DMD Fast Forward. Published on March 7, 2018 as DOI: 10.1124/dmd.118.080663
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Title Page

Beyond Competitive Inhibition: Regulation of ABC Transporters by Kinases and Protein-Protein Interactions as Potential Mechanisms of Drug-Drug Interactions

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Running Title Page

Running Title: ***Kinases and protein-interactions impact on ABC transporters***

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Number of text pages:

Number of tables: 2

Number of figures: 4

Number of references: 164

Number of words in the *Abstract:* 143

Number of words in the *Introduction*: 412

Number of words in the *Discussion (Perspective)*: 212

Abbreviations: ABC transporters, ATP-binding cassette transporters; BCRP, Breast Cancer Resistance Protein; BSEP, Bile Salt Export Pump; MDR1, Multidrug resistance protein 1; MRP1, Multidrug Resistance Associated Protein 1; MSD, membrane spanning domains; NBD, nucleotide-binding domains; Pgp, P-glycoprotein;

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Abstract

ATP-binding cassette (ABC) transporters are transmembrane efflux transporters mediating the extrusion of an array of substrates ranging from amino acids and lipids to xenobiotics, and many therapeutic compounds, including anticancer drugs. The ABC transporters are also recognized as important contributors to pharmacokinetics, especially in drug-drug interactions and adverse drug effects. Drugs and xenobiotics, as well as pathological conditions, can influence the transcription of ABC transporters, or modify their activity or intracellular localization. Kinases can affect the aforementioned processes for ABC transporters as do protein interactions. In this review, we focus on the ABC transporters ABCB1, ABCB11, ABCC1, ABCC4 and ABCG2 and illustrate how kinases and protein-protein interactions affect these transporters. The clinical relevance of these factors is currently unknown, however these examples suggest that our understanding of drug-drug interactions will benefit from further knowledge of how kinases and protein-protein interactions affect ABC transporters.

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Introduction

Mammalian ABC Transporters are integral membrane proteins that actively extrude various endogenous and exogenous molecules across cell membranes in a unidirectional fashion. While these transporters can be found in many organelles including the mitochondria, lysosome, peroxisome, and endoplasmic reticulum, only those at the plasma membrane are discussed here because they can determine the intracellular concentration of drugs, endogenously synthesized molecules as well as compounds formed from our microbiome. Drugs may act as substrates and/or inhibitors of these transporters. Such direct interactions (so called “victim” (the drug) and “perpetrator” (the inhibitor)) are well known to attenuate or alter transporter function. Less well known, but possibly as important is the knowledge of how drugs can affect a transporter’s intracellular location (e.g. by phosphorylation or by affecting interactions with partner proteins) or how the transporter is synthesized or degraded. In this review, we will highlight some of these processes, and illustrate how this type of regulation of transporter activity could account for important drug-drug interactions or other adverse reactions that are distinct from, but just as important as the typical “victim-perpetrator” paradigm. Moreover, we suggest that these types of interactions may be especially important as targeted therapies can interfere with important signaling pathways (e.g. kinase regulated pathways).

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Structural studies have indicated that the minimal composition of an ABC transporter includes a minimum of four domains: two membrane-spanning domains (MSD) and two nucleotide-binding domains (NBD). The MSD consists of alpha-helices (TMD, transmembrane domains) that span the membrane and may determine substrate specificity. The energy for transport is provided by both binding and hydrolysis of ATP by the NBD. The NBD harbors crucial motifs, such as the Walker A (G-X_4-GK-)-T-S), with its conserved lysine contributing to both ATP-binding and hydrolysis, and Walker B (hhhhDE) with a catalytic glutamate that is indispensable for ATP hydrolysis; ABC transporters also contain a characteristic Q-loop that is responsible for communication with the TMDs. Some members of the ABC family, such as ABCG2, are “half-transporters” that contain only one MSD and NBD; therefore, these half-transporters require either homo- or hetero-dimerization for their transport function. There are also ABC transporters that have a third MSD, or an N-terminal extension (NTE), such as ABCC1.

This review will focus on the transporters ABCB1 (P-glycoprotein, Pgp; Multidrug resistance protein 1, MDR1), ABCB11 (Bile Salt Export Pump, BSEP), ABCC1 (Multidrug Resistance Associated Protein 1, MRP1), ABCC2 (MRP2), ABCC4 (MRP4), and ABCG2 (Breast Cancer Resistance Protein, BCRP). A brief overview of each of these transporters is found below.

ABCB1:

The best characterized of the drug transporting ABC proteins is the 170kDa P-glycoprotein (Pgp). Pgp, also referred to as ABCB1 or MDR1, rose to

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prominence in the mid-1970's due to the challenge to understand how cancer cells acquired resistance to multiple structurally and mechanistically unrelated therapeutic cytotoxins; as a mechanism, Pgp's poly-substrate specificity was especially attractive. Subsequently, through the efforts of many laboratories, Pgp, from multiple species, was cloned and characterized (Gottesman and Ling, 2006; Juliano and Ling, 1976). Today, overwhelming evidence supports the idea that Pgp expression is important in a subset of human tumors, in particular acute myeloid leukemia and breast cancer. The demonstration that Pgp was expressed at the plasma membrane in "barrier organs" (ie, those separating the blood from the organ) such as the intestines, the liver, the testis, and cerebral vasculature (Beaulieu et al., 1997; Thiebaut et al., 1987) coupled with no obvious endogenous substrate led to the hypothesis that Pgp was a general protector against xenobiotics; *in vivo* proof of this proposition did not emerge until the development of *Abcb1*-null mice, and the serendipitous discovery of Pgp's function at the blood-brain barrier by the Borst Laboratory (Schinkel et al., 1994; Schinkel et al., 1996; Schinkel et al., 1995). ABCB1 has been implicated in many drug-drug interactions because of its high expression in tissues relevant for drug absorption, disposition, excretion, and substrate overlap with CYP3A inducers and substrates (Aszalos, 2007; Schuetz et al., 1996a; Schuetz et al., 1996b). As such, the Food and Drug Administration and European Medicines Agency suggest that all new drug candidates be screened for *in vitro* ABCB1 substrate and inhibition potential. Known substrates used for clinical studies are digoxin,

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dabigatran, and fexofenadine, while known inhibitors include verapamil and carvedilol.

ABCB11:

ABCB11, bile salt export pump (BSEP), or Sister of P-glycoprotein (SPGP) belongs to the same sub-family as ABCB1; thus, these two proteins share high sequence and structural homology. Because ABCB1 is not highly expressed in liver, a prominent excretory organ with canalicular membranes displaying ATP-dependent transport of bile acids (Nishida et al., 1991), the search for a bile acid ABC transporter began. SPGP was identified by a low-stringency hybridization screen of a liver cDNA library, using as a probe a cDNA fragment from ABCB1; ABCB11 was almost exclusively expressed in hepatocytes (Childs et al., 1995), although low levels of mRNA and protein expression were detected in testes and adrenal glands (Langmann et al., 2003; Uhlen et al., 2010). Intriguingly, ABCB11 is present in subapical vesicles under basal conditions, which can then be mobilized to the plasma membrane at the canalicular (apical) membrane following stimulus by cAMP or taurocholate (Kipp et al., 2001). Unlike ABCB1, ABCB11 has a more restricted range of substrates which are mostly monovalent taurine- and glycine-conjugated bile salts (Byrne et al., 2002). However, some non-bile acid substrates have been reported such as pravastatin and fexofenadine (Hirano et al., 2005; Matsushima et al., 2008), as well as the fluorescent dye, calcein-acetoxymethyl ester (Lecureur et al., 2000), a substrate thought to be primarily a ABCB1 substrate.

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Thus, ABCB11's main physiological role is in the export of bile salts during enterohepatic circulation. The essential role for ABCB11 in bile salt secretion was revealed by studies of inherited cholestatic diseases; gene mutations in ABCB11 accounted for the autosomal recessive disease, Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2). Importantly drug-induced ABCB11 inhibition, possibly coupled with functionally impaired ABCB11might predispose one to Intrahepatic Cholestasis of Pregnancy (ICP), a condition that can lead to a neonatal death that is due to bile acid accumulation in the lungs (Zhang et al., 2015). Both PFIC2 and ICP involve liver damage, which appears due to bile acid disruption of mitochondrial function rather than bile acids damaging the liver (Zhang et al., 2012).

ABCC1:

ABCC1, is a 190 kDa protein that was discovered through efforts to understand drug resistance beyond the "classical resistance" provided by ABCB1. It is also known as multi-drug resistance associated protein 1 (MRP1), and is the founding member of the C subfamily, and contains two NBDs and three MSDs. ABCC1 is expressed in many normal tissues, including lung, skin, small intestine, colon, kidney, and placenta, and localizes to the basolateral membrane in polarized cells, its role in the disposition of drugs used in treating non-malignant disease is not well-defined. Nonetheless, ABCC1 appears to protect certain normal tissues, as Abcc1$^{-/-}$ mice show hypersensitivities to various xenobiotics including etoposide and vincristine (Wijnholds et al., 1997). Additionally, ABCC1 transports endogenous molecules including the

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inflammatory molecule leukotriene C_4 (LTC_4) and the pro-oxidant glutathione-disulfide (Leier et al., 1996; Leier et al., 1994; Loe et al., 1996; Wijnholds et al., 1997). Finally, ABCC1 exports many glutathione-, glucuronide-, and sulfate-conjugates that are synthesized during phase II metabolism (Jedlitschky et al., 1996; Muller et al., 1994).

ABCC2:

ABCC2, previously known as canalicular multispecific organic anion transporter (cMOAT), has broad substrate specificity that includes both endogenous compounds, such as bilirubin and epinephrine metabolites, and exogenous compounds, such as irinotecan and methotrexate (Nies and Keppler, 2007). ABCC2 likely has multiple substrate binding sites (Borst et al., 2006) as transport activity can occur in a cooperative manner – substrate transport is stimulated by the presence of other substrates. For example, indomethacin and sulfinpyrazone can stimulate glutathione efflux in MDCKII cells at low concentrations (Evers et al., 2000). In contrast to ABCC1, ABCC2 is expressed on apical membranes (Mottino et al., 2001; Paulusma et al., 1997; Schaub et al., 1997). Genetic mutations in *ABCC2* lead to Dubin-Johnson syndrome (Kajihara et al., 1998; Kartenbeck et al., 1996; Mor-Cohen et al., 2001; Pacifico et al., 2010; Paulusma et al., 1997; Toh et al., 1999; Wada et al., 1998), a condition characterized by conjugated hyperbilirubinemia and a dark pigmented liver (Dubin and Johnson, 1954), caused by the build-up of epinephrine metabolites (Kitamura et al., 1992). The connection between ABCC2 and Dubin-Johnson syndrome was illustrated by the transport-deficient rat strain (TR-), the TR^{-} rat

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used to model Dubin-Johnson syndrome (Paulusma et al., 1996). Using a fragment of rat MRP1 as a probe, a cDNA of 1541 amino acids was isolated that had high expression in the liver of Wistar rats, but reduced in expression in TR^{-} rat liver. The causative mutation was found to be a deletion of amino acid 393 that caused a frameshift and introduction of an early stop codon that decreased expression of the protein. The link between ABCC2 and Dubin-Johnson has been further confirmed in another rat strain, the Esai hyperbilirubinemic rat (Ito et al., 1997), *Abcc2*$^{-/-}$ mice (Chu et al., 2006) and human patients (Kajihara et al., 1998; Kartenbeck et al., 1996; Mor-Cohen et al., 2001; Pacifico et al., 2010; Paulusma et al., 1997; Toh et al., 1999; Wada et al., 1998).

