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# Trials and Tribulations in the Expression and Purification of ABCG2

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*May those who follow their fate be granted happiness;*
*May those who defy it be granted glory.*

# Abstract

ABC (ATP binding cassette) transporters are a diverse superfamily of membrane proteins, which couple the transport of substrate with the hydrolysis of ATP. ABCG2 (ATP binding cassette G2, also known as Breast Cancer Resistance Protein (BCRP), is a well-known multidrug efflux pump that transports a wide range of structurally unrelated chemotherapeutic drugs, such as mitoxantrone, methotrexate, topotecans, flavopiridol and tyrosine kinase inhibitors to name a few. Physiologically, ABCG2 is expressed and localized in different tissues of the body such as the placenta, gut and blood-brain-barrier where it plays a protective role and provides a first line of defence against environmental toxins. A single nucleotide polymorphism (SNP) in ABCG2 has been shown to be a significant causative aspect in the development of gout due to impaired urate transport.

Although several studies have been performed on this membrane protein, data regarding its structure is still missing. Thus, the main aim of this study was to bridge this knowledge gap by taking the first step and developing a robust purification method for heterologously expressed ABCG2. Initially, $His_6$-tagged ABCG2 was overexpressed in Sf9 insect cells, and solubilised using a novel detergent free nanodisc-forming polymer styrene maleic acid (SMA). Although, solubilisation was remarkably effective, with the majority of ABCG2 being solubilised, purification using Ni-NTA resins was unsuccessful, potentially due to occlusion of the His tag. Thus, ABCG2 constructs expressing Strep and Strep-$His_{13}$-tag were engineered in order to offer alternative affinity chromatography possibilities. Both constructs were well expressed in Sf9 cell membranes and again could be easily solubilized with SMA. However, purification using either metal affinity chromatography or streptactin affinity chromatography again gave the same negative result. Finally, a classical detergent based protein extraction using a mild detergent (dodecyl-β-D-maltopyranoside) along with *E. coli* total lipid extract was employed. This resulted in partial solubilisation of ABCG2, but notably the ABCG2 could bind to nickel resins and be enriched, laying the groundwork for optimization and further purification.

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# Declaration

**School of Life Sciences (SoLS)**

The work presented in this thesis entitled "Trials and tribulations in the Expression and Purification of ABCG2" is the outcome of my own work, except where due references has been made in the text. Technical assistance, and collaborations where ever relevant, has been acknowledged.

This work has been undertaken during my period of study for this degree, MRes in Molecular Cell Biology at The University of Nottingham, under the guidance of Drs. Ian Kerr and Robert Layfield.

I understand that the nature of plagiarism is a serious academic offence. Thus, I confirm that no material in this project has been plagiarized.

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# Abbreviation

| | |
|---|---|
| **ABC** | ATP-binding cassette |
| **ADP/ATP** | Adenosine di/tri-phosphate |
| **BSA** | Bovine serum albumin |
| **DDM** | dodecyl-β-D-maltopyranoside |
| **DMSO** | Dimethyl sulfoxide |
| **DNTP** | Deoxynucleotide solution mix |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EM** | Electron Microscopy |
| **FCS** | Foetal calf serum |
| **GFP** | Green fluorescent protein |
| **H33342** | Hoechst 33342 |
| **HEK293T** | Human embryonic kidney 293 cells with SV40 large T antigen |
| **MalK** | ABC subunit of maltose transporter |
| **MDR** | Multidrug resistance |
| **MRP1** | Multidrug resistance protein 1 |
| **NBD** | Nucleotide-binding domain |
| **NCBI** | National Centre for Biotechnology Information |
| **P-gp** | P-glycoprotein |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **SDS** | Sodium dodecyl sulphate |
| **SMA** | styrene maleic acid |
| **SBP** | Solute Binding Protein |
| **TBE** | Tris/Borate/EDTA buffer |
| **TMD** | Transmembrane domain |
| **TPE** | Tris-Phosphate EDTA |
| **UV** | Ultraviolet |
| **WT** | Wildtype |

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# Chapter 1: Introduction

Cell membranes are important as they protect and organize cells; they not only enclose the cell and define its boundaries but also maintain the differences between the cytosol and the extracellular environment which is essential for its functions. The hydrophobic lipid membranes play a key role in the integrity of the cells by limiting the movement of substances across the living cells. However, transport of specific substances across this barrier is essential for cell survival. For this purpose, membranes have specialized proteins associated with membrane transport functions. These transport systems allow uptake of specific molecules and removal of unwanted compounds from the cell. Such transport systems render the membranes selectively permeable for the substances. A transport system associated with the removal of compounds from cells is the focus of this thesis.

## 1.1 Multidrug transporters

Cells or organisms can develop the ability to resist the cytotoxic effects of a diverse range of chemical structures, including therapeutic drugs with different intracellular targets, by developing multiple mechanisms. These mechanisms include enzymatic metabolism of the drug, up-regulation of a compensatory, drug counteracting cellular process, increased repair of damage caused by the drug or alteration of the molecular target site to prevent drug binding (Gottesman, 2002, Wong et al., 2014). But the most common mechanism often used by cells is the direct export of the drugs or toxic compounds out of the cell. This phenomenon of acquiring resistance to many distinct drugs due to the direct physical export of the compound from within the cell through an efflux pump mediated transport process is known as multidrug resistance (MDR) (Leonard et al. 2003; Szakács et al., 2008).

One of the main concerns of MDR is the associated failure of cancer chemotherapy. Many of these efflux pumps are known to be overexpressed in cancerous tissues which play a major role in reducing the anticancer drug accumulation resulting in the poor prognosis along with reduced rates of remission and low efficacy of conventional chemotherapy treatments (Chan et

al., 1991; Hornicek et al., 2000; van den Heuvel-Eibrink et al., 2000). Among the well-known MDRs, several human ATP binding cassette (ABC) proteins have been well described to play a vital role. Three of the most significant ones, ABCB1 (P-glycoprotein), ABCC1 (multidrug resistance protein 1) and ABCG2 (breast cancer resistance protein) have been investigated critically.

## 1.2 ATP binding cassette transporters: an introduction

### 1.2 ATP binding cassette transporters

ATP-binding cassette (ABC) transporters are a large superfamily of membrane proteins which includes a variety of active transporters with diverse functions (Higgins 1992). They are ubiquitous in biology and power the translocation of large number of structurally different substrates across the membrane using the energy derived from ATP hydrolysis (Linton et al., 2007).

ABC transporters function as either importers (Rice et al., 2014) or exporters (Fath et al., 1993). The importers bring necessary nutrients and other molecules into cells whereas the exporters pump toxins, drugs and lipids outside across membranes. Whereas exporters are commonly found in both eukaryotes and prokaryotes, importers seem to be largely present in prokaryotic organisms (Rees, Johnson et al. 2009). However, in plants, AtABCB14 a novel ABC importer are present for transporting malate that modulates stomatal closure on transition to elevated $CO_2$. And in mammals ABCA4, found in retinal photoreceptor transports *N*-retinylidene-phosphatidylethanolamine from the lumen to the cytoplasmic leaflet of disc membranes thereby facilitating the removal of potentially toxic retinoid compounds from photoreceptors (Quazi et al., 2012, Lee et al., 2008).

Recently, two ABC transporters in yeast associated with transport of sterols were shown to promote either influx or efflux of a cholesterol derivative depending on the sterol context of the cell. This is a rare example of an ABC transporter mediating bi- directional transport, suggesting that direction of transport is not a static inherent property of the transporter, but rather that it is mutable and influenced by surrounding sterols and proteins (Gulati, et al., 2011).

