics *via* the rab11a-positive, recycling endosomal pool [99,100], which can be mobilized by bile acids in an LKB1- or PKA-dependent manner [101,102]. MDR1/ABCB1 has been reported to take both direct route [103,104] and the endosomal pathway, involving not only rab11a-, but rab6-, EE1-, and rab5-positive endosomes [99,105,106]. In addition to these normal cellular routes, an unconventional, a Golgi reassembly stacking protein-dependent pathway has been reported, in which a portion of CFTR in its immature, core-glycosylated form traffics directly from the ER to the plasma membrane, bypassing the Golgi apparatus [107]. In polarized cells, apical ABC transporters, such as MRP2 and BSEP, are not transcytosed, but delivered directly or *via* the recycling endosomal to the cell surface [98,99]. Although substantial information has been accumulated on cellular routing of various ABC transporters, how ABCG2 is delivered from the Golgi apparatus to the cell surface is yet to be studied.

There is an additional quality control mechanism at the cell periphery. This peripheral quality control system involves internalization, endosomal sorting, recycling, and lysosomal degradation. It has been shown that ABC transporters, such as MDR1, BSEP, or CFTR, continuously shuttle between the plasma membrane and the endosomal pool [108–110]. However, contradictory data have been published on the involvement of various rab small GTPases in this process, most likely due to its dependence on the cell type, the experimental conditions, and the polarization status of the cell [99]. Various stimuli have been reported to trigger internalization of ABCG2. Endocytosis and subsequent lysosomal degradation are induced by certain drugs, which inhibit ABCG2 transport activity [111]. Also, mild oxidative stress has been shown to stimulate a reversible internalization of ABCG2 in human pluripotent stem cells [112]. Similarly, oxidative stress was attributed to the reduced cell surface expression of ABCG2 observed in human umbilical vascular endothelial cells under hyperuricemic conditions [113]. In these cells, urate-derived reactive oxygen species production blocked the PI3K/Akt signaling pathway; consequently, diminished Akt phosphorylation led to intracellular accumulation of ABCG2. Similar translocation of the transporter was found in neurospheres and glioma cells, expressing ABCG2 in response to inhibition of PI3K and Akt, but not that of mTOR [114]. Whether the reduction in ABCG2 cell surface

expression in these cases is due to accelerated internalization or reduced recycling to plasma membrane remains to be clarified.

Interestingly, labeling of ABCG2 with the specific antibody 5D3, which locks the transporter in a certain conformation, induces rapid internalization [115]. This phenomenon allows studying the mechanisms of ABCG2 endocytosis, which led to the conclusion that ABCG2 is internalized by a dynamin-dependent manner *via* both clathrin-dependent and cholesterol-dependent/caveolin-independent pathways. Only a fraction of the endocytosed ABCG2 undergoes lysosomal degradation, a substantial portion remains in the endosomal compartment with the potential for recycling to the plasma membrane. Inhibition of the lysosomal degradation results in two- to fourfold increase in the steady-state expression level of ABCG2 [69,85,92,116], indicating that ABCG2 is continuously degraded by the lysosomes even under normal conditions.

ABCG2 variants with trafficking defect—Modulators and inhibitors of the ABCG2 trafficking pathways

Hundreds of mutant or polymorphic variants of ABCG2 have been described thus far (recently reviewed in Ref. [30]). Some mutations markedly reduce mRNA or protein expression. Mutations leading to a protein with substantial structural and folding problems are targeted to instantaneous degradation by the central quality control system. This group of mutants includes R113X, Q126X, Q236X, R246X, G262X, E334X, S340del, and Q531X. Individuals homozygous or compound heterozygous for these mutations have no ABCG2 present in the membrane of their red blood cells, comprising the Jr(a-) blood group [117,118]. New additions to this list include the R147W and the R383C variants [119,120]. Other mutations, such as S248P, P269S, and F431L, do not affect protein expression, but impair transport function. Several mutations in ABCG2 attenuate both expression and function. However, mild mutations or polymorphisms can also result in a transporter with preserved functionality, normal surface expression, and unaffected protein stability. These are exemplified by the frequent V12M and the rare K360del variants [67,121,122]. D620N-ABCG2 and N590Y-ABCG2 exhibit even elevated surface expression levels, although conflicting data have been published about the functionality of these mutants,. The relatively rare I206L variant (MAF = 0.0003) is a gain-of-function mutant, which exhibits approximately twofold transport activity as compared to wild-type [68].