ABCC4:

ABCC4 is the shortest member of the ABCC subfamily and like ABCB1, has a typical ABC transporter structure with four domains: two NBDs and two MSDs. Originally it was identified as the first mammalian ABC transporter capable of exporting nucleotide analogs (Adachi et al., 2002; Schuetz et al., 1999; Wielinga et al., 2002), a phenomenon that had been originally described in the 1960's, but had not been accounted for mechanistically. ABCC4 also contains a C-terminus PDZ interaction motif (Li et al., Cell 2007). ABCC4 can be found in many tissues, including the blood-brain and blood-CSF barriers, liver, kidneys, Leydig cells and platelets. ABCC4 is unique in that it can be found on either the basal or apical membrane in polarized cell types. For example, in hepatocytes and the choroid plexus, ABCC4 is found on the basolateral membrane, while in the renal proximal tubule and blood-brain barrier, ABCC4 is

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located on the apical membrane (Leggas et al., 2004). The mechanisms accounting for the basolateral versus apical localization of ABCC4 are unknown. ABCC4 has been shown to transport many drugs including nucleoside analogues (Imaoka et al., 2007; Ray et al., 2006), loop and thiazide diuretics (Hasegawa et al., 2007) and cephalosporins (Ci et al., 2007) with sulfate conjugated drugs favored over glucuronyl conjugates. The preference for sulfate conjugates is notable because it is coordinately upregulated with Sult2a by the nuclear receptor, CAR (Nr1i3) (Assem et al., 2004). ABCC4 is also capable of exporting endogenous substrates including cAMP (Chen et al., 2001), urate (Van Aubel et al., 2005), and prostaglandin PGE_2 (Reid et al., 2003). Transport of such signaling molecules suggests ABCC4 may play a key regulatory role in cell signaling, in addition to drug transport.

ABCG2:

ABCG2 (breast cancer resistance protein, BCRP) is a half-transporter that homodimerizes for transport function (Kage et al., 2002), although some evidence suggests it is found in cells as an oligomer (Bhatia et al., 2005; Dezi et al., 2010; Litman et al., 2002; McDevitt et al., 2006; Ni et al., 2010; Xu et al., 2004), cross-linking studies indicate that the predominant functional form is as a dimer (Bhatia et al., 2005). ABCG2 is ubiquitously expressed, but is most abundant in the placenta (for this reason, it was once referred to as “ABCP”(Allikmets et al., 1998)) small intestine, liver, and mammary tissue (Maliepaard et al., 2001). Like ABCB1, ABCG2’s apical localization and ubiquitous expression suggests that it plays a role in the protection of tissues

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from environmental insults; however, it is also highly up-regulated in many cancers, both solid and hematological (Fukuda et al., 2017; Wijaya et al., 2017), leading to multidrug resistance. Substrates of ABCG2 include the topoisomerase inhibitor mitoxantrone, tyrosine kinase inhibitors such as sorafenib (Agarwal et al., 2011; Shukla et al., 2012), the anti-metabolite methotrexate (Chen et al., 2003) and the endogenous substrate protoporphyrin IX. Besides being isolated as a new drug resistance gene (Doyle et al., 1998), ABCG2 was also established as key protein that was both a marker of stem cells (the so called “side-population”, a transport phenotype typified by a characteristic export signature of Hoechst 3382 dye), and had an intrinsic role in protection of hematopoietic stem cells (Kim et al., 2002; Scharenberg et al., 2002). During hematopoiesis, ABCG2 is highly expressed in primitive bone marrow stem cells and is reduced dramatically following differentiation (Bunting, 2002; Scharenberg et al., 2002; Zhou et al., 2001), providing further support that ABCG2 both protects and helps to maintain hematopoietic stem cells. This role is further supported by studies in *Abcg2*-/- mice. Hematopoietic cells reside in a hypoxic niche and *Abcg2*-/- hematopoietic stems cells have impaired survival under hypoxic conditions; moreover, ABCG2 is up-regulated by the hypoxia responsive Hif1-α transcription factor (Krishnamurthy et al., 2004). ABCG2 may play a similar role in cancer stem cells in a variety of solid tumors (Alvi et al., 2003; Chen et al., 2006; Haraguchi et al., 2006; Hirschmann-Jax et al., 2004; Ho et al., 2007; Mohan et al., 2006; Olempska et al., 2007; Raaijmakers et al., 2005; Seigel et al., 2005; Wang et al., 2007).

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Due to the role these transporters play in regulating the cellular concentrations of endogenous and exogenous molecules, as well as being located in the barrier tissues of drug distribution and excretion, the opportunity for drug-drug interactions is quite high. Screening of new drugs (e.g. kinase inhibitors – **Table 1**) for direct interactions with ABC transporters is performed to minimize potential drug-drug interactions. However, interference with ABC transporter function may be more complex than the simple victim and perpetrator competitive interactions. Review of the literature reveals three key additional mechanisms of drug-transporter interaction, beyond a direct interaction with the transporter (**Figure 1**): 1) regulation of protein expression via changes in transcription, translation, or degradation, 2) interruption of trafficking to or from the membrane, or 3) modulation of activity of the transporter via phosphorylation. Each of these potential mechanisms is explored with examples within this review.

Regulation of ABC Transporter Expression by Growth Factor Receptor Kinases:

Epidermal Growth Factor (EGF) is a growth factor that when bound to its receptor (EGFR), a tyrosine kinase receptor, stimulates a signaling cascade through two main pathways: the mitogen activated protein kinase-extracellular signal regulated kinase (MAPK-ERK) pathway and the phosphoinositide 3-kinase (PI3K)-Akt pathway. Each of these pathways has been shown to regulate the expression of ABC transporters (**Figure 2**).

Induction of ABCG2 expression following EGF treatment has been demonstrated in several cell lines and under various conditions. Treatment of

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cultured BeWo (human trophoblasts) and MCF-7 (human breast adenocarcinoma) cells led to increased ABCG2 mRNA and protein expression (Meyer zu Schwabedissen et al., 2006). This induction was not due to a global increase in transporters, as there was no change in ABCC2 expression following EGF treatment. The increase in expression was paralleled by increased drug resistance to mitoxantrone and topotecan, known ABCG2 substrates, but not to doxorubicin, which is not an ABCG2 substrate, except when it acquires a mutation producing an amino acid substitution at arginine 482 (Allen et al., 2002). Pharmacological inhibition of the EGFR signaling cascade with a tyrosine kinase inhibitor (AG1478) or a MEK inhibitor (PD98059) abrogated EGF increase in ABCG2 mRNA and protein expression in and as expected re-sensitized cells to mitoxantrone. The ability of the MEK inhibitor to block the increase in ABCG2 expression does suggest that the MAPK cascade regulates ABCG2 expression at the mRNA level following EGF activation of its receptor.

Similar results were found in kidney and liver cells, though these studies also suggest a role for the Akt pathway downstream of EGFR in regulation of ABCG2 expression. Treatment of conditionally immortalized human proximal tubule epithelial cells (ciPTEC) cells with EGF increased the expression of ABCG2 (Caetano-Pinto et al., 2017). Treatment of these cells with either an Akt inhibitor (LY294002) or ERK inhibitor (U-0126) led to a reduction in ABCG2 expression, though it is unclear whether these inhibitors suppressed basal ABCG2 expression or blocked EGF induction of ABCG2. These data suggest regulation of ABCG2 expression is through both Akt and MAPK pathways in

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response to EGF stimulation. An *in vivo* extension, hepatocyte-specific knockout of Egfr ($Egfr^{hep}$) reduced ABCG2 protein in both total lysate and membrane-enriched fractions (Traxl et al., 2017). Monitoring the clearance of the substrate [^{11}C] erlotinib via positron emission tomography in the $Egfr^{hep}$ mice demonstrated a reduction in hepatic clearance and shift to renal excretion. These data suggest the shift in clearance of [^{11}C] erlotinib is due to the loss of ABCG2 protein in hepatocytes caused by the knockout of Egfr, whereas Egfr and ABCG2 expression are unchanged in the kidney, leading to renal excretion. Overall, it is clear that activation of the EGF pathway enhances of ABCG2 expression at the mRNA and protein levels in many relevant tissues. However, further data is needed to determine the mechanism by which this occurs.

Data suggest that EGF signaling can also regulate the expression of ABCB1 and ABCC4, but in more complex ways. Treatment of ciPTEC cells with EGF decreases the mRNA level of both *ABCB1* and *ABCC4* (Caetano-Pinto et al., 2017). Interestingly, pharmacological blockade of ERK increased ABCB1 and ABCC4. Paradoxically, Akt inhibition decreased expression of these two transporters at the mRNA level. Thus, unlike ABCG2, the MAPK and Akt pathways seem to have opposing roles in the control of the expression of ABCB1 and ABCC4. In the $Egfr^{hep}$ mice mentioned above, ABCB1 protein expression increased, in addition to the changes in ABCG2 (Traxl et al., 2017). Finally, treatment of human colorectal cells, HCT-15 and SW620-14, or breast cancer cells, MCF-7/MDR and MDA-MB-231/MDR, with EGF or basic fibroblast growth factor (bFGF) enhanced ABCB1 expression at the protein level with no effect on

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mRNA expression (Katayama et al., 2007). Both siRNA-mediated knockdown and pharmacological inhibition of ERK decreased ABCB1 protein expression (**Figure 2)**. Further pulse-chase experiments determined that ERK inhibition promoted degradation of ABCB1 with no effect on its biosynthesis. Thus, these data suggest that the MAPK pathway (downstream of growth factor signaling) increases ABCB1 by preventing its proteasomal degradation.

Overall, these data illustrate how disruption of growth factor signaling by kinase inhibitors (**Table 1**) can produce unanticipated alterations in transporter protein expression; such changes can be due to alterations in transcription or protein stability. More broadly, these changes could impact drug disposition and clearance, consequently increasing adverse effects, drug-drug interactions, and non-optimal plasma concentrations of drug.

Kinase Modifications of ABC Transporters:

Phosphorylation sites have been identified in many ABC transporter (Stolarczyk et al., 2011) (see “PHOSIDA” searchable database http://141.61.102.18/phosida/index.aspx). Phosphorylation of proteins changes their conformation, and is reversible; however, unlike our knowledge of the impact on transcription factors (e.g. TP53), the evidence that specific phosphorylation changes ABC transporter activity is still nascent. Further, the relationship between phosphorylation sites and the kinases mediating the modifications is virtually unknown. **Figure 3** illustrates the predicted phosphorylation sites for ABCB1, C1, C4, and G2 discussed in this review, and **Table 2** lists experimentally identified phosphorylation sites for some of these

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proteins and the putative effect of phosphorylation at these sites. In addition to direct phosphorylation of ABC transporters, kinases may contribute to signaling cascades that ultimately affect the trafficking and/or activity of transporters. Given that kinase inhibitors may ultimately be deployed in a variety of therapeutic settings, and also given the well-known, off-target effects of kinase inhibitors (Anastassiadis et al., 2011), it is likely that alterations in ABC transporter function may occur secondary to altered transporter phosphorylation. The importance and identity of kinases affecting ABC transporter activity is emerging, but some examples are discussed below.

Kinases and Trafficking:

In addition to the transcriptional changes associated with EGF treatment discussed above, there is evidence that EGF downstream signaling may also regulate the trafficking of ABCG2. Side population cells, first identified in the lab of Richard Mulligan, (Goodell et al., 1996)), express high levels of ABCG2 and are enriched in hematopoietic stem cell activity. Brief treatment of side population cells with the PI3K inhibitor LY294002 led to the translocation of ABCG2 to the cytosol (**Figure 2)**, as visualized via immunofluorescence detection of endogenous protein (Mogi et al., 2003). There was no change in total fluorescence intensity suggesting no change in the total protein expression, only localization. This phenomenon was independent of ABCG2 transcription because LLC-Pk1 cells that heterologously express ABCG2, when treated with EGF, display a two-fold increase in cell surface expression of ABCG2 with no change in total protein expression (Takada et al., 2005). Accordingly, treatment with PI3K

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inhibitors to disrupt this pathway showed ~50% decreased surface expression as measured by surface biotinylation and confocal microscopy. Finally, PPARγ agonists, which activate PTEN, a negative regulator of PI3K activity, also reduced surface expression and transport activity of ABCG2 with no change in mRNA or protein expression in MCF-7 FLV1000 cells (To and Tomlinson, 2013). Thus, while EGF affects ABCG2 transcription (**Figure 2**), evidence indicates EGF, through the PI3K/Akt pathway, stabilizes ABCG2 at the membrane of cells. It is unknown if this pathway produces specific phosphorylation of ABCG2 to facilitate its membrane localization or retention. Although speculative, one could imagine phosphorylation altering ABCG2 membrane recycling or its interaction with the actin network.