ABC transporters are widespread in archaea, eubacteria and eukaryotes and play a wide variety of physiological roles in all species of living organisms such as maintenance of osmotic homeostasis, nutrient uptake, resistance to xenotoxins, antigen processing, cell division, bacterial immunity, pathogenesis and sporulation, cholesterol and lipid trafficking and in the development of stem cells (Jones and George, 2004). Apart from these physiological roles, ABC transporters are also associated with a variety of clinical problems such as cystic fibrosis (a chronic, life-threatening lung disease) and multidrug resistance (chemotherapy drugs) (Higgins, 1992).

Despite the large variety of substrates and physiological processes in which they are involved, for proper cellular function, ABC transporters contain a common core functional unit, comprising of at least 4 domains, which consists of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). The TMDs are made up of typically (but not exclusively) six hydrophobic α-helices, which are essential for the formation of a pathway for vectorial transport of solutes (Mao Q., & Unadkat J.D., 2015). The two NBDs are peripherally located at the cytoplasmic face of the membrane and are linked to the TMDs, either covalently within the same polypeptide or non-covalently in many bacterial ABC systems on a different polypeptide. In prokaryotes, the four domains are often encoded by different genes, whereas in higher organisms these domains are frequently (but not exclusively) encoded in the same gene (Schmitt and Tampe 2002).

The NBDs are hydrophilic by nature and function in the binding and hydrolysis of each ATP molecule (Aronica et al., 2005). The structure of the NBD is further divided into the two fundamental domains: a catalytic core domain and a more structurally diverse α-helical domain. The NBDs of all the unrelated ABC transporters, have a high level of sequence similarity is present specifically in 7 conserved regions, critical for defining these transporters function (Lawson and Kerr, 2008). These consists of the P-loop or Walker A motif, the Walker B motif, Q-loop, H-motif, C- signature motif, A- loop and D- loop.

The conserved P-loop or Walker A motif (GXXGXGK(S/T) where X could be any amino acid residue), that interacts with the phosphate groups of the

nucleotide while the Walker B motif (φφφφD, of which φ is a hydrophobic residue), contains a glutamate residue that act as a general base to activate a water molecule for a nucleophilic attack on the γ- phosphate of the ATP. These distinguishing motifs, are present in all nucleotide binding proteins and are involved in the ATP hydrolysis by the formation of hydrogen bonds with nucleotide phosphates, and the nucleophilic attack via coordination of $Mg^{2+}$ ions (Zhang et al., 2003).

The Q-loop that consists of a conserved glutamine residue, functions by sensing the γ phosphate of ATP and contributes to the contact interface with the TMD, the H-motif (or switch region) contributes to the catalytic reaction due to the presence of a histidine residue (Beis, 2015; Lawson et al*.,* 2008, Aronica et al., 2005). The α-helical domain also contains a conserved LSGGQ sequence also known as "C" signature motif which is characteristic for the ABC ATPases involved in the binding of the nucleotide (Higgins and Linton 2004; Deeley, Westlake et al. 2006; Rees, Johnson et al. 2009). An A- loop, containing a conserved aromatic acid residue, which in the bound state stacks against ATP adenine and a D- loop, containing a conserved aspartic acid residue which is involved in the contact interface between the 2 NBDs (Beis, 2015, Lawson et al*.,* 2008).

Unlike the NBDs, TMDs of the ABC transporters are not conserved, the primary sequences are evidently variable compared to those of the NBDs. Since these membrane spanning α helical segments provide a translocation pathway through which the substrate crosses the membrane, their variable sequences are also believed to be involved in the substrate binding thus explaining the enormous substrate diversity of the transporters.

Apart from this typical four domain structure, sometimes additional elements can fuse to the TMDs and/or NBDs of ABC transporters. Most of them are believed to be associated with some regulatory functions although the exact function of these extra domains is mostly unclear (Biemans-Oldehinkel, Doeven et al., 2006). For example, the R domain of the ABCC7 (CFTR) which contains multiple protein kinase A sites is thought to be responsible for channel gating, but the exact mechanism is still unknown (Riordan et al., 1989; Winter and Welsh, 1997, Hegedűs et al., 2009). Also the extra cytoplasmic

solute binding proteins (SBPs) in bacteria are important in recognizing substrates (mostly nutrients) with high affinity, for example the bacterial maltose and vitamin $B_{12}$ importer systems (Davidson et al., 2008).

There are 48 known human ABC proteins, which have been placed into 7 distinct subfamilies (A-G) depending on their primary sequences. They also play various physiological roles due to the vast number of substrates that are collectively transported by them which includes charged molecules, hydrophobic compounds, sugars, lipids, amino acids, small proteins and metabolites (Higgins et al., 2001, Theodoulou et al., 2015, Vasiliou et al., 2009).




**Figure 1.1 Sequence alignment of the NBDs of human ABCG2, human and mouse P-gp (MDR1), human MDR3, mouse MDR1a (MDR3), Sav1866 and rat TAP1.** Consensus motifs of the NBDs are coloured as follows: A-loop Green; Walker-A orange; Q-loop pink; C-signature motif blue; Walker-B yellow; D-loop purple and H-loop red. Sequences were aligned by using Clustal Omega and the multiple alignment was not manually adjusted. Figure has been produced by the author, with reference to Lawson and Kerr 2008.

### 1.2.1 ABCG2 structure and oligomerization

The ABCG family in humans (with 5 members, ABCG1, G2, G4, G5 and G8) is involved in the transport of lipids, urate, haem, drugs and plant sterols. The ABCG2 protein is the focus of this work and so will be discussed in more detail. ABCG2 (also known as human breast cancer resistance protein BCRP) protein is composed of a single cytoplasmic, nucleotide binding domain (NBD) at the N-terminus where ATP hydrolysis occurs and a single transmembrane domain at the C terminus, containing six transmembrane α helixes responsible for substrate recognition and transport (Kerr, et al., 2011). The domain topology of ABCG family members is opposite to the other ABC subfamilies (in which the NBD is C-terminal to the TMD) and, furthermore, ABCG only has half the number of domains in a canonical ABC transporter (2 NBDs and 2TMDs are required for function), shown in Figure 1.2. Therefore, ABCG transporters are often called "half-transporters" and are believed to undergo homo-dimerization or oligomerization to larger species, giving them a functional transporter structure for ATP binding and hydrolysis (Wong, et al., 2015). ABCG2 is 655 amino acids in length and approximately 72 kDa in size depending on the extent of glycosylation present.

When it comes to understanding the ABCG2 structure, the six transmembrane helices of ABCG2 were identified by consensus topology prediction algorithms followed by confirmation by epitope tagging. When this experimental data was compared to the predicted data there were some differences in the topological location of TM2 and TM5 (Wang et al., 2008) which may be both shifted C-terminally with respect to the computer-based predictions. As ABCG2 lacks a high resolution structural data, homology modelling has been used to assist in the interpretation of experimental data. However, there has been very low sequence identity, often less than 20 % (Xu et al., 2015). This makes it harder to obtain a reliable homology model of the ABCG2 as for instance the 120 amino acid NBD-TMD linker region which is unique to the ABCG family of transporters cannot be modelled. Analysis of the ABCG5 G8 crystal structure (Yeuan Lee et al., 2016) will allow for a better delineation of the transmembrane segments of ABCG2 and will allow for better homology models to be built of this transporter.