Nevertheless, several mutations and polymorphisms lead to impaired trafficking (recently reviewed in Ref. [63]). Among these, Q141K-ABCG2 is the most frequent and most well-studied, since this polymorphic variant assumed to be responsible for at least 10% of gout cases (see next section). The transport function of Q141K-ABCG2 is reduced to some extent [45,123], but this variant also has a trafficking defect. Recently, we have described a novel polymorphic variant (V71M) with properties similar to Q141K, exhibiting somewhat reduced transport function and impaired trafficking [73]. Other mutants that fall in this category include the T153M, F208S, S441N, and F489L variants. It has been demonstrated that a large fraction of the Q141K-ABCG2 is ubiquitinated and targeted to proteasomal degradation, while another portion accumulates in aggresomes and is subsequently proteolysed *via* the autophagic pathway [45,69,92,116]. Interestingly, the not degraded Q141K-ABCG2 is present in its fully glycosylated form, sensitive to PNGase F, but resistant to endo-H deglycosylation. This indicates that the residual transporter underwent Golgi-mediated glycoprocessing, thus reached the Golgi apparatus. Therefore, the model, proposing that a majority of misfolded Q141K-ABCG2 sequestered in the aggresomes originated from the ER, should be revised.

Several pharmacological substances are used to influence the cellular trafficking of membrane proteins. Cycloheximide, an inhibitor of protein synthesis, is frequently applied to make trafficking studies independent of biosynthesis, by preventing feeding of the available protein pool with newly synthesized species. The commonly used trafficking inhibitor brefeldin A, prevents ER to Golgi transfer. Protein trafficking can also be blocked by impeding organelle acidification by V-ATPase blockers, such as concanamycin A and destruxin B, or by inhibiting GTP synthesis, for example, with mycophenolic acid. Disruption of the cytoskeleton by various drugs, such as nocodazole, cytochalasin, or colchicine, also leads to altered protein trafficking. For instance, treatment with colchicine, a blocker of microtubule polymerization, results in accumulation of Q141K-ABCG2 in the plasma membrane rather than in aggresomes, demonstrating the involvement of retrograde transport along the microtubules in trafficking of Q141K-ABCG2 into aggresomes [92]. Endocytosis and recycling can be blocked by dynasore, nystatin, chlorpromazine, or monodansylcadaverine [124].

Nonspecific chemical chaperones, such as glycerol or DMSO, as well as small molecules with more specific effect, called pharmacologic chaperones or correctors, are able to promote folding and surface delivery of

membrane proteins, including ABC transporters (reviewed in Ref. [125]). Lumacaftor (VX-809) was developed to stimulate trafficking of CFTR mutants with folding defect to the cell surface [126,127], but was proven to be also effective with other ABC transporters, such as ABCA4 [128,129]. Similarly, the histone deacetylase inhibitor (HDI) 4-phenylbutyrate (4-PBA) has been reported to improve trafficking of variants of several ABC transporters, including CFTR [130], BSEP [131], ABCB1 [132], ABCB4 [132,133], ABCC6 [134], and ABCG2 [45,46,73]. The underlying mechanism, which has been proposed for this phenotype rescue, is that 4-PBA elevates Hsp70 chaperone protein expression, which in turn prevents misfolded proteins from being targeted to the ERAD. Other HDIs, such as romidepsin, panobinostat, vorinostat, and to a lesser extent valproic acid, promoted relocalization of Q141K-ABCG2 from aggresome to the cell surface without affecting chaperone protein expression [92]. This rescue was attributed to reduced aggresome targeting by the inhibition of dynein/microtubule retrograde transport, as in the case of colchicine. Interestingly, the cytotoxic drug mitoxantrone has also been reported to increase the processing of various ABCG2 variants [76,92]. For instance, targeting of the Q141K-ABCG2 to aggresomes was halted by mitoxantrone [92].