The PI3K/Akt pathway and PKC have been implicated in the retrieval of ABCC2 and ABCB11 from the membrane during cholestasis induced by estradiol 17β-D-glucoronide (E_2-17G)-induced cholestasis (Boaglio et al., 2010; Crocenzi et al., 2008), an endogenous metabolite of estrogen. The endocytic retrieval of both ABCC2 and ABCB11 from the membrane occurs following treatment with E_2-17G (Crocenzi et al., 2003; Meyers et al., 1980; Mottino et al., 2002). Pharmacological inhibition of cPKC isoform with Gö6976, partially rescued the E_2-17G-induced reduction in biliary secretory function and ABCC2 and ABCB11 transport activity and was able to maintain membrane expression of both transporters (Crocenzi et al., 2008). However, since cPKC activity only partially prevents the effects of E_2-17G, further work was required. Inhibition of PI3K with wortmannin or LY294002 also significantly rescued the activity and membrane of

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ABCC2 and ABCB11 following E_2-17G treatment (Boaglio et al., 2010). Downstream of PI3K, Akt inhibition also partially rescued ABCC2 and ABCB11 activity in response to E_2-17G treatment; however, this was not to the same magnitude as PI3K inhibition, suggesting there are other PI3K downstream pathways that are involved in transporter trafficking. The PI3K and cPKC signaling was shown to be additive, suggesting complementary, rather than overlapping pathways. Thus, both PI3K and PKCs contribute to maintaining plasma membrane ABCC2 and ABCB11 expression in hepatocytes.

Preliminary evidence suggests that the Src kinase pathway may regulate transporter protein trafficking downstream of VEGF (Hawkins et al., 2010). VEGF reversibly decreases ABCB1 transport activity with no changes in protein expression in isolated brain capillaries. In fact, the effect of VEGF could be reversed within 30 minutes of washout. Both Flk1 and Src activity are needed for this signaling pathway, as pre-treatment with inhibitors abolished the VEGF-induced reduction in transport activity. This effect was specific for Src downstream of Flk1 as treatment with PI3K inhibitor and PKC inhibitor had no effect. Intracerebroventricular injection of VEGF increased the brain distribution of ABCB1 substrates morphine and verapamil to the ipsilateral cerebral hemisphere with no change in sucrose distribution, indicating no general change in blood brain barrier permeability. This increase in brain distribution of verapamil could be abrogated by intraperitoneal injection of a Src kinase inhibitor. Finally, to determine the mechanism by which VEGF affects transporter activity, nocodazole, a microtubule polymerization inhibitor, blocked the VEGF-induced

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loss of ABCB1 activity in isolated brain capillaries but the proteasome inhibitor lactacystin does not. These data together, suggest a change in ABCB1 trafficking, but whether it is related to altered endosomal trafficking or loss of membrane retention remains the subject of future investigation. In another report, ABCB1 and Src were found to co-immunoprecipitate from MCF-7 drug resistant cells (Zhang et al., 2014), further supporting a role for Src signaling in ABCB1 membrane localization; however, these results are preliminary and further work is needed to verify and determine the clinical relevance, if any, of this interaction.

Pim1-kinase, an oncogenic serine/threonine kinase, is overexpressed in many cancers and has diverse substrates that include pro-apoptotic proteins (Aho et al., 2004), cell cycle regulatory proteins (Bachmann et al., 2006; Mochizuki et al., 1999; Morishita et al., 2008; Zhang et al., 2007), and transcription factors (Aho et al., 2006; Chen et al., 2002; Glazova et al., 2005; Kim et al., 2008; Peltola et al., 2004; Zhang et al., 2008). ABCB1 and ABCG2 have been identified as putative substrates for Pim1-kinase, where phosphorylation is necessary for the plasma membrane localization of both transporters (Darby et al., 2015; Natarajan et al., 2013; Xie et al., 2010; Xie et al., 2008).

Pim1 directly phosphorylates ABCG2, and the 44kDa isoform of Pim1 co-immunoprecipitates ABCG2 when exogenously expressed in HEK293T cells or endogenously in CWR-R1 prostate cancer cells (Xie et al., 2008). This interaction requires the Pim1 kinase domain and its N-terminus proline-rich domain, which is present only in the 44kDa isoform. siRNA-mediated knockdown of Pim1 in CWR-

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R1 cells reduced mitoxantrone or docetaxel resistance, while overexpression of Pim1 in cells that express very little ABCG2 (LNCaP cells) increased drug resistance. ABCG2 has a Pim1 substrate consensus sequence located in the cytoplasmic linker region. To determine if ABCG2 was a substrate of Pim1, they were co-expressed in HEK293T cells, and ABCG2 was phosphorylated at T362. This phosphorylation could be abrogated by Pim1 knockdown. Overexpression of a T362A ABCG2 mutant, which blocks phosphorylation at this site, in LNCaP cells did not change drug sensitivity, unlike wild-type ABCG2, which strongly enhanced drug resistance. Accordingly, a phospho-mimicking T362D mutant increased drug resistance even when Pim1 had been knocked down, suggesting this specific phosphorylation event is important for ABCG2 transport activity. Finally, the wild-type or T362D ABCG2 in LNCaP cells could be observed on the plasma membrane of cells by immunofluorescence and cell fractionation, but the T362A mutant ABCG2 could only be observed in the cytosol, suggesting the phosphorylation site is required for membrane localization. This was further validated, by either T362A mutant overexpression or by Pim1 knockdown, each of which exhibited a decrease in oligomerization of ABCG2. These data suggest phosphorylation by Pim1 at T362 is necessary for proper localization and function of ABCG2.

Pharmacological inhibition of Pim kinase with the inhibitor SGI-1776 decreased the surface expression of ABCG2 and increased the uptake of substrate drugs in cells that expressed high levels of ABCG2 (Natarajan et al., 2013). Additionally, use of novel Pim kinase inhibitors in MCF-7 drug-resistanct

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cells over-expressing ABCG2 increased the potency of flavopiridol, mitoxantrone, topotecan, and doxorubicin, while also decreasing the protein expression of ABCG2 (Darby et al., 2015). While preliminary, these results suggest that Pim kinase inhibition could regulate ABCG2 activity, localization, and expression.

Pim1 phosphorylation is also required for ABCB1 membrane expression, likely due to stabilization of immature ABCB1 to allow glycosylation to occur (Xie et al., 2010). Total and plasma membrane expression of ABCB1 is decreased when Pim1 is knocked-down via shRNA or siRNA. This decrease in protein expression is probably due to an increase in degradations; cyclohexamide treatment in cells with Pim1 knockdown decreased the half-life of ABCB1 in comparison to cells with Pim1 present. A consensus Pim1 sequence exists in ABCB1 between NBD1 and MSD1, and an in vitro kinase assay demonstrates ABCB1 phosphorylation following incubation with Pim1. Therefore, the stabilization of ABCB1 appears to be through its phosphorylation. Pharmacological inhibition of Pim1 with SGI-1776 demonstrated similar effects as genetic inhibition, namely a decrease in mature ABCB1 and a decrease in ABCB1 half-life. Co-treatment of cells with SGI-1776 and MG132, a proteasome inhibitor, maintained ABCB1 protein stability, suggesting that phosphorylation of ABCB1 prevents its proteasomal degradation. Finally, it was determined that this stabilization appears to be required for effective glycosylation of immature ABCB1. Inhibition of glycosylation by 2-deoxyglucose led to accumulation of 150kDa ABCB1 protein, while co-treatment with SGI-1776 decreased this accumulation. Thus, these data suggest that Pim1-phosphorylation of ABCB1

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enhances its maturation and membrane trafficking. However, another report suggests that treatment of $NCI^{ADR/Res}$ cells, which overexpress ABCB1, with novel Pim kinase inhibitors does not affect sensitivity to doxorubicin, a well known ABCB1 substrate (Darby et al., 2015). That these reports do not agree suggests that further work is needed to fully determine what, if any effect Pim-1 kinase activity may have on ABCB1 trafficking.

Kinases and Transporter Activity:

ABCC1 can be regulated by several kinases including GSK3 and Casein Kinase 2 (CK2). GSK3 is a serine/threonine kinase that has two isoforms (GSK3α and β) that have been shown to modulate the PI3K-Akt pathway. The GSK3's have a diverse array of substrates that do not depend on mitogenic signaling. Phosphorylation of a serine (Ser9 for GSK3α, or Ser21 for GSK3β) within the basic pocket near the catalytic site leads to auto-inhibition of kinase activity (Hermida et al., 2017).

Changes in ABCC1 expression in response to cadmium exposure are complex, but GSK3 inhibitors provided insight. ABCC1 is both induced and re-distributed from the nuclear-rich membrane fraction to the cytosolic fraction following cadmium exposure, which can be suppressed by the GSK3 inhibitor (Kim et al., 2015). GSK3 knockout decreased ABCC1 mRNA and protein expression in response to cadmium. Notably, ABCC1 and p-Ser GSK3 can be co-immunoprecipitated, indicating a physical interaction. While it is clear that GSK3 regulates ABCC1 expression following cadmium treatment, the consequence, or role of GSK3 in normal regulation of ABCC1 is unknown.

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Casein Kinase 2 (CK2), a ubiquitous and constitutively active serine/threonine kinase, also modulates ABCC1. In yeast, the homolog of CK2 phosphorylates Ser251 in the intracellular loop that connects MSD_0 to MSD_1 producing an increase in activity. (Paumi et al., 2008). This CK2 consensus site is conserved in human ABCC1 as Thr249. In a variety of cancer cells, treatment with CK2 inhibitors decreases ABCC1 dependent efflux of doxorubicin, thereby increasing doxorubicin cytotoxicity (Stolarczyk et al., 2012). How this specific phosphorylation site changes ABCC1 activity is unknown, one could speculate that this occurs by altering degradation, localization, or protein-protein interactions. Further work is needed to determine the consequences of ABCC1 phosphorylation by either GSK3 or CK2.

ABCB1 activity can be acutely modulated at the blood-brain barrier through PKC signaling. Freshly isolated brain capillaries, exposed to subnanomolar–to-nanomolar concentrations of Endothelin-1 (ET1) reduced ABCB1 transport activity within 15 minutes (Hartz et al., 2004), with ET1 washout returning ABCB1 activity to baseline within 60 minutes. An inhibitor of the ET_B receptor prevented the effects of ET1 on ABCB1 transport activity, suggesting signaling occurs through this receptor. Signaling through iNOS and PKC are required for the effect of ET1 on ABCB1; blocking these downstream targets, iNOS with l-NMMA or PKC with BIM prevents ET1's suppression of ABCB1 function. Furthermore, increasing nitric oxide with the donor SNP mimics ET1's reduction of ABCB1, and BIM also blocks this activation, suggesting PKC is downstream of iNOS. Further work showed that upstream of ET1, TNFα

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exposure also reduces ABCB1 activity, by signaling the release of ET1. This effect is both acute, reduction of activity is seen within 30 minutes, and reversible, washout of TNFα returns activity to baseline within 150 minutes, suggesting it is not a change in protein stability (Hartz et al., 2006). Reduction of ABCB1 activity could also be replicated by treatment with lipopolysaccharide (LPS), a component of Gram negative bacterial cell walls that activates an inflammatory response that includes release of TNFα. Intraperitoneal injection of LPS in mice increased brain uptake of [^{3}H]-verapamil 18 hours after injection, indicative of decreased ABCB1 activity (Salkeni et al., 2009). Increased brain uptake was evident until 36 hours after injection and occurred despite an increase in ABCB1 protein expression. In mice, inhibition of NO signaling did not restore ABCB1 transport activity, suggesting there may be factors present in a live animal that are not in isolated brain capillaries that are a part of this signaling cascade.