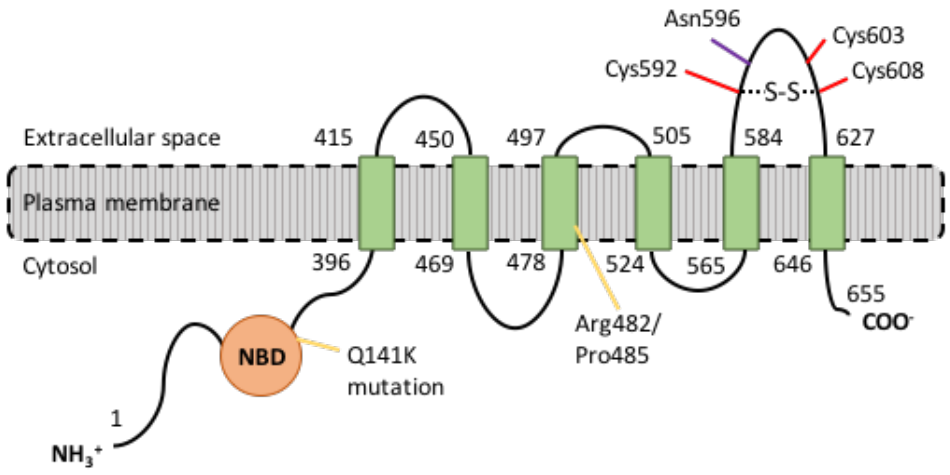



**Figure 1.2 Topology model of the ABCG2 within the membrane.** The ABCG2 monomer consists of 655 amino acid residues, with a 250 amino acid intracellular N-terminal NBD (orange), an uncharacterized linker region followed by six TMDs (green) and associated intra and extracellular loops. N-linked glycosylation site is shown in between the TM5 and TM6 extracellular loop (purple), stability affecting sites (red) and putative drug binding sites (yellow) in addition to the gout associated Q141K mutation are shown. Figure has been produced by the author.

Although based upon other ABC transporters it was plausible that ABCG2 would require at least dimerization to form a functional unit, it was also evident that higher order oligomers can also be formed. Native gel electrophoresis and Electron Microscopy (EM) data has demonstrated the formation of tetrameric species (Dezi et al., 2010, Xu et al., 2004) and other EM studies identified the formation of stable homo-octameric association (McDevitt et al., 2009). Studies by Wong et al., (2015) on the oligomeric state of the protein in intact membranes in live and fixed cells; (by both fluorescence correlation spectroscopy and stepwise photobleaching) supported the observation that in the presence and absence of drug substrates, ABCG2 is predominantly found in a tetrameric organization. Overall, it is still problematic to understand the structural basis of oligomerization. Studies have proposed that TM5-6 contains sufficient motifs to homo-oligomerize (Mo et al., 2012). Within this region C603 has been shown to form an inter-molecular covalent disulphide bond to another ABCG2 molecule but this is not essential for dimer formation (Haider et al., 2011; Kage et al., 2005). Other papers have proposed TM1 as important in dimer formation mediated by TMD: TMD interactions (Polgar, et al., 2004), and the required interaction of the NBDs for ATP hydrolysis (see below) will also contribute to dimer formation.

### 1.2.2 Substrate transport mechanism

So far different models have been proposed for the transport mechanism of various types of ABC transporters. Although, the exact mechanistic details for the mechanism of ABC transporters are still lacking, and have caused controversy amongst experts (Al-Shawi et al., 2011) all of them share some common predictions owing to the structural similarities of all ABC transporters. Even though having the conserved sequences present within the NBD, that is signature for all ABC transporters, there are differences between members forming at least 3 different groups: small importers, large importers and exporters. Here, given the focus of this thesis on ABCG2, I will examine the proposed transport mechanism for export ABCs (Figure 1.3).

In a typical ABC transporter, the two NBDs assemble such that the Walker A (P- loops) of one NBD and the LSGGQ motif of the other come closer and generate two ATP binding and hydrolysis sites (Higgins and Linton 2004; Jones and George 2004). In the absence of a nucleotide, this association is believed to be "loose" with a gap at the NBD domain interface of disputed size (Aller et al., 2009; Dawson & Locher, 2007 show two quite different NBD: NBD interfaces).

Clark et al., (2006), worked on the pharmacology study of substrate binding affinity in ABCG2, in the absence of ATP using radiolabelled daunomycin as the trace ligand and insect cell membranes expressing ABCG2 (R482G mutant, gain-of-function mutation which mediates the transport of doxorubicin, daunomycin and rhodamine 123, whereas it has a loss of function with respect to methotrexate transport). Binding affinities of several substrates of ABCG2 in competition binding experiments were established, where it was seen that the binding was high (100 mM) in the absence of nucleotide. Mitoxantrone (substrate for both wild type and R482G ABCG2) was shown to be more potent than unlabelled daunomycin in displacing radiolabelled daunomycin. However, the displacement of the total binding was incomplete (approximately 60 %). With the employment of Hoechst 33342 (less potent than mitoxantrone) together with the incomplete displacement result it was suggested that multiple daunomycin binding sites are present in ABCG2, i.e. mitoxantrone and Hoechst 33342 could displace the radio ligand from one

binding site, but not the other. The kinetic and equilibrium data support the presence of at least two symmetric drug binding sites on ABCG2, while in P-gp there were the two distinct asymmetric sites.

When considering the mechanism of substrate transport, the transporters are usually in its open dimer form at basal state, so that the two nucleotide binding sites are accessible. In step 1, as the substrate binds with high affinity at the TMDs (Martin et al., 2000), it initiates the conformational changes in the NBDs increasing affinity for ATPs by 30-fold (Linton et al., 2007). When two ATPs are bound, the NBD: NBD interface closes and the nucleotides are sandwiched between the NBDs, namely by the Walker A and Walker B motifs being contributed from opposing domains (Locker et al., 2009).

As of consequence, the TMDs are transformed from a high affinity state (presumably facing inward) to a low affinity (facing outward) conformation at step 2 (Higgins et al., 2004). This "power stroke" contributed from the ATP-binding allows for substrate release prior to ATP hydrolysis (Martin et al., 2000). At step 3, the NBD dimer closes and facilitates ATP hydrolysis, subsequently, with the release of the Pi and ADP which causes destabilisation of the closed dimer (Linton et al., 2007). This resets the transporter to its basal state and restores high affinity binding for substrate again (Martin et al., 2000). However, work done by McDevitt et al., (2008) on $ABCG2^{R482G}$ isoform showed that, when bound to ATP reduces the drug binding site to low affinity, it does not generate an outward-facing conformation. However, immediately post-hydrolysis with the release of Pi, ADP-bound $ABCG2^{R482G}$ isoform adopts a conformation capable of supporting the binding of radiolabeled [$^3$H] daunomycin.