Blocking the various protein degradation mechanisms allows assessing which variant and what fraction of a given protein is degraded by the particular proteolytic pathways. Inhibiting the proteasome activity by MG132 or bortezomib hardly elevates the wild-type ABCG2 expression, whereas substantially increases the expression of the naturally occurring Q141K and M71V variants [73,92], as well as of the artificially generated C592G, C608G, and N596Q mutants [69,85,116], indicating that these variant are considerably degraded *via* the ubiquitin-proteasomal pathway. Similarly, lysosomal degradation of various ABCG2 variants, including the wild-type, Q141K-ABCG2, and N596Q-ABCG2, is demonstrated by their elevated expression upon inhibition of lysosomal degradation by bafilomycin A1 [69,92,116]. In contrast, the expression level of certain ABCG2 mutants, exemplified by the C592G or C608G variant, remains unaffected by bafilomycin treatment [85]. 3-methyladenine, which blocks autophagosome formation, substantially elevates the expression of Q141K-ABCG2; inversely, rapamycin, an effective inducer of autophagy, decreases the level of this variant, demonstrating a role of autophagy in its degradation [92]. Interestingly, the expression of the wild-type ABCG2 is not affected by these treatments, suggesting that ABCG2 is normally not degraded *via* the autophagic pathway.

Diseases connected to the altered expression and function of the ABCG2 protein

ABCG2 expression and function in general pharmacology, tissue barriers, and stem cells

ABCG2 has been found a key player in the absorption, distribution, metabolism, and toxicity of numerous clinically applied compounds [4,135]. Accordingly, based on the guidance of the international transporter consortium, both FDA and EMA require the preclinical testing of the interaction of new drugs with this transporter [13], http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf [15]. In addition, the role of polymorphic and mutant ABCG2 variants, causing reduced expression and/or function, has also been recognized in investigations of drug metabolism. Pharmacogenomics is a relatively new field of studying drug responses based on the individual, genetically modified responses, including lower or increased drug efficiency and toxicity in a given patient or in a cohort of patients. This kind of response, resulting in some cases major adverse reactions in a large group of patients (e.g., malignant hyperthermia, epidermal necrolysis, or drug-induced liver diseases—for review, see https://www.pharmgkb.org), resulted in the withdrawal of numerous compounds and becomes more and more important in the development of new drugs. Additional genetic diagnostics for the personalized or stratified use of several advanced drug therapies have already been suggested or even required to predict specific responses (see USFDA [136]).

In databases, among thousands of SNPs in the *ABCG2* gene, hundreds of missense variants can be found, affecting the coding region of the protein (see the previous sections). However, it is still not clear which are the variants with a significant effect on protein expression and function [30]. There is a relatively frequent mutation, resulting in an early stop codon in the *ABCG2* gene (Q126X—rs72552713, with a minor allele frequency, MAF value of 0.002) present in Asian populations [137]. Several further rare ABCG2 variants, caused by nonsense, splice site, or frameshift mutations, for example, Q236X, 264LhfsX14, or R383C, result in truncated proteins or major structural and folding problems; thus, these proteins undergo immediate degradation at the central quality control (see above). The two most frequent missense polymorphic ABCG2 variants are V12M (rs2231137, c.34G>A, with MAF values of 0.2 in Asia and 0.06 in Europe)

and Q141K (rs2231142, c.421C>A, with MAF values of 0.17 in Asia and 0.098 in Europe). Thus, in Asian populations all these variants are more frequent and may affect a significant number of individuals (for the detailed MAF values, see Ref. [28,137]).

According to most studies, the V12M variant has no appreciable effect on the expression, localization, or function of the transporter [67,121,122]. In contrast, Q141K-ABCG2, as discussed above, has a reduced function and a trafficking problem in the human cells. Some of the mutations causing impaired variants in homozygous or compound heterozygous forms result in the practical absence of the ABCG2 protein in the plasma membrane, which in the red cells leads to the rare Jr- blood group [117,118]. Still, individuals carrying these mutations are generally healthy, while may have altered responses to drugs or endogenous metabolites.

Because of its complex quality control and trafficking, in order to evaluate the functional protein expression, it is important to examine both the activity and the cell surface level of ABCG2. Interestingly, the determination of the ABCG2 protein expression in the red cell membrane may help to decipher the cellular fate of the missense variants. When comparing the missense SNPs in the coding region and the level of ABCG2 cell membrane expression, several variants were found to be unaltered, while other rare variants, such as Q141K, M71V, or R383C, in the heterozygous individuals, resulted between 30% and 50% decrease in the ABCG2 red cell membrane levels [67,73].