TNFα signaling stimulates sphingosine-1-phosphate (S1P) from sphingosine by activating sphingosine kinase 1 (SK) (De Palma et al., 2006; Xia et al., 1999). S1P signals through its receptor, S1P receptor (S1PR1). Treatment of brain capillaries with SK or S1PR1 antagonists block the TNFα- and PKC agonist-mediated reduction in ABCB1 transport activity, suggesting SK signaling is downstream of TNFα and PKC (Cannon et al., 2012). An approved small molecule used for the treatment of multiple sclerosis, FTY720, is a non-selective S1PR agonist. Treatment of capillaries with this S1PR agonist rapidly reduces ABCB1 activity. Brain perfusion with FTY720 increased accumulation of ABCB1

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substrates verapamil by ~4-fold and loperamide and paclitaxel by ~5-fold, which is indicative of decreased ABCB1 activity. Downstream of S1P, AFAP-1 is required for TNFα-induced reduction of ABCB1 activity, for siRNA mediated knockdown of AFAP-1 maintains ABCB1 activity following treatment with TNFα, lipopolysaccharide (LPS), and S1P (Hoshi et al., 2017). Interestingly, long-term (3-6 hours) treatment with ET1 or TNFα produces a two-fold increase in protein in isolated rat brain capillaries, which correlates with an increase in ABCB1 activity through a NF-κB-mediated cascade (Bauer et al., 2007). These data illustrate a signaling cascade, mediated by multiple phosphorylation events, can profoundly affect ABCB1 activity, suggesting a potential mechanism for ABCB1 mediated drug-drug interactions with drugs that effect ET1, iNOS, PKC, inflammatory, or sphingosine signaling.

PKC also regulates ABCB1 activity in other contexts (**Table 2**). Phosphorylation of the linker region of ABCB1 by PKC has been well documented at Ser661, Ser 671, and one or more of Ser667, Ser675, and Ser683 (Chambers et al., 1990; Chambers et al., 1994; Chambers et al., 1993; Orr et al., 1993) and this phosphorylation increases the activity of ABCB1 (Chambers et al., 1990; Chambers et al., 1992; Idriss et al., 2000; Masanek et al., 2002; Sachs et al., 1996; Szabo et al., 1997). This phosphorylation site is similar to the location of phosphorylation sites within the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a member of the ABC transporter “C” family. While the fact that ABCB1 is phosphorylated by PKC is well-established, the mechanism by which this phosphorylation event increases

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transporter activity is less obvious. Co-expression of PKCα with ABCB1 increased the ATPase activity in insect and ovarian cells (Idriss et al., 2000), while PKC inhibitor treatment did not alter ATPase activity in MCF-7 cells (Sachs et al., 1996). Perhaps this difference is due to the expression systems or differences between mammals and insects, but it may also be an isoform specific effect as co-expression of PKCε with ABCB1 in insect ovarian cells had no effect on ATPase activity (Idriss et al., 2000). Mutation of Ser661, Ser667, and Ser671 from serine to alanine creates a protein that is not phosphorylated and required an increased substrate concentration to achieve half-maximal activity (Szabo et al., 1997). This suggested a change in substrate binding. However, this effect was only true for verapamil, vinblastine, and rhodamine 123, but not valinomycin or calcein acetoxymethylester. These data suggest phosphorylation changes the substrate binding site selectively. However, it is important to note that there is some discrepancy in the role PKC plays in ABCB1 activity because inhibition of PKC failed to reverse drug resistance in some human cancer cells (Scala et al., 1995). Notably, in fish proximal tubule cells activation of PKC decreased ABCB1 transport activity (Miller et al., 1998). Similarly, activation of the PKCβ1 isoform at the blood-brain barrier in rats led to the rapid decrease in ABCB1 transport activity (Rigor et al., 2010). Thus, the effects of PKC on ABCB1 function vary, and may depend on the phosphorylation sites, as other inputs from upstream stimuli, or perhaps the species and/or cell-type.

It is clear that phosphorylation is an attractive mechanism to control ABC transporter activity, and may contribute to drug-drug interactions or adverse

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events by changing ABC transporter protein activity, although the clinical consequences remain unexplored.

Protein-protein interactions:

Direct interactions between proteins may be another source of regulation, as these interactions might contribute to the proper localization and stabilization of transporters at the plasma membrane. To determine the interactome of non-mitochondrial ABC transporters, Snider and colleagues used the unbiased membrane yeast two-hybrid (MYTH) method, which allows interrogation of protein-protein interactions of membrane proteins in their native state (Snider et al., 2013). The MYTH system is more effective for determination of protein interactions with membrane-bound proteins than traditional yeast two-hybrid methodologies. It is based on the split-ubiquitin protein complementation assay and allows for detection of novel protein-protein interactions (Lentze and Auerbach, 2008). When combined with the BioGrid database (see thebiogrid.org), 537 unique binary interactions across 366 proteins involving the 19 non-mitochondrial ABC transporters encoded by the yeast genome were identified. Interactions were classified using gene ontology and included interactions between transporters and proteins involved with metabolism, cell cycle, growth and division, cytoskeleton, DNA replication, maintenance, and repair, nuclear function, protein degradation, folding, and modification, protein synthesis and ribosome, RNA processing and regulation, stress response, and transport, trafficking, and secretion. Additionally, the transporters were found to interact with one another, not only known half-transporter interactions, but also

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interactions between full transporters (e.g. Pdr5p and Snq2p – members of the PDR family that has homology to the ABCG family in mammals). Thus, there are many diverse protein-protein interactions that if disrupted may have an effect on transporter activity. Specific examples and the consequences of the protein interactions between ABC proteins and ERM or PDZ proteins are discussed in more detail below as examples of protein-protein interactions that modulate ABC protein activity.

ERM Proteins:

Ezrin-radixin-moesin (ERM) family proteins are scaffolding proteins that crosslink actin filaments and integral membrane proteins (McClatchey, 2014). ERM proteins may be important for ABCB1 stabilization at the membrane; ABCB1 has been shown to interact with each of the ERM proteins. In HepG2 cells with radixin (Rdx) knocked-out there is decreased ABCB1 at the membrane and decreased transport activity (Kano et al., 2011). This interaction has also been found in *Rdx-/-* mouse small intestines where the amount of ABCB1 at the membrane was reduced compared to wild-type mice (Yano et al., 2013). Ezrin co-immunoprecipitates with ABCB1 and is required for optimal ABCB1 membrane localization and transporter activity (Brambilla et al., 2012). ABCB1 and Ezrin also co-localize with the lipid raft marker G_{M1}, and Ezrin is required for ABCB1 localization to lipid rafts. In both cells and in mice, the importance of an interaction between ABCB1 and ERM proteins has been shown to be important, for stabilization at the membrane. However, the nature of this interaction and the domains mediating it remain to be defined.

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That the proper subcellular localization of ABCC2 (which modulates bile acid-independent bile flow) in the bile canalicular membranes (BCM), required an interaction with the major liver ERM, Rdx, was unknown, until the *Rdx*-/-) mouse was developed (Kikuchi et al., 2002). The *Rdx*-/- mouse appeared normal at birth; however, by age four weeks, concentrations of conjugated bilirubin began to rise in the serum, and reached 15-fold higher levels than wild-type by 16 weeks. This correlated with histological evidence for mild liver injury and increased serum levels of alkaline phosphatase and aspartate aminotransferase. This is similar to Dubin-Johnson syndrome, a human genetic disease, producing hyperbilirubinemia associated with ABCC2 loss of function. The expression of ABCC2 in the *Rdx*-/- mice was determined in the BCM by immunofluorescence and Western Blot; a decrease in Abcc2 relative to other BCM-resident proteins was evident in *Rdx*-/- mice as compared to wild-type mice. In the bile canaliculi, co-localization of Abcc2 and Rdx was histologically demonstrated in liver frozen sections suggesting a direct interaction that was then confirmed by co-immunoprecipitation from wild-type liver. In MDCK cells transiently expressing FLAG-ABCC2, ezrin, the dominant isoform of ERM proteins in these cells, was co-immunoprecipitated, and this interaction was prevented by the removal of the C-terminus of ABCC2. To map the location of ABCC2's interaction with Rdx, *in vitro* studies were conducted whereby a purified fusion protein composed of the C-terminus of ABCC2 fused to GST was incubated with the recombinant N-terminus half of Rdx. The complex between these two proteins suggested that the N-terminus of Rdx interacted with the C-terminus of ABCC2. However,

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ABCC2 lacks the prototypical PDZ-motif at its C-terminus, thus the exact nature of this interaction is unknown. In combination, the studies from the MDCK cells and *Rdx*-/- mice suggest that the ERM proteins are required for the expression of ABCC2 at the membrane, and that in BCMs, radixin interacts with ABCC2. At this point, it is unknown if ERM proteins like radixin interact due to their affinity or abundance.

PDZ (Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)) Proteins*:*

PDZ domains are the most common protein-protein interaction domains in humans with over 250 PDZ-domains in 150 unique proteins (Feng and Zhang, 2009; Ponting et al., 1997; Walsh et al., 2015). An individual protein may contain from one to multiple PDZ domains that facilitate the formation of multi-protein complexes. The canonical PDZ domain is composed of a stretch of 80-90 amino acid residues that are arranged as 6 β-strands and 2 α-helices in a globular structure (Karthikeyan et al., 2001). Although some PDZ-motifs can be found throughout the target protein, PDZ motifs are typically C-terminal sequences and are generally three to four residues long. These protein-protein interactions are mediated through binding of the PDZ-motif to a hydrophobic binding pocket in the PDZ-domain protein that is formed by a β-strand, an α-helix, and the loop that connect these two (Walsh et al., 2015).

PDZ motifs are found in ABCC2 (-STKF) and ABCC4 (-ETAL). ABCC2 interacts with the PDZ-domain containing proteins NHERF1, NHERF3 (aka PDZK1), and NHERF4 (Hegedus et al., 2003; Kocher et al., 1999). Interestingly,

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the serine present in the PDZ motif of ABCC2 is also a PKC consensus phosphorylation site. In Sf9 cells, phospho-mimicking mutants of this serine (S1542E) interacted with NHERF1 and -4 more strongly than WT MRP2, while a dephosphorylated mimicking mutant (S1542A) had no effect on the interaction with NHERF4 but decreased the interaction with NHERF1 (Hegedus et al., 2003). These data suggest that the phosphorylation of the serine in the PDZ domain of MRP2 may regulate its protein-protein interactions. PDZ protein interactions appear to be important for ABCC2 protein localization. In Nherf1-KO mouse liver, Abcc2 mRNA expression was unchanged, but the total lysate and membrane fraction protein expression of Abcc2 was significantly decreased as compared to WT mice (Li et al., 2010). Abcc2 was still localized to the apical membrane, detected by immunofluorescence, suggesting that trafficking to the membrane was unchanged when Nherf1 was absent. Functional outputs of Abcc2, glutathione and glutathione-methylfluorescein excretion, were also decreased in Nherf1-KO mice, further supporting a decrease in Abcc2 function. Using immunofluorescence, the requirement for NHERF1 was further confirmed and refined in WIF-B cells. Mutation of the first PDZ domain or the radixin-binding domain of NHERF1 decreased ABCC2 membrane localization and function (Karvar et al., 2014). These data suggest that the interaction between ABCC2 and NHERF1 is important for the proper membrane localization of ABCC2 in hepatocytes.

The interaction between ABCC2 and NHERF3 was the first to be discovered, using a yeast two-hybrid system (Kocher et al., 1999), and was

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further confirmed in later studies (Emi et al., 2011; Hegedus et al., 2003). However, in Nherf3-KO mouse kidney proximal tubule cells, there was no difference in the expression or localization of Abcc2 when compared to wild-type cells (Kocher et al., 2003). In contrast, in HepG2 cells, overexpression of the PDZ domain of NHERF3 can destabilize ABCC2's membrane localization (Emi et al., 2011). Thus, the importance of NHERF3 on ABCC2 localization may be dependent on cell type and other PDZ-containing proteins present.