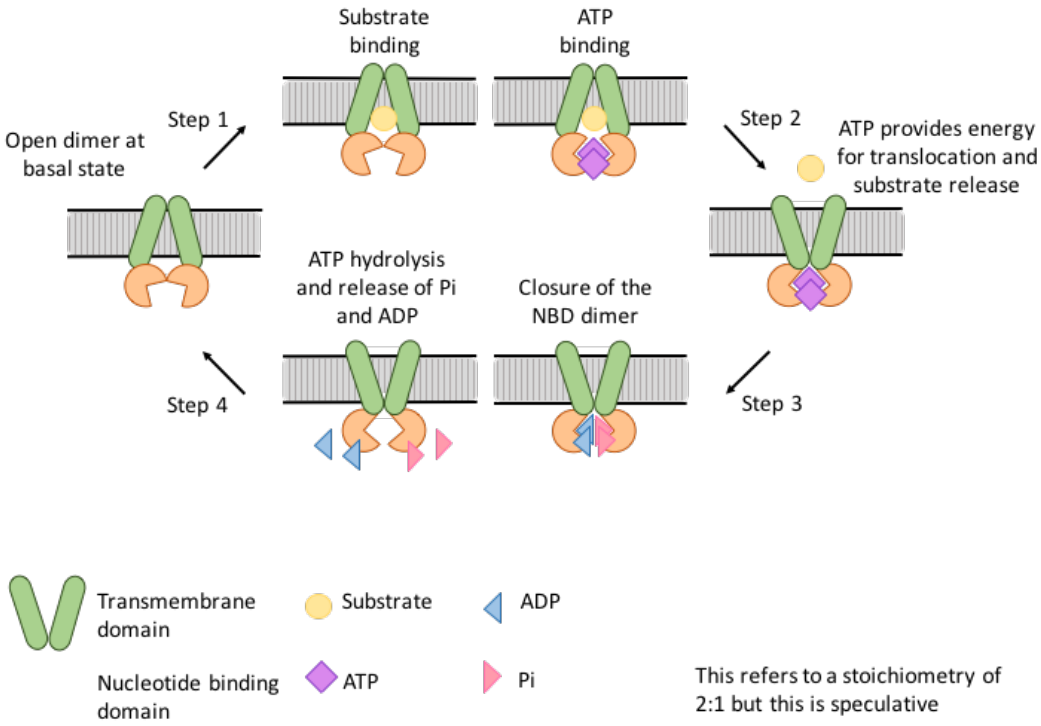



**Figure 1.3 The substrate transport by ABC transporters following the "ATP" switch model.** In step 1, binding of substrate, induces the conformational change in the transporter that stimulates the binding of ATP. Step 2, binding of 2 ATP molecules, causes the conformational changes in the TMDs thereby changing the state of the transporter from high affinity binding to low affinity binding state thus releasing the substrate on the opposite side of the membrane. In step 3 ATP is hydrolysed, and ADP and Pi is released followed by step 4, where the transporter returns to its basal state due to dissolution of the closed NBD dimer which is then reset to the high affinity open conformation ready to begin the cycle of transportation again. Figure has been produced by the author, with reference to Linton and Higgins 2007.

### 1.2.3 ABCG2 in vivo: localization and physiological function

ABCG2 although appears to play a major role in MDR of human cancer cells, as it was first isolated from MDR cancer cell (Doyle et al., 1998), its expression and distribution pattern in normal tissues implies that it must play some important physiological purposes, such as protecting the organism as a first line of defense against environmental toxins. Among some of these substances that are effluxed by ABCG2 include antibiotics, sterols, immune-suppressants (including anti-HIV drugs), fluorescent dyes (e.g. Hoechst 33342), photosensitizers (pheophorbide A and protoporphyrin IX). However, increased expression of ABCG2 has been linked to MDR in cancer and numerous studies have describe ABCG2 to mediated transport of chemotherapeutic drugs including mitoxantrone, methotrexate, topotecans, flavopiridol and tyrosine kinase inhibitors (imatinib, gefitinib and nilotinib)

(Basseville et al., 2016). A study done by Jonker et. al., (2002) on ABCG2-null mice helps us to understand this defensive role of ABCG2, where the ABCG2-null mice are more susceptible to diet-induced phototoxicity, protoporphyria, and possibly other porphyrin-related toxicities and disorders, which are caused by accumulation of pheophorbide A, a chlorophyll degradation product found in food and supplements.

Being a membrane transport protein ABCG2 is primarily localized to the plasma membranes of cells in different types of tissues, several of which serve the purpose to protect secretory or barrier function (Rocchi et al., 2000). In different types of human and rodent tissues, the localization of ABCG2 can play a vital role in limiting absorption (in the small intestine), mediating distribution (e.g., in the blood–brain and blood–placental barriers), and facilitating biliary and renal elimination and excretion (in the liver and kidney) of drugs or xenobiotics that are ABCG2 substrates. This specific ABCG2 distribution profile among the different types of tissues is closely related to the physiological role it assumes in the body (Horsey et al., 2016).

For instance, in the blood-brain barrier, ABCG2 is expressed highest on the luminal side of brain endothelial cells. Here it serves as a crucial barrier to drug access, significantly limiting the penetration of drugs or xenobiotics into the brain (Zhang et al., 2003, Aronica et al., 2005). Apart from this, along with ABCG2, ABCB1 is also co-localized at the same site, and possibly has a synergistic affect in their function for protection of drug toxicity in the brain (Agarwal et al., 2011).

In addition, ABCG2 is physiologically expressed in the apical membrane of epithelial cells in the gastrointestinal (GI) tract, with highest expression in the duodenum and a gradual decrease along the GI tract to the rectum. Here, ABCG2 play an important role in limiting the absorption of orally administered anticancer drugs and ingested toxins (Gutmann et al., 2005). In the liver canalicular membranes, where ABCG2 is constitutively expressed, a possible protective role is adopted against xenobiotic absorption and toxic metabolite excretion (Maliepaard et al., 2001). In the human kidney ABCG2 is expressed on the apical membrane of proximal tubular cells, however with a lower expression level than that in the liver and the small intestine, with

mostly responsible for urate export (Wei Mo et al., 2012). Kruijtzer et. al., (2002), in his study showed that when GF120918, a dual inhibitor of ABCB1 and ABCG2 is administrated, a significant increase occurred in the bioavailability and systemic concentration of topotecan after oral administration. Thus, in both the liver and kidney, ABCG2 facilitates biliary and renal elimination of drugs and xenobiotics.

Among normal human tissues, ABCG2 is expressed the highest on the apical membrane of the placental syncytiotrophoblasts. Here ABCG2 expels drugs or xenobiotics from the fetal compartment back to the maternal circulation, limiting fetal exposure of the toxic substances and playing a major role in protecting fetus against toxic material ingested by mother (Litman et al., 2000, Zhou et al., 2007). ABCG2 is also expressed in the mammary gland being induces strongly during the lactation phase in mice, cows, and humans. In the mammary gland, it has been shown that ABCG2 actively transport drugs (e.g., topotecan and cimetidine), xenobiotics (e.g., 2-amino-1-methyl-6-phenylimidazo [4, 5- b] pyridine or PhIP), and vitamins (e.g., riboflavin) into breast-milk (Jonker et al., 2005, Herwaarden et al., 2007). Although passing of important nutrients like vitamins are helpful for feeding babies, but taking medication that are ABCG2 substrates can contribute to side-effects or toxicity (Ishikawa et al., 2005).

Hirschmann-Jax et. al., (2004) identification of ABCG2 as a cancer stem cell marker has led to many studies on ABCG2 and stem cell research. The hematopoietic stem cells have the ability to actively extrude Hoechst 33342, a fluorescent dye. Fluorescence-activated cell sorting (FACS) of the low Hoechst 33342-staining cells are termed as ‘side population’ (SP), which possess characteristics similar to stem cell in a variety of tissues (Wei Mo et al., 2012). Studies with ABCG2-null mice further showed that ABCG2 is necessary for SP phenotype, since loss of ABCG2 expression resulted in a radical decrease in SP cells in the bone marrow and skeletal muscle (Zhou et al., 2002). Thus, ABCG2 has also been proposed to play a role in protecting putative cancer stem cells.