The ABCG2 transporter, because of its numerous variants and its important role in ADME-tox, is a candidate gene to be examined before therapeutic application of potential substrates and/or inhibitors [15,138]. It has been shown that the presence of the Q141K-ABCG2 variant affects the pharmacokinetics, the blood plasma levels, and the clinical responses to several ABCG2 substrates, including atorvastatin, simvastatin, and fluvastatin [139], or result in toxic side effects, for example increased risk of rosuvastatin-induced myopathy [140,141]. In fact, the clinical studies showing the effect of ABCG2 Q141K polymorphism on pharmacokinetics of clinically applied drugs provided additional evidence for medically important substrate interactions of the human ABCG2 protein [142].

Among the tissue barriers, in the human BBB, ABCG2 has a pronounced role in limiting the drug exposure of the CNS. While based on animal (mouse and rat) knock-out experiments, the role of ABCB1 has long been emphasized in this regard, recent results indicate that ABCG2 expression levels in the primate and human brain capillary endothelial cells are as high or even higher than those of ABCB1, and according to advanced human PET-CT studies, ABCG2 function may be even crucial [143–147]. In aging individuals and in neurodegenerative diseases, the altered ABCG2 expression or the presence of the transporter variants may cause greater CNS entry and toxicity of drugs normally extruded by ABCG2 [143,148].

As mentioned above, ABCG2 expression levels are relatively high in the liver canalicular membranes, the intestinal and kidney epithelial cells, the placenta, the mammary glands, and progenitor and stem cell populations. However, there is little information as yet on the effects of genetic variants of this transporter in the endo- and xenobiotic extrusion in these tissues [112]. In addition, a significant regulation of the *ABCG2* gene expression has been shown by several metabolic, hormonal, and environmental factors, as well as by drugs. The three major ABCG2 promoter variants, differently expressed in various tissues, all contain cis-regulatory elements [149–155], and extensive microRNA regulation of ABCG2 expression has also been described [156–160]. Hormones, stress conditions, hypoxia, and antioxidants have been found to alter ABCG2 mRNA and protein expression [149,161–167], thus potentially modulating ADME-tox properties of a number of drugs, while the potential effects of genetic variations in the regulatory regions have not been fully explored yet. Detailed pharmacogenomic studies should help to decipher the role of regulatory elements in the drug transport functions of ABCG2.

The role of alterations in ABCG2 expression in cancer drug resistance

In several types of cancer, especially in advanced stages and metastatic forms, chemotherapy is still the main approach of treatment. Cytotoxic chemotherapy is by now efficiently extended or replaced by targeted therapies, directly affecting the relevant driver oncogenes. However, in all small-molecule cancer therapies targeting intracellular effectors, multifactorial drug resistance is likely to occur, and the drug extrusion function of three major ABC multidrug transporters, ABCB1 (Pgp), ABCG2, and ABCC1 (MRP1), has been clearly shown to be involved in a number of multidrug-resistant cancer types [168–171]. The variable expression and the wide, overlapping substrate spectra of these transporters in the actual tumor tissues make it difficult to select the appropriate drugs, which can avoid or inhibit these proteins.

The expression of ABCG2 has been found especially relevant in the suggested cancer stem cells, or the corresponding DPT cells; thus, this transporter may have

a major role in the drug resistance of these highly tumorigenic cell populations with an increased metastatic potential [5,9–12,171–174]. In fact, the side-population phenotype of these cells is based on the increased Hoechst dye extrusion feature caused by ABCG2 expression [6,10,171]. There are convincing data showing that the presence of ABCG2 variants may directly affect clinical tumor cell resistance against sunitinib [175] and gefitinib [17], or increase the toxic side effects of certain anticancer drugs [63,142,176]. In addition, in the cancer cells ABCG2 expression may be significantly upregulated either indirectly by hormonal or stress responses [161,162,164], or following a treatment with anticancer drugs, whether these are ABCG2 substrates or not [168,177,178].

The frequently occurring polymorphic and mutant forms of ABCG2 may directly modulate the effects of the therapeutic agents in the cancer cells [66,71,121]. The Q141K variant was found to be associated with reduced transport of gefitinib, erlotinib, and lapatinib, suggesting that Q141K, in addition to its effect on pharmacokinetics, may also directly modulate the cellular effects of these drugs [179,180]. Also, the presence of the ABCG2 minor variants may be related to increased drug toxicity [22,28,92,181].