The C-terminal PDZ motif of ABCC4 (-ETAL) is highly conserved from fish to humans (Pitre et al., 2017). In an attempt to catalog the extent of PDZ domain proteins capable of interacting with this ABCC4 motif, we used an in vitro strategy and interrogated the Panomics' PDZ Domain Arrays (**Figure 4)**. These commercial arrays include a total of 123 different human PDZ domains representing recombinant conserved binding sites from the individual PDZ domain proteins that have been fused with glutathione-S-transferase (GST). These proteins were affinity-purified, immobilized onto a membrane, spotted in duplicate and then probed with a biotinylated peptide harboring the ABCC4 PDZ-motif (see legend for description and supplement for methods). We discovered almost 30 PDZ domain-containing proteins that interact with the ABCC4 PDZ-motif. Among these, some were already known, such as PDZK1 and NHERF1, but many have never been reported before.

As mentioned above several PDZ-domain proteins have been reported to interact with ABCC4's PDZ-motif. Initial studies evaluated several PDZ domain proteins, and pull-down assays demonstrated that ABCC4 binds with the highest

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affinity to PDZK1 (aka NHERF3) (Li et al., 2007). PDZK1, a protein with four PDZ-domains, mediated a functional coupling between ABCC4 and another ABC transporter protein in the C subfamily, the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP activated (via PKA mediated phosphorylation of CFTR) Cl^- channel. Genetic deletion and pharmacological inhibition of ABCC4, a cAMP exporter (Li et al., 2007), potentiated CFTR Cl^- currents, likely due to an accumulation of cAMP near CFTR, resulting in increased transport activity of CFTR. In gut epithelia, CFTR, ABCC4, and PDZK1 were co-immunoprecipitated suggesting that formation of this macromolecular complex, facilitated by a PDZ protein, functionally couples CFTR and ABCC4. Disruption of this macromolecular complex with competitive ABCC4 PDZ peptides attenuated the potentiation of CFTR conductance suggesting that the extent of complex formation can regulate CFTR activity. Unanswered questions include what signals promote the formation or disassociation of this macro-molecular complex?

PDZK1 is reportedly required for ABCC4 transport activity in kidney, presumably through this PDZ interaction (Park et al., 2014); however, given that ABCC4 only interacts with one of the four PDZ domains, other interacting proteins could modulate the activity. Nonetheless, when co-expressed in HEK293 cells, PDZK1 increased ABCC4 expression, membrane stability, and drug transport activity, and reduced the internalization of the transporter and lysosome-mediated degradation. These results were extended to in vivo studies in the kidney of Pdzk1-knockout mice, which had decreased Abcc4 expression,

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membrane localization, and plasma clearance of [^{3}H]-adefovir, a measurement of Abcc4 function in the kidney. However, given PDZK1 has the potential to bind more than one PDZ-motif containing protein, it remains to be determined if another PDZ-motif protein interacts with ABCC4 and PDZK1 in this context.

An interaction between ABCC4 and NHERF1 has also been defined (Hoque and Cole, 2008; Hoque et al., 2009; Li et al., 2007), although the consequence of this interaction is unclear. In HeLa cells, NHERF1 knockdown by siRNA increased ABCC4 total and membrane protein abundance and decreased cellular accumulation of ABCC4 substrates [^{3}H]-bis(POM)PMEA and [^{14}C]6-mercaptopurine, indicative of increased transport activity (Hoque and Cole, 2008). It has been proposed that NHERF1 promotes ABCC4 internalization, as NHERF1 siRNA-mediated knockdown reduced ABCC4 internalization. The role of NHERF1 is likely cell context dependent and dictated by the proteins occupying its two PDZ-domains because, unexpectedly, NHERF1 was also found to be required for membrane localization of ABCC4 (Hoque et al., 2009). ABCC4 is unique in that it can locate to both basolateral and apical membranes in polarized cell types and it has been proposed that NHERF1 might contribute to polarized cellular localization of ABCC4. In MDCKI cells, ABCC4 is expressed on the basolateral membranes. In contrast, ABCC4 is found predominantly on the apical membrane of LLC-PK1 cells. Interestingly, NHERF1 protein could be detected only in LLC-PK1 cells, suggesting NHERF1 contributes to the membrane localization of ABCC4. High ectopic expression of NHERF1 in MDCKI cells appeared to redirect ABCC4 away from the basolateral toward the apical

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membrane. Thus, the localization of ABCC4, by interactions with NHERF1, seems to depend on either the cell type and/or a cell's ability to polarize. These data suggest that in non-polarized cells, NHERF1 promotes transporter internalization, but in polarized cells, NHERF1 facilitates ABCC4 trafficking to the apical membrane. However, what happens to ABCC4 localization when NHERF1 is knocked-down in polarized cells and where it is expressed is currently unknown. Moreover, given NHERF1 has more than one PDZ, and the ABCC4 PDZ-motif binds to a single PDZ-domain, it would be important to know the other proteins in the NHERF1, ABCC4 complex. These might influence ABCC4 function or activity.

Another PDZ adaptor protein, sorting nexin 27 (SNX27) was also identified in a pull-down assay to bind to ABCC4 (Hayashi et al., 2012). Knockdown of SNX27 in HEK293 cells led to increased plasma membrane expression and transport activity of ABCC4. SNX27 siRNA-mediated knock-down leads to a decrease in ABCC4 internalization; this combined with SNX27's localization to endosomes suggests that SNX27 promotes ABCC4 internalization from the membrane.

To determine if single-domain PDZ-domain proteins impacted ABCC4 function in relation to disease (in this case acute myeloid leukemia), we elucidated a new functional relationship between ABCC4 and membrane palmitoylated protein 1 (MPP1), a gene associated with poor survival in acute myeloid leukemia; this interaction enhanced both leukemogenesis and ABCC4 chemoresistance (Pitre et al., 2017). ABCC4 and MPP1 reciprocally

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immunoprecipitated one another in myeloid leukemia cell lines, and removal of the C-terminal PDZ motif from ABCC4 prevented this interaction. In vitro mapping studies indicated ABCC4 interacted only required the MPP1 PDZ domain. This interaction stabilized ABCC4 at the plasma membrane, as degradation of surface ABCC4 was reduced when MPP1 was co-expressed, and transport activity increased as measured by cell sensitivity to ABCC4 substrates 6-mercaptopurine (6MP) and cytosine arabinoside (Ara-C). Loss of MPP1 via CRISPR-Cas9 genetic ablation decreased 6MP resistance and ABCC4 surface expression, further validating that MPP1 is crucial for endogenous ABCC4 plasma-membrane localization, and function in myeloid leukemia. Subsequently, a small molecule (TR-FRET) screen was developed to identify drugs capable of disrupting this interaction; 11,297 chemicals were tested and 144 unique disrupting compounds were identified. Further refinement led to the characterization of 17 compounds that displayed dose-response activity with Antimycin A being the most potent. Antimycin A treatment re-capitulated the effects of Mpp1 gene deletion or competition with ABCC4-PDZ peptides, - i.e., Antimycin A sensitized cells to the ABCC4 substrate 6MP and blocked growth of leukemic progenitors. These data demonstrate that drugs can disrupt protein-protein interactions can alter membrane localization and function of ABCC4 and suggest that other reported inhibitors might act through a similar mechanism.

Perspective:

ABC transporters impact the cellular concentrations of drugs, xenobiotics and endogenous compounds. Direct interactions between drugs, acting as

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substrates and/or inhibitors, and ABC transporters, often referred to as “victim” and “perpetrator” interactions, are well-known to affect therapeutic outcomes, generally in a negative manner. In this review, we have tried to briefly provide an overview that illustrates how biological factors alter the function of transporters; these pathways regulating transporter expression, trafficking, and activity are less well understood. By focusing on kinases, we have illustrated how drug-induced alterations in receptor kinase activity, can have myriad effects, from altering transcription (and possibly translation), to altering the phosphorylation of the transporters themselves. The effects of phosphorylation on a transporter are idiosyncratic to the transporter, some transporters have increased expression and function, while others are diminished in both. These pathways, by-and-large have not been previously elucidated or are understudied. Further, we have shown how protein-protein interactions via specific domains can alter the function of ABC transporters. Post-translational regulation of ABC transporters requires further study, as relatively little is known about how many of these clinically relevant proteins are regulated and/or trafficked. By extension, we propose that the clinical implications of regulation of transporter expression, degradation, trafficking, phosphorylation, and protein-protein interaction need to be considered in drug development.

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Acknowledgements:

We would like to thank all of the members of the Schuetz lab for the suggestions to improve this manuscript.

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Contributed to new reagents or analytic tools:

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Footnotes:

a) This work was supported by the National Institutes of Health National Cancer Institute [Grants R01CA194057, P30 CA21745, CA21865, CA194057, and CA096832] and the American Lebanese Syrian Associated Charities (ALSAC).

b) Person to receive reprint requests: John D. Schuetz, Ph.D., Department of Pharmaceutical Sciences, 262 Danny Thomas Place, MS 313; St. Jude Children's Research Hospital, Memphis, TN 38105; Phone: (901) 595-2205; Fax: (901) 595-3125.

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Figure Legends:

Figure 1: Indirect Mechanisms of Drug Interactions with ABC Transporters

Drugs influencing the activity of ABC transporters by factors distinct from direct binding to the transporter. These mechanisms include (A) regulation of mRNA and protein expression through influences on transcription, translation, and/or proteasomal degradation, (B) regulation of protein trafficking leading to changes in stabilization of the transporter on the membrane and/or internalization, or (C) regulation of transporter activity through protein interactions or phosphorylation by kinases.

Figure 2: EGFR Effects on ABC Transporters

EGFR signals through both MAPK and PI3K pathways to effect ABC transporters, differentially, at the transcriptional, translational, and post-translational levels. Following ligand binding, EGFR dimerizes and signals through its tyrosine kinase domain to lead to the phosphorylation and activation MEK, which in turn phosphorylates and activates ERK. ERK signaling increases the mRNA and protein expression of ABCG2, decreases the mRNA and protein expression of ABCC4, and decreases the mRNA expression of ABCB1 while preventing the degradation of ABCB1 protein by the proteasome. PI3K can also be activated downstream of EGFR tyrosine kinase activity leading to the activation of Akt. Akt signaling then increases the mRNA expression of ABCG2, ABCB1, and ABCC4, and also increases the protein expression and membrane localization of ABCG2. PI3K signaling can be inhibited by PTEN; PTEN is

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activated by PPARγ signaling. Pharmacological inhibitors used to elucidate this pathway have been noted at the level of their inhibition. mRNA changes induced by the ERK pathway are noted in blue while changes induced by the Akt pathway are noted in orange.

Figure 3: Predicted Phosphorylation Sites on ABCB1, ABCC1, ABCG2 and ABCC4

Potential post-translational modifications of the indicated proteins were performed using the default parameters at the following website: www.phosphosite.org

Figure 4: ABCC4 PDZ Motif Binds to a Variety of PDZ-domain Containing Proteins

(**Upper panel**): Overview of the workflow for PDZ arrays. The purified PDZ domains from the indicated PDZ-domain proteins were been immobilized, in duplicate, on the array membrane (Panomics). Biotin-containing ABCC4 purified peptide harboring the ABCC4 PDZ motif was incubated with the array membrane. Unbound ABCC4 was then washed away and bound ABCC4 detected using streptavidin-HRP; The ABCC4 was peptide: biotin-KSGSG-STLTIFETAL$_{COOH}$ (Blue = linker, orange= spacer; green=PDZ motif) (**Lower panel**): Results of three different PDZ arrays. Proteins that have been immobilized on the membrane are noted in table to the right, with positive binding indicated by the bold font.