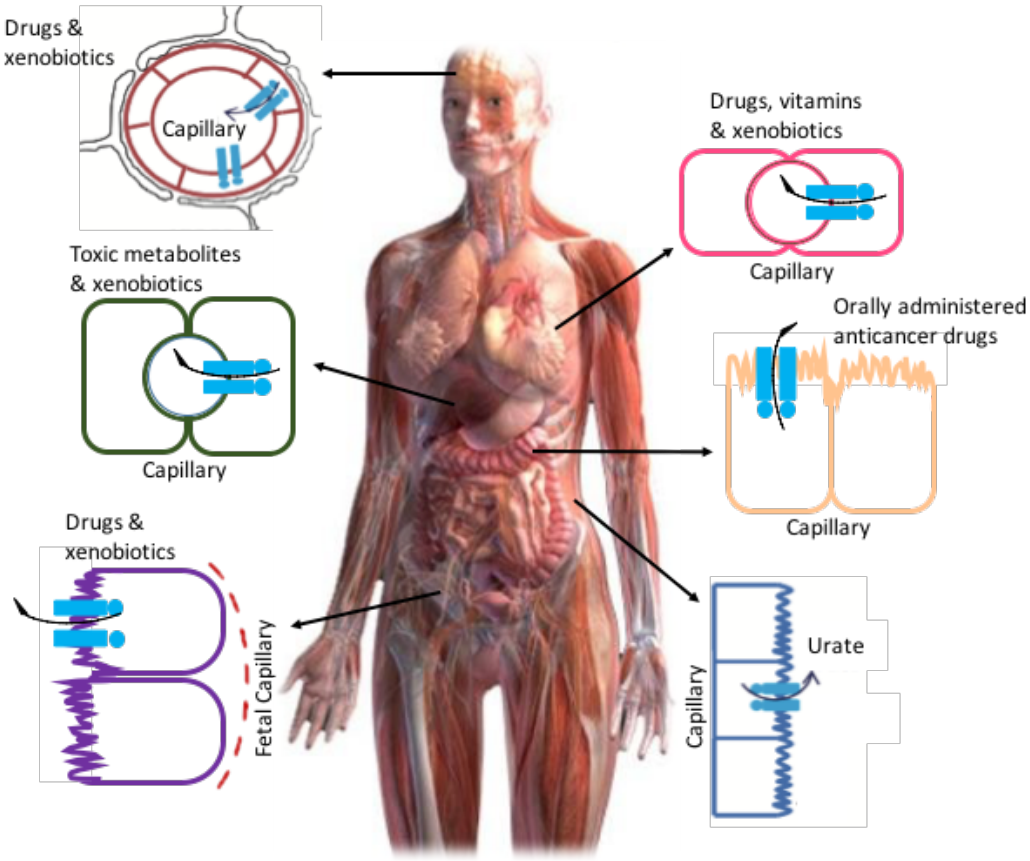



**Figure 1.4 ABCG2 localization and distribution**. From top left clockwise: ABCG2 homodimer (blue) exports drugs into capillary lumen, preventing their crossing the blood–brain barrier. ABCG2 also transports drugs, vitamins and xenobiotics from mammary gland, into breast-milk. In the GI tracts ABCG2 is expressed in the apical surface of the cells to limit the absorption of orally administered anticancer drugs. In the human kidney ABCG2 is expressed on the apical membrane of proximal tubular cells, to export urate. In the placenta, ABCG2 expels drugs or xenobiotics from the fetal compartment back to the maternal circulation. Finally, in the liver canalicular membranes, ABCG2 is constitutively expressed, to export xenobiotic and toxic metabolite. Figure adapted from Haider et al., 2011.

### 1.2.4 ABCG2 in vivo: Single nucleotide polymorphisms (SNPs)

A common recurring theme is the link between ABCG2 single nucleotide polymorphism and disease disposition. Single nucleotide polymorphisms (SNPs) of the ABCG2 gene have been suggested to play a critical role in affecting patients' responses to medication and/or the risk of diseases. After sequencing the ABCG2 gene from human samples it was revealed that there are over 80 different, naturally occurring sequence variations within its gene coding region. Among which, a total of 17 non-synonymous polymorphisms have been reported (Ishikawa et al., 2010, Zhou et al., 2014). The effect of ABCG2 polymorphisms on clinical pharmacology is a crucial area of current research; as the promiscuity of the transporter towards its substrate makes it

inevitable that some polymorphisms will impact on the pharmacokinetics of particular drugs.

But the most extensively studied among the SNPs with potential clinical relevance is the rs2231142 polymorphism, which results in the glutamine to lysine substitution (Q141K). This polymorphism affects the ATP-binding domain, between the Walker A motif (amino acid residues 83–89), (Ishikawa, 2005). This polymorphism is common associated with the prevalence of gout (Zhou et al., 2014). ABCG2 as mentioned above is expressed in kidney proximal tubular cells where it exports urate (product of purine metabolism) out of the cells. *In vitro* studies conducted by Woodward et. al., (2009) showed that single nucleotide polymorphism revealed the Q141K mutation resulted in reduced function (ATP activity and drug efflux) and impaired surface expression of ABCG2. As when expressed in *Xenopus* oocytes and the accumulation of radiolabelled urate in oocytes and urate efflux rates from oocytes were measured, there is a significant decrease by 75.5 % in oocytes expressing ABCG2 compared with water-injected control oocytes. When they also used Genome-wide association studies to identify SNPs in a genomic region on chromosome 4 that is associate with serum urate levels and gout, introducing the mutation Q141K encoded by the rs2231142 allele by site-directed mutagenesis showed 54 % reduced urate transport rates compared with those expressing wild-type ABCG2 at similar levels. This showed that Q141K as a causal loss-of-function variant, showing reduced transport of chemotherapeutic agents and causing hyperuricemia and gout. This was also demonstrated using transport and ATPase assays and immunohistochemistry fluorescence technique (Kondo et al., 2004; Mizuarai et al., 2004; Morisaki et al., 2005). Following this two other studies suggested that using the histone deacetylase inhibitor 4-phenylbutyrate, which is known to protect misfolded proteins from endoplasmic reticulum degradation, the processing defect caused by Q141K mutation could be liberated (Sarankó et al., 2013; Woodward et al., 2013).

Also apart from this, a SNP at nucleotide position 421 (cytosine to adenine, counting from the start of the ABCG2 gene) in the ABCG2 genome has been found to increase the bioavailability and clinical efficacy of topotecan

(topoisomerase I inhibitor used as second-line therapy in patients with advanced ovarian cancer or small cell lung cancer) (Sparreboom et al., 2005). In the same study, it was shown that mice lacking a functional ABCG2, had 6-fold higher bioavailability of topotecan, suggesting that ABCG2 limits the uptake of this drug. It was also shown that patients with defective allele 421C-A all have elevated plasma concentration of topotecan as compared to those patients with two wild-type alleles. The same polymorphism was also reported to significantly affect the pharmacokinetics of atorvastatin and potentially influence the efficacy and toxicity of rosuvastatin commonly used in the treatment of hypercholesterolaemia. ABCG2 plays an important role in limiting the absorption of these statins in the gut although the affect was greater in the case of rosuvastatin than in atorvastatin, indicating that differences exist in the impact of ABCG2 polymorphism on the pharmacokinetics of different statins (Keskitalo et al., 2009).

## 1.3 ABCG2 *in vitro:* Heterologous expression to better understand structure and function

When it comes to functional characterization of ABCG2, several biochemical and functional assays are involved. Although extensive research on this transporter is still ongoing, still there are several questions left to be solved. These include understanding how drugs are bound and transported by this MDR pump, what are the drug binding sites and how the drug binding and ATP hydrolysis are coupled. This requires getting more functional and structural data so that the information can be correlated and interpreted. These things could be investigated *in vitro* if we have sufficient ABCG2 protein in hand. Although, the protein of interest does not necessarily have to be a highly expressed protein in its native organism.

Expression of a particular protein can sometimes be induced by specific substances. However, in most cases such increase in the overall protein expression can be toxic to the native host, thereby making the use of native host system for overexpression to be fairly unsuitable (Khlistunova, Biernat et al. 2006; Xie, Nair et al. 2008). In such cases, expression of the protein of interest is required to be expressed in another suitable host system. Several prokaryotic and eukaryotic expression systems have been developed over the last decades for such heterologous expression of proteins.