To further explore both the transcriptional- and the protein-level regulations of ABCG2, recently we have generated fluorescence-based reporter tumor cell lines by using the CRISPR-Cas9 engineering system [178]. In A549 lung carcinoma cells, we have targeted an eGFP-coding sequence to the translational start site of *ABCG2*. When the eGFP-expressing cells contain a stop codon within the following coding sequence, the ABCG2 knock-out cells exclusively report the transcriptional regulation of the transporter. In another construct, the engineered cells express an N terminally GFP-tagged ABCG2 protein; thus the expression, trafficking, and transport activity of the full-length transporter can be all followed in the reporter cells. Using the engineered cell lines, we demonstrated the upregulation of ABCG2 by a number of anticancer drugs, HDAC inhibitors, hypoxia-mimicking agents, and glucocorticoids. Thus, the drug- and stress-dependent increase in the ABCG2 transporter expression directly in the tumor cells could be medically important when considering cancer drug resistance.

Box 1. Uric acid metabolisms and gout

In humans, uric acid, the end product of purine metabolism, is generated mainly in the liver, while excreted in the kidney and the intestine. Hyperuricemia under certain patient-specific and environmental conditions leads to gout, an extremely painful inflammatory arthritis with redness and swelling, caused by the deposition of sodium urate crystals in the synovial fluid of the joints. Chronic gout may lead to permanent disability and often coincides with metabolism-based heart and kidney diseases, or type 2 diabetes. Numerous famous individuals suffered from gout (from king Henry VIII, Benjamin Franklin, or Isaac Newton to Luciano Pavarotti, see www.naturelife.co.za/gout-and-the-famous-people-who-suffer-from-it/), and this painful condition is still frequent, as over 8 million people have it.

The major forms of hyperuricemia and gout are caused by overproduction or undersecretion of uric acid, in some cases in a mixed form. The key uric acid transporters are SLC22A12 (URAT1), SLC2A9 (GLUT9), and ABCG2. These transporters in the kidney perform a carefully regulated secretion and reabsorption of uric acid, while in the intestine ABCG2 has a key role in uric acid excretion [139,183].

Gout affects mostly men, and up to 30 human genetic loci are involved in modulating serum uric acid levels, urate crystal formation, and the inflammatory response in gout [26,184,185]. A main genetic factor found in GWAs predisposing to gout is the ABCG2 locus [45,186–188].

The treatment of chronic hyperuricemia and prevention of gout are based on the use of urate-lowering drugs, decreasing the formation of uric acid through inhibition of xanthine oxidase (allopurinol, febuxostat), or uricosuric agents, reducing the reabsorption of uric acid in the kidney (probenecid, lesinurad). Acute gout is treated with drugs fighting inflammation and probably also affecting uric acid excretion (e.g., nonsteroidal anti-inflammatory drugs, steroids, and colchicine [183,189]).

The role of ABCG2 in uric acid metabolism and gout

The relatively recent discovery regarding an important physiological and pathological function of ABCG2 is related to the uric acid transport activity of this protein. In 2008, in GWA studies, reinforced by several following publications, the ABCG2-Q141K variant has been found to be associated with gout, and this finding led to the recognition of uric acid as an important endogenous substrate of this transporter [27,29,182]. The reduced membrane expression of the Q141K variant causes hyperuricemia and gout by a

reduced ABCG2-dependent excretion of uric acid, especially in the intestine by this transporter (see Box 1 for uric acid metabolism and gout).

Individuals homozygous for the alleles coding for the Q141K ABCG2 variant may have up to 50% reduction in intestinal uric acid extrusion capacity [75,120,190,191], and early-onset gout is especially connected to this polymorphism [192]. In addition, hyperuricemia may directly downregulate ABCG2 expression [113]. The higher allele frequency of the mutant variants of ABCG2 makes the Asian populations more vulnerable for the development of gout [193,194]—the minor allele frequency of the Q141K variant is 0.094 in European, while in East Asia it is up to 0.29 [28]. In addition, the most frequently used xanthine oxidase inhibitor, allopurinol, and its metabolites have been reported to be substrates of ABCG2, and an association between the presence of the Q141K variant and a reduced response to allopurinol was found, with a still not fully explained mechanism [195–197].

Conclusions and Perspectives

- ABCG2 is a key protein in the extrusion of endo- and xenobiotics
- Recent structural and molecular dynamic information allows to delineate the transport pathway, the role of protein variants, and the mechanistic features of ABCG2
- Trafficking to the cell membrane is a multifactorial process resulting in variations of cell surface localization of ABCG2
- Polymorphisms and mutations in ABCG2 are important in several human diseases

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