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Tables

Table 1. FDA-Approved Tyrosine Kinase Inhibitors and Known Interactions with ABC Transporters.

Generic Name	*Brand Name*	*Kinase Target*	*Use*	*In vitro Transporter interactions*
Afatinib	Gilotrif	EGFT, HER2, HER4	NSCLC	ABCB1 substrate and inhibitor
Alectinib	Alecensa	ALK, RET	ALK-positive, metastatic NSCLC	May inhibit ABCB1 and ABCG2
Axitinib	Inlyta	VEGFR 1-3	Renal Cell Cancer, advanced	ABCB1 inhibitor, but not at therapeutic levels
Bosutinib	Bosulif	BCR-ABL, Src, Lyn, Hck	CML, resistant	
Cabozantinib	Cometriq, Cabometyx	RET, MET, VEGFR 1-3, KIT, TRKB, FLT-3, AXL, ROS1, TYRO3, MER, and TIE2	Metastatic medullary thyroid cancer, Renal cell cancer	MRP2 Substrate
Ceritinib	Zykadia	ALK, IGF-1R, InsR, and ROS1	ALK-positive, metastatic NSCLC	ABCB1 substrate
Cobimetinib	Cotellic	MEK1 and MEK2	Melanoma with BRAF mutations V600E or V600K	ABCB1 substrate
Crizotinib	Xalkori	ALK, HGFR, c-Met, c-Ros, RON	Metastatic NSCLC	ABCB1 inhibitor
Dabrafenib	Tafinlar	BRAF, CRAF, SIK1, NEK11, LIMK1	Melanoma or NSCLC with BRAF mutation	ABCB1, ABCG2 substrate, ABCG2 inhibitor
Dasatinib	Sprycel	BCR-ABL, Src, LCK, YES, FYN, c- KIT, EPHA2, and PDGFRβ	Philadelphia chromosome positive CML and ALL	ABCB1 substrate
Erlotinib	Tarceva	EGFR	Metastatic NSCLC, Pancreatic Cancer	
Gefitinib	Iressa	EGFR, IGF, PDGF	Metastitic NSCLC with EGFR mutation	ABCB1 substrate
Ibrutinib	Imbruvica	BTK	Mantle Cell Lymphoma, CLL, SLL, Waldenström's macroglobulinemia, marginal zone lymphoma, chronic Graft versus host disease	May inhibit ABCB1 and ABCG2

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Idelalisib	Zydelig	PI3Kδ	Relapsed CLL, Follicular B-cell non- Hodgkin lymphoma, SLL	ABCB1, ABCG2 substrate, ABCB1 inhibitor
Imatinib	Gleevec	BCR-ABL, c-Kit, PDGF, SCF	Philadelphia chromosome positive CML and ALL, myelodysplastic/myeloproliferative diseases associated with PDGFR gene rearrangements, aggressive systemic mastocytosis, hypereosinophilic syndrome, chronic eosinophilic leukemia, dermatofibrosarcoma protuberans, GIST	
Lapatinib	Tykerb	EGFR, HER2	Breast Cancer, Her2 positive	ABCB1 and ABCG2 inhibitor
Lenvatinib	Lenvima	VEGFR 1-3, FGFR 1-4, PDGFR , c- Kit, RET	Differentiated Thyroid Cancer, Renal Cell Cancer	ABCB1 and ABCG2 substrate, ABCB11 inhibitor
Nilotinib	Tasigna	BCR-ABL, PDGFR, c-KIT, CSF-1R,and DDR1	Philadelphia chromosome positive CML	ABCB1 inhibitor
Niraparib	Zejula	PARP1 and -2	Ovarian, fallopian tube, or primary peritoneal cancer	Weak ABCG2 inhibitor; ABCB1 and ABCG2 substrate
Olaparib	Lynparza	PARP 1-3	Recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer, advanced ovarian cancer	ABCG2 and ABCB1 inhibitor, ABCB1 substrate
Osimertinib	Tagrisso	EGFR, HER 2-4, ACK1, and BLK	Metastatic, EGFR mutated, NSCLC	ABCG2 and ABCB1 substrate, ABCG2 inhibitor
Palbociclib	Ibrance	CDK4/6	Breast Cancer, Hormone Receptor positive, Her2 negative	Weak ABCB1 and ABCG2 inhibitors
Pazopanib	Votrient	VEGFR 1-3, FGFR 1,3, Kit, Itk, Lck, c-Fms, PDGFRβ	Renal Cell Cancer, Soft tissue sarcoma	ABCB1 and ABCG2 substrate
Ponatinib	Iclusig	ABL, BCR-ABL, VEGFR, PDGFR, FGFR, SRC, KIT, RET, TIE2, and FLT3	CML, ALL	ABCB1, ABCG2, and ABCB11 inhibitor

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Regorafenib	Stivarga	RET, VEGFR 1-3, KIT, PDGFR , PDGFRβ, FGFR1-2, TIE2, DDR2, TRKA, Eph1A, RAF-1, BRAF, SAPK2, PTK5, Abl, CSF1R	Colorectal Cancer, GIST, hepatocellular carcinoma	ABCG2 inhibitor
Ribociclib	Kisqali	CDK4/6	Breast Cancer, Hormone Receptor positive, Her2 negative	ABCG2, ABCB11 inhibitor
Rucaparib	Rubraca	PARP 1-3	Ovarian Cancer, Advanced	ABCB1, ABCG2 substrate and inhibitor, ABCC4 inhibitor at ultra-therapeutic concentrations
Ruxolitinib	Jakafi	JAK1 and 2	myelofibrosis, polycythemia vera	
Sorafenib	Nexavar	c-CRAF, BRAF, KIT, FLT-3, RET, RET/PTC, VEGFR 1-3, PDGFRβ	Renal Cell carinoma, hepatocellular carcinoma, refractory thyroid carcinoma	ABCB1 inhibitor
Sunitinib	Sutent	PDGFR and β, c-Ki, FLT3, CSF- 1R, RET, VEGFR2	GIST, renal cell carcinoma, pancreatic neuroendocrine tumors	
Tofacitinib	Xeljanz	JAK1-3,	Rheumatoid arthritis	
Trametinib	Mekinist	MEK1-2	Malignant Melanoma or NSCLC with BRAF mutation	ABCB1, ABCB11 substrate
Vandetanib	Caprelsa	EGFR, VEGFR, RET, BRK, TIE2, EPHR, Src	Medullary thyroid cancer	ABCB1 inhibitor
Vemurafenib	Zelboraf	BRAF, CRAF, ARAF, SRMS, ACK1, MAP4K5, and FGR	Melanoma or Erheim-Chester Disease with BRAF V600 mutation	ABCB1 and ABCG2 substrate and inhibitor

Table 1. FDA-Approved Tyrosine Kinase Inhibitors and Known Interactions with ABC Transporters. All information is from labeling information that has been submitted to the FDA by the manufacturer.

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Table 2: Experimentally Identified Phosphorylation sites on ABCB1, ABCG2, and ABCC1

Gene	Species	Modification	Effect of Modification	Reference
ABCB1	H. sapiens	S661	PKC Regulation of transport activity	Chambers TC, Pohl J, Raynor RL and Kuo JF (1993) Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268**:4592-4595.
		S667	PKA; PKC Regulation of transport activity	Chambers TC, Pohl J, Glass DB and Kuo JF (1994) Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. *Biochem J* **299 (Pt 1)**:309-315. Chambers TC, Pohl J, Raynor RL and Kuo JF (1993) Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268**:4592-4595.

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		S671	PKA; PKC Regulation of transport activity	Chambers TC, Pohl J, Glass DB and Kuo JF (1994) Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. *Biochem J* **299 (Pt 1)**:309-315. Chambers TC, Pohl J, Raynor RL and Kuo JF (1993) Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268**:4592-4595.
		S683	PKA Regulation of transport activity	Chambers TC, Pohl J, Glass DB and Kuo JF (1994) Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. *Biochem J* **299 (Pt 1)**:309-315.

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				Chambers TC, Pohl J, Raynor RL and Kuo JF (1993) Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268**:4592-4595.
		*ni	Pim-1 Regulation of membrane localization	Xie Y, Burcu M, Linn DE, Qiu Y and Baer MR (2010) Pim-1 kinase protects P-glycoprotein from degradation and enables its glycosylation and cell surface expression. *Mol Pharmacol* **78**:310-318.
ABCG2	H. sapiens	T362	Pim-1 Modulation of dimerization	Xie Y, Xu K, Linn DE, Yang X, Guo Z, Shimelis H, Nakanishi T, Ross DD, Chen H, Fazli L, Gleave ME and Qiu Y (2008) The 44-kDa Pim-1 kinase phosphorylates BCRP/ABCG2 and thereby promotes its multimerization and drug-resistant activity in human prostate cancer cells. *J Biol Chem* **283**:3349-3356.
ABCC1	H. sapiens	T249	CK2α Negative regulation of transport activity	Stolarczyk EI, Reiling CJ, Pickin KA, Coppage R, Knecht MR and Paumi CM

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				(2012) Casein kinase 2alpha regulates multidrug resistance-associated protein 1 function via phosphorylation of Thr249. *Mol Pharmacol* **82**:488-499.

*ni= not identified

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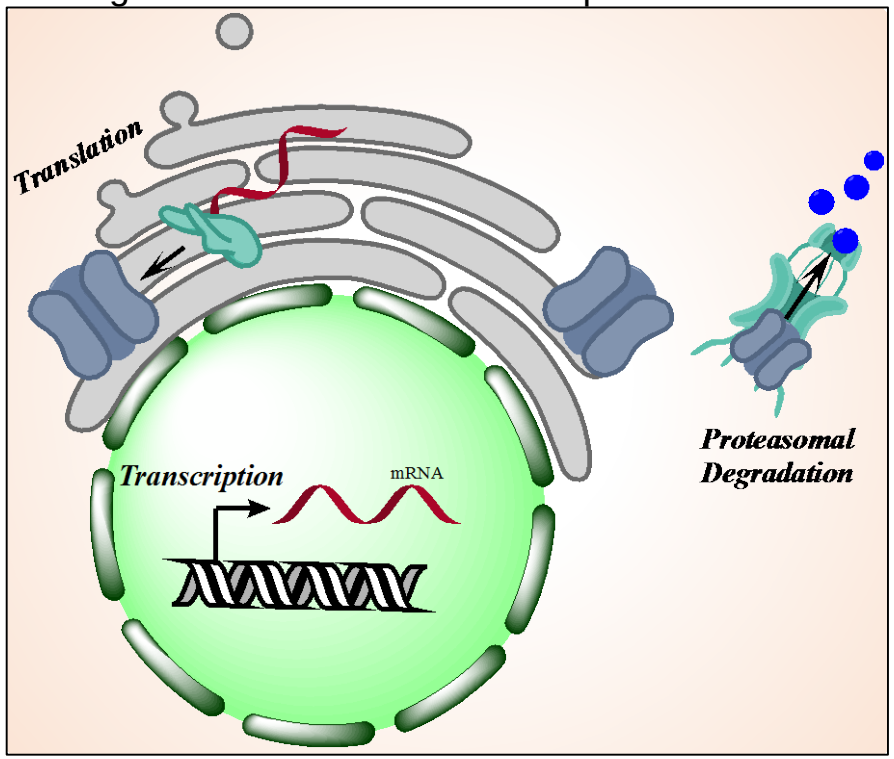
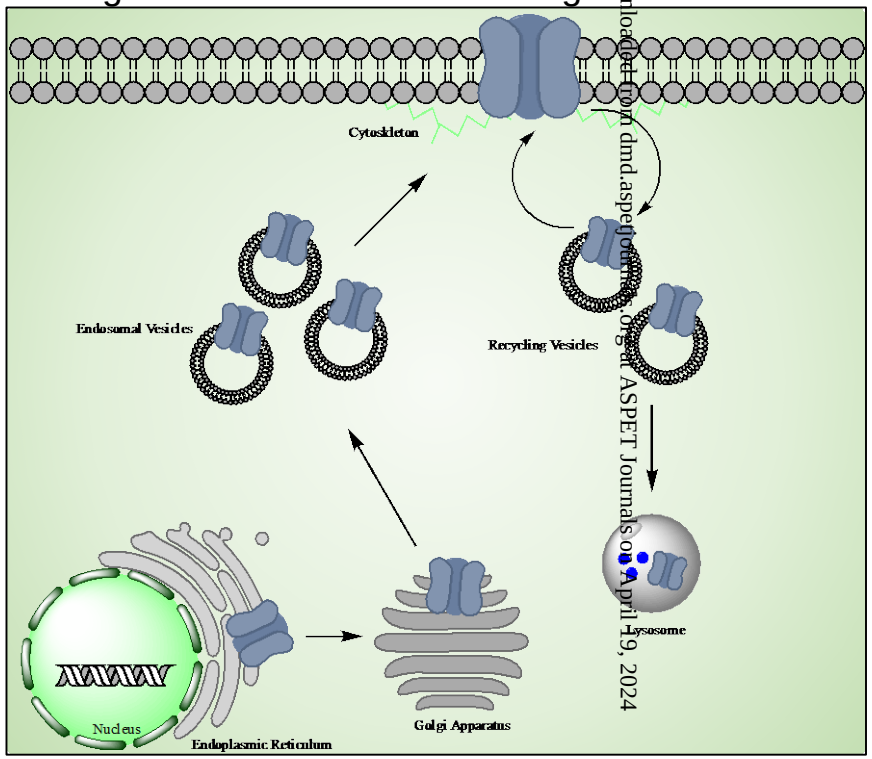
A. Regulation of mRNA/Protein Expression