*Escherichia coli* has been the most frequently used prokaryotic expression system for the high-level production of heterologous proteins (Makrides 1996; Baneyx 1999). Easy methods for genetic manipulation, low cost of production, high yield of protein, ease of scaling and short turnaround time are the advantages that make this system a first choice for any recombinant protein expression (Sorensen and Mortensen 2005). However, most eukaryotic proteins such as large multimeric membrane proteins, when expressed in *E.coli* (and other prokaryotic systems) are not correctly folded (Baneyx and Mujacic 2004). Moreover, prokaryotic expression systems cannot perform the post-translational modifications like glycosylation (addition of sugars to the protein), phosphorylation (addition of phosphate group), lipidation (addition of lipid group), sulfation (addition of sulfate group) etc., which are essential for

the activity and/or trafficking of the eukaryotic proteins. These facts limit the types of protein(s) that can be expressed heterologously in the prokaryotic systems (Brondyk 2009, Schlegel et al., 2014). In other words, bacterial cells protein expression is often inducible but that over-expression of eukaryotic membrane proteins in bacteria is not often successful.

Thus, the choice of the host for overexpression is of major importance, as the expressed protein must be in the right conformation and active after the purification. In order to overcome the disadvantages of the prokaryotic expression system, many eukaryotic expression systems have been developed having the capability of performing post translational modifications and expressing the protein in its active form. These include mammalian cell expression systems, yeast cell expression system and insect cell expression system. Although expression of ABCG2 in mammalian cells (HEK-293) is possible but the expression levels are typically much lower in comparison to insect cell line (confirmed) and also harder to scale up into larger volume cultures. As for attempts of expression of ABCG2 in the yeast cell so far has been unsuccessful (Personal communication Ian D. Kerr). Therefore, this study it will mainly focus on the expression of ABCG2 in insect cell.

### 1.3.1 Insect cells

Protein production in insect cells such as *Spodoptera frugiperda* (Sf9) using Baculovirus-mediated infections is popular method for the production of proteins. Insect cells can be grown in suspension in serum free media and optimized expression vectors have been designed for easy scale up and high yield of protein (Mus-Veteau 2002; Hitchman, Locanto et al., 2011). Apart from the expression of intracellular, surface bound or secreted proteins, this system has also been utilized for the production of membrane proteins (Possee, Thomas et al. 1999; Hitchman, Possee et al., 2009). Several mammalian transporters and receptors are functionally overexpressed by using this system (Sievert, Thiriot et al., 1998).

However, the post translational modifications done by the insect systems are not as complicated and precise as seen in human cells (Geisse, Gram et al., 1996). For instance, ABCG2 has a molecular weight of about 72 kDa when expressed in mammalian cells (Mao & Unadkat, 2005). But when expressed in

insect cells due to different level of glycosylation, the molecular size of the protein is ~65 kDa, under reductive conditions (using mercaptoethanol) (Ishikawa et al., 2003, McDevitt et al., 2006). ABCG2 expression in insect cells allowed for production of protein suitable for (EM) analysis. 3D structural data of purified ABCG2 expressed in insect cells revealed a possible octameric complex of ABCG2 formed by four dimers, using single particle electron microscopy analysis and modelling (McDevitt et al., 2006). Clark et al., (2006) worked on the pharmacological analysis by studying the substrate binding affinity in ABCG2, using radiolabelled daunomycin as the trace ligand and insect cell membranes expressing ABCG2$^{R482G}$.

## 1.4 Membrane Protein Solubilisation

One of the most difficult step working with membrane proteins is finding an environment with optimal properties that can allow the solubilisation of the protein (i.e. extraction from the membrane) and also to perform variety of downstream studies. Preferably, this environment should stabilize the protein, so that it stays in its most native form, allow for its purification, and enable the study of its structural and functional properties (Dörr et al., 2016). Previously, ABCG2 in insect cells could only be solubilized with hugely expensive fos-choline-16 detergents (McDevitt et al., 2006). In this study one of the focus was to investigate whether we could obtain ABCG2 without recourse to these. We have considered two different forms of membrane protein solubilisation: the classical detergent solubilisation and the novel non-detergent Styrene maleic acid (SMA) solubilisation.

### 1.4.1 Membrane protein solubilisation using detergents

Detergents are amphipathic molecules, consisting of a polar head group and a hydrophobic chain (or tail). When they are in aqueous solutions, they spontaneously form (generally) spherical micellar structures. One crucial factor to account for when working with membrane proteins is the critical micelle concentration (CMC) of the detergent. The CMC can be defined as the minimum concentration of detergent for individual detergent molecules to cluster and form micelles around the membrane protein, such that there is a

sudden change in surface tension and other physical properties. It is the point where the entropy of water favours aggregation over monomerisation.

Depending on the conditions such as pH, ionic strength, temperature as well as the presence of protein, lipid and other detergent molecules, the CMC can vary (Maire et al., 2000). It decreases with the length of the alkyl chain of the detergent and increases on the introduction of double bonds and branch points (for example, those present in bile salts, which consequently have a high CMC). When ionic detergents are involved, the CMC is determined by the combined effect of the head group repulsive forces and the hydrophobic interactions of the tails. Usually at lower temperatures, detergents remain in a crystalline insoluble form that is in equilibrium with small amounts of solubilized monomers. As the temperature is increased, more monomer dissolves until the CMC is reached.

Although all detergents have some common properties, there are some factors to account for when working with membrane proteins, such as the charge on the detergent, the chain length or the CMC of the detergent, particularly if needed to remove the detergent later. However, depending on the choice of detergents used, as it is usually a trial and error the downstream work can influence the work that will be carried out. As previously, fos-choline-16 and fos-choline-14 detergents were the only ones that solubilised ABCG2 in a buffer with osmotically neutral salt concentrations (McDevitt et al., 2006).

Amphiphilic detergents are most widely used to solubilise membrane proteins by creating a mimic of the natural lipid bilayer environment normally inhabited by the protein. Depending on the structure they fall under, detergents are classified into four major categories: ionic detergents, nonionic detergents, zwitterionic detergents and bile acid salts (Seddon et al., 2004).

Ionic detergents contain a hydrophobic hydrocarbon chain or steroidal backbone and a head group with a net charge that can be either cationic or anionic. Examples include sodium dodecyl sulfate (SDS), which is extremely effective in the solubilisation of membrane proteins however almost always denatured the protein to some extent (Booth et al., 1995). Some proteins can be renatured from sodium dodecyl sulfate by transferring the protein to a renaturing detergent or lipid environment.

Nonionic detergents as their name says, contain uncharged hydrophilic head groups of either polyoxyethylene (e.g. Tween) or glycosidic groups. They are generally considered to be mild and relatively non-denaturing, since they usually break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions (which causes denaturation). Due to this, membrane proteins structural features are not affected and can be isolated in its biologically active form. However, short chain (C7-C10) nonionic detergents, such as Octyl- β -Glucoside (OG), can often lead to deactivation of the protein, whereas longer chains (C12-C14) chain derivatives do not (Lund et al., 1989). Other alkyl-glucosides, such as n-Dodecyl-β-D-Maltoside (DDM), are increasingly popular and common among membrane protein solubilisation, since many proteins can be readily solubilised in a functional state (Lund et al., 1989, Aken et al., 1986, Fleming et al., 1997).

Bile acid salts (e.g. CHAPS) are ionic detergents, but differ from SDS as they contain a backbone of steroidal groups. Due to this, the bile salts have a polar and apolar face, instead of a well-defined head group like in the case of ionic detergents. When soluble, they form small kidney-shaped aggregates unlike the spherical micelles formed by traditional ionic linear chain detergents. Bile acids are relatively mild detergents and are often less deactivating than linear-chain detergents with the same head group (Lund et al., 1989).