B. Regulation of Protein Trafficking



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C. Regulation of Transporter Activity

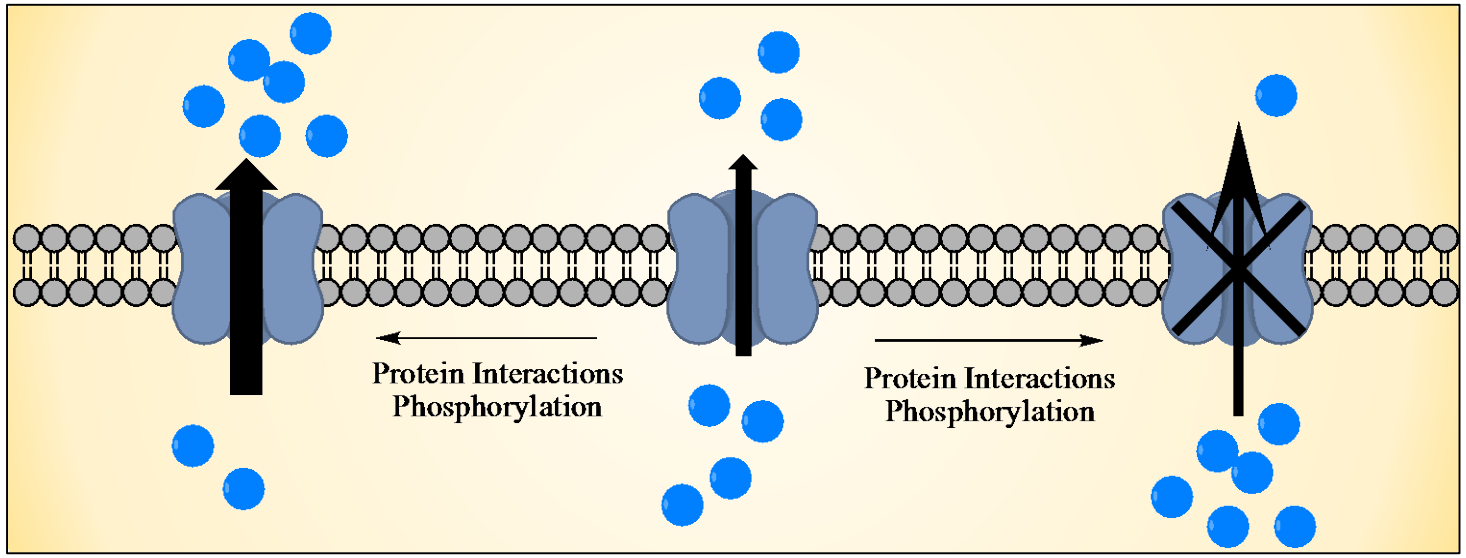

Figure 1: Indirect Mechanisms of Drug Interactions with ABC Transporters

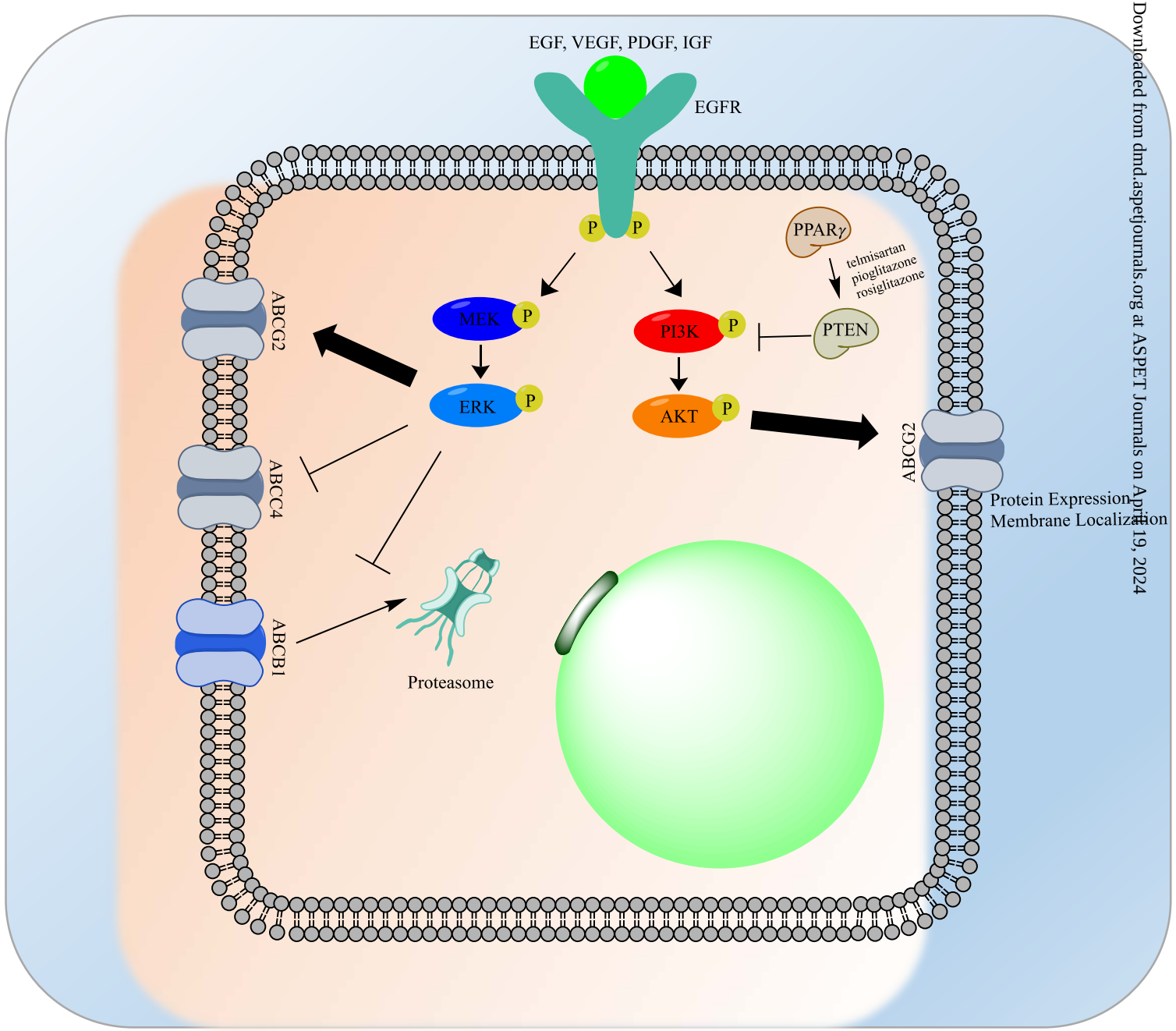

Figure 2: EGFR Effects on ABC Transporters

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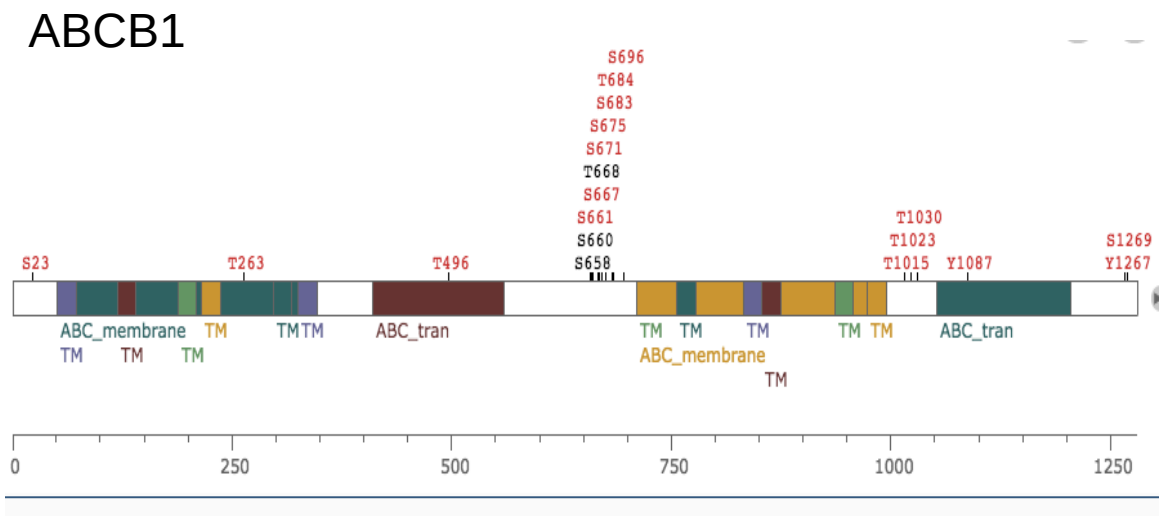

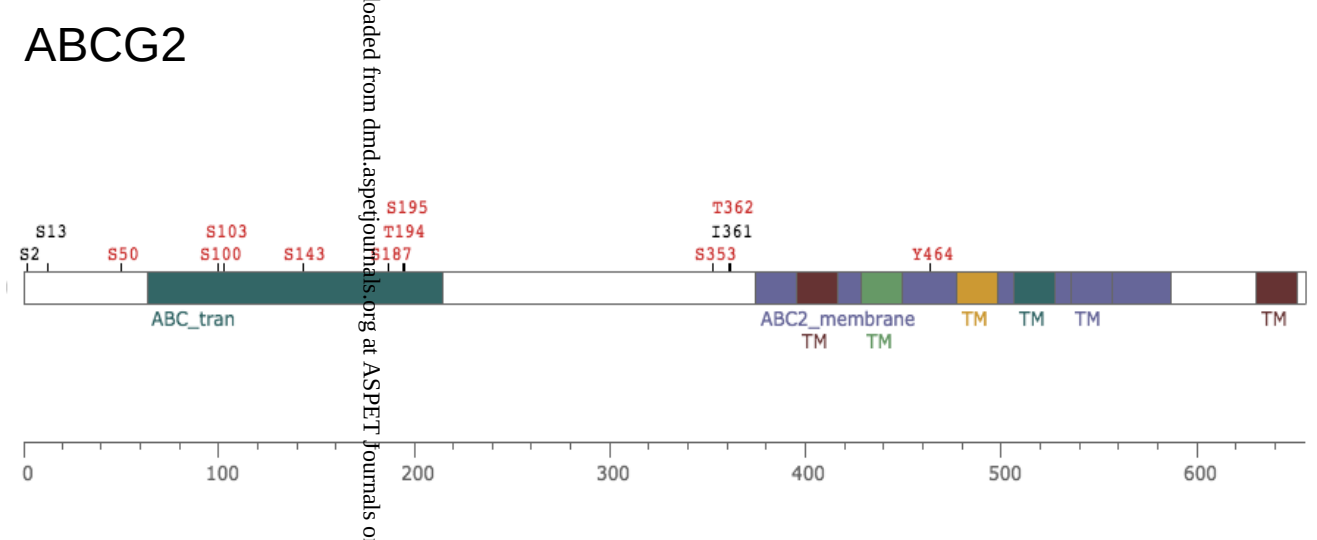

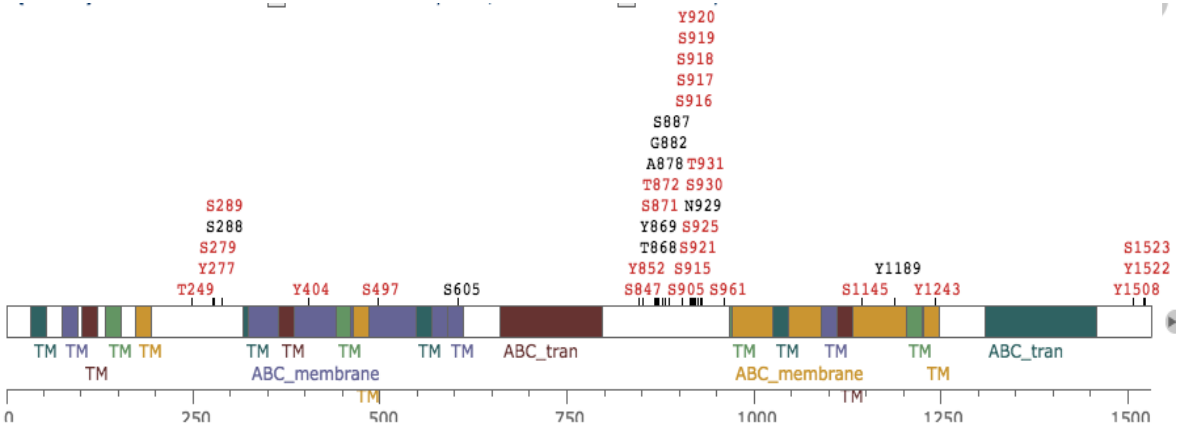

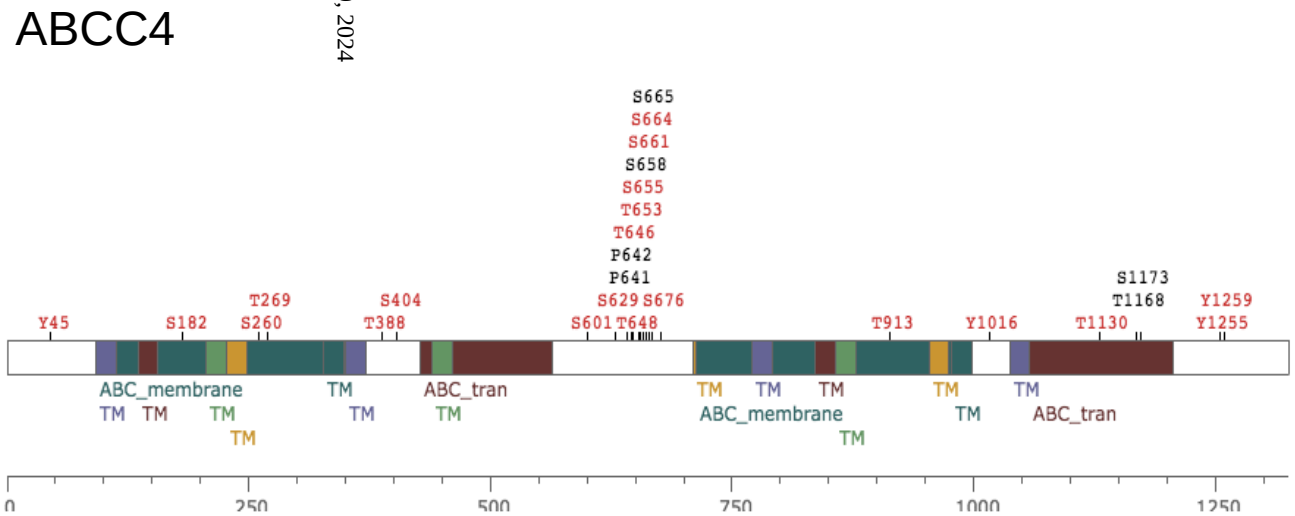

Figure 3: Predicted Phosphorylation Sites on ABCB1, ABCC1, ABCG2 and ABCC4

https://www.phosphosite.org/proteinAction.action?id=1819900&showAllSites=false

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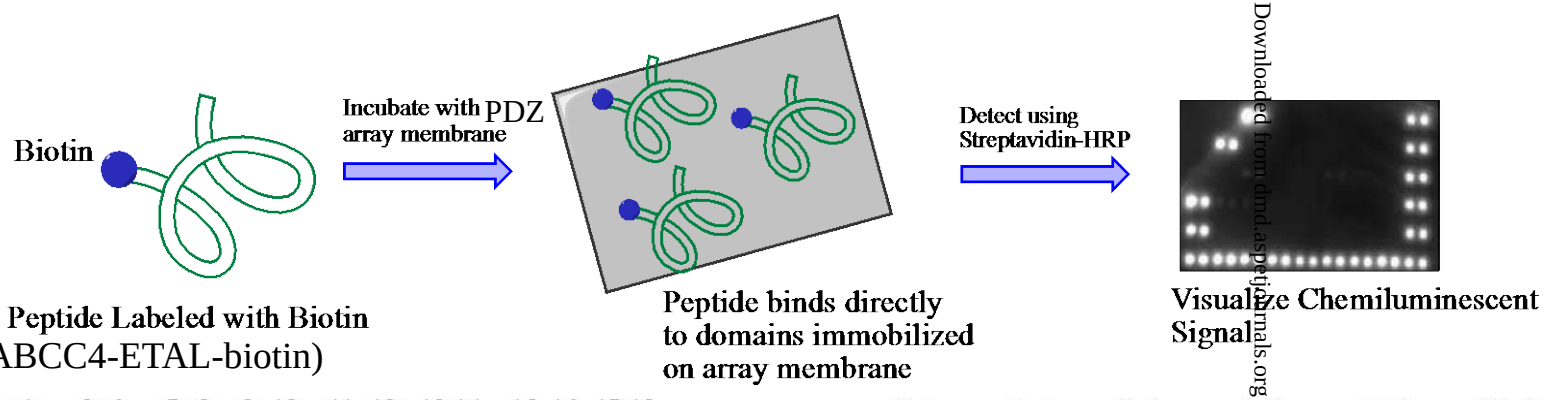

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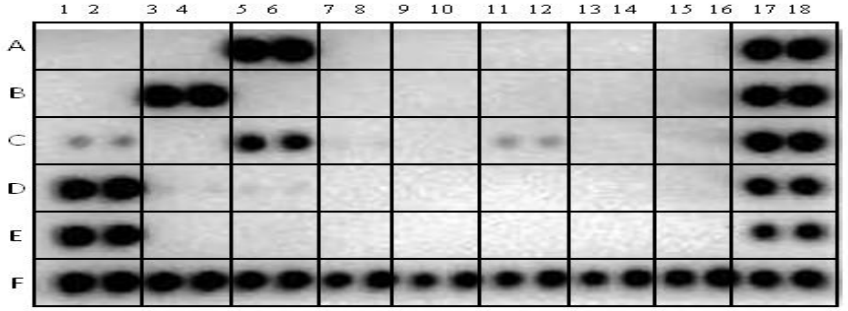
A



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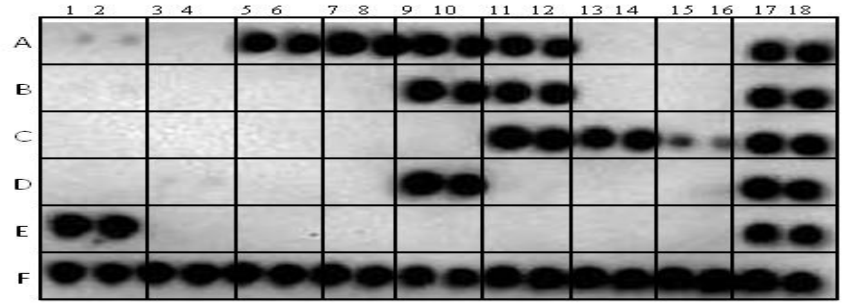

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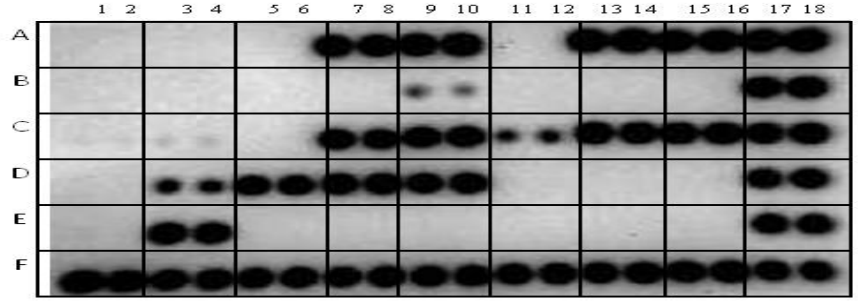

D

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18
A	KIAA0300-D6	KIAA0303	**KIAA0316**	KIAA0559	KIAA0613	KIAA1719-D7	KIAA1719-D4	KIAA1719-D3	**POS**
B	KIAA1526-D3	**MAST205**	KIAA1849	PALS1-D8	PALS1-D9	PALS1-D1	PALS1-D2	PALS1-D5	**POS**
C	**PALS1-D3**	MGC5395	**FLJ23209-D1**	**FLJ23209-D2**	KIAA1719-D6	**FLJ00011**	E3KARP-D1	E3KARP-D2	**POS**
D	**NHERF1-D1**	**ZO1-D1**	**ZO1-D2**	SDB-D1	SDB-D2	IL16(2)-D3	LNX1-D3	NOS1	**POS**
E	**PDZ-pos**	GST							**POS**
F	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**

E

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18
A	**MAGI3-D2**	MAGI3-D4	**MAGI3-D5**	**MAGI3-D6**	**BAI1-D2**	**BAI1-D3**	BAI1-D4	BAI1-D1	**POS**
B	BAI1-D6	AIP-D2	AIP-D3	AIP-D4	**AIP-D5**	**AIP-D6**	hPTP1E-D2	hPTP1E-D3	**POS**
C	hPTP1E-D4	PTPN4	GRIP 1 –D4	GRIP 1 –D3	GRIP 1 –D2	**SCRIB1-D1**	**SCRIB1-D2**	**SCRIB1-D4**	**POS**
D	PARD3-D2	**PARD3-D3**	HARM-D3	MLL4	**TIP1**	SDB2-D2			**POS**
E	**PDZ-pos**	GST							**POS**
F	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**

F

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18
A	MUPP1-D6	MUPP1-D12	MUPP1-D2	**MUPP1-D3**	**MUPP1-D13**	MUPP1-D1	**DLG3-D2**	**DLG3-D3**	**POS**
B	DLG5-D1	DLG5-D4	DLG5-D3	DLG5-D2	**DLG2-D3**	PAR6B	LIK1	LOMP	**POS**
C	RIL	**A2LIM**	TIAM1	**LIN7C**	**LIN7B**	**LIN7A**	**GEF11**	**GEF12**	**POS**
D	PDKZK1-D1	**PDZK1-D2**	**SNB1**	**SNA1**	**SHK1**	MPP6	PIST	GEF2	**POS**
E	RIM2	**PDZ-pos**	GST						**POS**
F	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**

Figure 4: ABCC4 PDZ Motif Binds to a Variety of PDZ-domain Containing Proteins