Zwitterionic detergents have properties of both the ionic and nonionic detergents, but generally more deactivating than nonionic detergents. Examples include the use of N, N-Dimethyldodecylamine N-oxide (DDAO) in the crystallization of the reaction centre of *Rhodopseudomonas sphaeroides* (Deisenhofer et al., 1985) and structural work on rhodopsin.

### 1.4.2 Membrane protein solubilisation using SMA

Membrane protein solubilisation have been classically performed by using detergents. However, their use often has proven to be inadequate, this is mainly due to loss of native lipid environment surrounding the membrane proteins and also no single detergent has been capable of solubilising all membrane proteins. This has led to inefficient and expensive screening procedure followed by downstream studies performed in a non-physiological environment (Garavito et al., 2001). Following the solubilisation of the

protein, lipids are needed to be added back, and detergent to be removed so that the protein can be reconstituted into a lipid bilayer (Rigaud and Levy, 2003). Some of these reconstituted systems include proteoliposomes and planar membranes, and more recently several forms of lipid nanoparticles, such as bicelles or nanodiscs.

The last of these, nanodiscs, is a non-covalent assembly of a genetically engineered "membrane scaffold protein" (MSP) based upon the sequence of human serum apolipoprotein AI and a phospholipid. The phospholipid part of it associates as a bilayer domain into which membrane proteins are inserted, while two molecules of MSP wrap around the edges of the discoidal structure in a belt-like configuration, one MSP covering the hydrophobic alkyl chains of each leaflet (Bayburt and Sligar, 2010). Several ABC transporters have been successfully reconstituted into nanodiscs, to enable many downstream applications, ranging from biochemical and functional studies of MsbA and P-gP (Ritchie et al., 2011; Kawai et al., 2011) to also structural dynamics studies (Alvarez et al., 2010). However, in majority of the cases, the membrane protein was completely pre-solubilized with a compatible detergent and mixed with the Nanodisc assembly components. Detergent solubilisation requires an expensive, trial and error process, even as the protein appears to be solubilized it can still be aggregated or undergo time-dependent aggregation and detergents that work well for solubilisation may not be compatible with downstream purification or analytical methods (McDevitt et al., 2006; Telbisz et al., 2013; Bayburt and Sligar, 2010). Regardless, nanodics reconstitution methods has been demonstrated to be responsive to many different techniques (Schuler et al., 2013).

Styrene maleic acid (SMA) is a copolymer of styrene and maleic acid groups which is an alternative method of solubilising membrane protein directly from the membrane itself. It is the hydrolysed form of the styrene–maleic anhydride (SMAnh) copolymer, synthesised by the co-polymerisation of styrene and maleic anhydride monomers. SMA molecules exhibit a particularly different mode of action than detergents. When the polymer is added to synthetic or biological lipid membranes, it spontaneous forms discoidal particles with diameters of ~10 nm. surrounding the protein, by encircling the whole lipid:

protein complex, as opposed to the micelle forming detergents (Figure 1.5 A & B) (Knowles et al., 2009; Broecker 2016, personal communication). When this type of polymer-bounded SMA lipids particles (SMALPs) are formed, the bilayer organization is conserved (Jamshad et al., 2015; Orwick et al., 2012, Knowles et al., 2009, Lee et al., 2016). This retention of lipid environment is important as the composition of membrane environment can affect studies on protein structure and function (Dörr et al., 2016). SMALP are destabilised by divalent metal ions thus reconstitution of the protein from the lipid disc structure into proteoliposomes is often required to fully characterise the proteins for ATP hydrolysis kinetics (Gulati et al., 2014).

SMA according to Scheidelaar et al., (2015), solubilises membrane protein in three steps. In the first step SMA binds to the surface of the lipid bilayer. This step is dependent on the concentration of SMA used, as if more amount of SMA is used more will be solubilised but the only problem here is the electrostatic interactions: the presence of anionic lipids causes repulsion and thus impairs binding of the negatively charged polymer. In the following step, SMA inserts into the hydrophobic core of the membrane and like a "cookie cutter" it solubilises the membrane protein and takes portion of the lipid bilayer along with it. In the final step, which is the actual solubilisation of the bilayer, SMALP particles are formed.

One of the most common membrane protein that was solubilised using SMA was the G-Protein Coupled Receptor (GPCR, human A2aR) which was expressed in both yeast and mammalian cells. Jamshed et al., (2008) showed that A2aR–SMALP exhibited increased thermostability and suitable for receptor-based functional assays/screens. Furthermore, it was also shown that, problems with the interferes in spectrophotometric studies of membrane protein reconstituted using nanodiscs, which stabilizes the protein by an annulus of scaffolding proteins were not faced when solubilised using the non-proteinaceous nature of the SMA polymer, as it does not interfere with biophysical characterization of the embedded receptor (Jamshed et al., 2008).

Several other proteins have been solubilised and purified using SMA, such as the tetrameric potassium channel KcsA directly from the membrane of *Escherichia coli* (Dörr et al., 2014). SMA polymer here directly lysed *E. coli*

whole cells with overproduced His-tagged KcsA, followed by isolation of the KcsA containing SMALPs nanodics by Ni-affinity and size-exclusion chromatography. It was shown that sufficient protein can be purified in nanodiscs for extensive biophysical characterization, where to study the thermos-stability of KcsA, CD and fluorescence spectroscopy was used (Dörr et al., 2014).

Apart from this, three other bacterial membrane proteins, BmrA, LeuT and ZipA has also been successfully solubilised from *E. coli* cell membranes using SMA (2.5 % (w/v)). C-terminal $His_6$ tagged BmrA and ZipA, and N-terminal $His_8$ tagged LeuT were effectively purified using Ni-affinity and further quantified to perform substrate binding assays using Tryptophan fluorescence quenching assay. Thermo-stability of the purified proteins was also observed through aggregation to see how proteins within the SMALPs are behaving with increase in temperature (Morrison et al., 2016).

The Bacterio-rhodopsin (7 transmembrane α-helical bundle) and PagP (8-stranded β-barrel) proteins were also solubilised and purified using SMA. It was shown that these proteins were integrated into SMALPs by adding 2.5 % (w/v) SMA to protein in dimyristoyl phosphatidylcholine (PC) containing liposomes. The resulting nanoparticles were purified by Nickel-affinity chromatography or optimized to minimize vacant nanoparticles for further functional and biophysical characterisation such as CD thermal denaturation assays (Knowles et al., 2009, Orwick et al., 2012) and EPR spectroscopy (Orwick et al. 2012). This allowed for accurate distance measurements that revealed similar dynamics of bacteriorhodopsin as compared to its state in the plasma membrane (Orwick et al. 2012).

When it comes to ABC transporters, an extensive amount of work was done by Gulati et al., (2014) on several different types of eukaryotic ABC transporters such as P-glycoprotein (ABCB1), MRP1 (ABCC1), MRP4 (ABCC4), ABCG2 and CFTR (ABCC7). Each one of these proteins were expressed in different cell-lines including mammalian cells, insect cells and yeast. It was demonstrated in this study that the solubilisation efficiency varied between detergent extraction and SMA extraction. Variation were also seen when the same protein was solubilised with SMA when expressed in different cell lines

(Gulati et al., 2014). ABC transporters within the SMALPs were not only capable of being purified using standard affinity chromatography methods but also retained their ability to bind to substrates and nucleotide. This was contrasting in a sense that when solubilised with detergents, all the downstream procedures required the addition of detergents which is expensive, but with SMA all the procedures can be performed in a single step. Also, SMA method doesn't disrupt the lipid environment surrounding a membrane protein in the way that detergents do. Instead a segment of membrane is extracted, and the native lipid bilayer surrounding a membrane protein is maintained. It was also demonstrated that SMA solubilised proteins were more thermostable, less prone to aggregation than detergent solubilised were suitable for EM structural data analysis (Gulati et al., 2014).

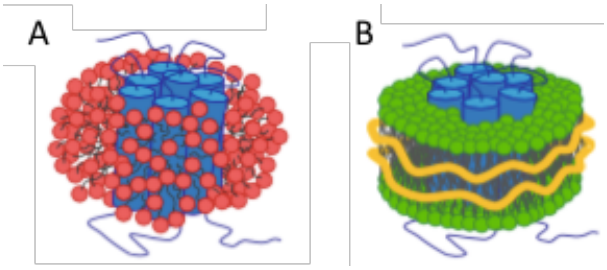



**Figure 1.5 Different types of membrane protein solubilisation**. **(A) Detergent solubilisation** shows by the formation of micelle (show in red) and **(B) SMA solubilisation** by the formation of nanodisc (yellow) with retention of native lipids in the membrane (green), around an indicative membrane protein (blue). Figure adapted from Dörr et al., (2016)

## 1.5 Aims and Rationale

To date, our understanding about the ABCG2 structure and drug binding sites are still not complete. One of the biggest challenge of working with this membrane protein is the solubilisation and purification, as so for data regarding this has been very conflicting. Thus, the aims of this projects can be stated as such:

1. High level expression of ABCG2, thus in this research *Spodoptera frugiperda* (Sf9) cells have been employed as they are a widely-used expression system due to their ability to produce foreign proteins with high expression (Marheineke et al., 1998).
2. Solubilisation of the membrane protein. In this research two different methods have been perceived into, the classical detergent solubilisation and the non-detergent novel styrene maleic acid (SMA) solubilisation.
3. Purification of ABCG2. Following the detergent and SMA extraction purification by Nickel/Cobalt ion affinity chromatography and Streptactin chromatography was employed to purify the protein in preparation for downstream studies, helping us to more accurately define the unknown molecular features around ABCG2, particularly regarding drug binding sites.

# Chapter 2: Materials and Methods

## 2.1 Molecular biology

### 2.1.1 Materials and reagents

The starting DNA vectors which had the main backbone of the pFastBac_HTC were obtained from Dr. Ian Kerr, University of Nottingham. The vectors contain ampicillin™ (amp) and gentamycin™ (gen) resistance genes for selection purpose. Apart from this the vector also contain strong Polyhedrin (PH) promoter for high-level protein expression, an N-terminal 6×His (Invitrogen) or 12×His tag (Haider et al., 2011) for purification of recombinant fusion proteins using metal-chelating resin and a TEV protease cleavage site for removal of the His tag subsequent protein purification (Polayes et al., 1996).

All molecular biology reagents were purchased from New England Biolabs (NEB, Hitchen, UK) and other reagents were obtained from Sigma-Aldrich (Poole, UK), unless stated otherwise.

### 2.1.2 Cloning

#### 2.1.2.1 Restriction digestion

Typically, restriction digests were set up in 20 µL volumes containing the appropriate reaction buffer (selected by consulting the enzyme manufacturer's website), 200-500 ng DNA, and 5-20 units of enzyme(s). Following mixing in a pipette tip the reactions were incubated for 90 minutes at 37 °C. Where indicated in the results the experimental conditions were varied to optimize digestion without concomitant non-specific "star" activity.

#### 2.1.2.2 Primer Annealing

Oligonucleotide primers were designed manually to contain the Strep and Strep-$His_6$ tag sequence (Table 2.1). Following design, the primers were checked using Netprimer to ensure that there were no unwanted secondary structures (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html).

**Table 2.1: List of the Primers designed used to insert the tag sequences**. Each of the primers contains sequences complementary to the 5' or 3' overhang that would be available following digestion of plasmid DNA with the indicated restriction enzymes.

| **Primer/oligomer sequences** | | **Restriction sites** |
|---|---|---|
| Strep tag | 5′-GTCCGATGGCCTGGAGCCACCCGCAGTTC GAAAAAGAAAACCTGTATTTTCAGGGCG-3′ | ½ RsrII and ½ KasI |
| Strep-$His_{13}$ tag | 5′-GTCCGATGGCCTGGAGCCACCCGCAGTTC GAAAAACATCACCATCACCATCATCA-3′ | ½ RsrII and ½ NdeI |

The forward and reverse oligomers for both Strep and Strep-$His_{12}$ tag (Table 2.1) were annealed together using a PCR machine (SensoQuest, Göttingen, Germany) to obtain a constant cooling programme. 100-200 pmoles of each oligonucleotide (i.e. forward and reverse) were combined and the reaction was heated to 95 °C for 1 min, and then cooled slowly to 50 °C in a linear fashion over a 10-minute period, i.e. 4.5 °C/min. The cycle was repeated 5 times and then the PCR product i.e. annealed primers was separated on a 1 % (w/v) agarose gel.

### 2.1.2.3 Ligation

For ligation, the digested DNA products were purified from agarose gel slices using the Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit (Düren, Germany) according to the manufacturer's requirement. The purified vector (plasmid backbone) was incubated with 2.5 units of shrimp alkaline phosphatase (Promega, Southampton, UK) at 37 °C for 30 min to prevent self-ligation, and then re-purified using the Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit (Düren, Germany).

Typically, ligation reactions were set up in 20 µL volumes containing the appropriate reaction buffer (T4 ligase buffer), 200-500 ng vector, 100-300 ng insert (i.e. annealed oligonucleotides) and 400 cohesive end units of T4 DNA ligase. Amount of vector and insert used was estimated at a vector: insert molar ratio of 2:1 for optimal ligation conditions. Ligation reactions were performed overnight at 16 °C or 2 h at room temperature.

### 2.1.3 Transformation of competent DH5α E. coli and DH10 Bac cells

Chemically competent DH5α *E. coli* cells were used for transformation of plasmid DNA and competent DH10Bac were used for generation of bacmid DNA. Aliquots (100) µL of competent DH5α *E. coli* were thawed on ice and DNA was then added (typically 5 µL of a ligation reaction or 100-500 ng of circular DNA). The competent cells were then left on ice for 30 min, heat shocked at 42 °C for 90 s and again left on ice for further 2 min. 900 µL of Luria-Bertoni, LB medium (1 % (w/v) NaCl, 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract) was added and the cells then incubated with shaking (200 rpm) for 60 min at 37 °C. the transformed competent cells were then spread plated onto pre-warmed LB agar plates (1.5 % (w/v) agar; supplemented with 100 µg/mL ampicillin). For ligated products transformation, $1/10^{th}$ (i.e. 100 µL of the transformation) and $9/10^{th}$ (i.e. the remaining 900 µL of the transformation was centrifuged (10,000 *g*) to pellet the bacteria which were re-suspended in 100 µL for convenient plating) of the transformation reaction volumes were plated. For circular plasmid transformation, $1/100^{th}$ of the transformation reaction volume was plated. Transformed competent cells were incubated at 37 °C overnight.

For DH10Bac transformation the same protocol was applied with slight modifications; following transformation cells were incubated for 4 hours at 37 °C and then were plated onto "X-gal plates", which comprised LB-agar (1.5 % (w/v) agar) supplemented with kanamycin (50 µg/mL), tetracyclin (10 µg/mL), gentamycin (7 µg/mL), X-Gal (100 µg/mL) and IPTG (40 µg/mL), and incubated for 36-48 hours until clear blue/white screening was evident.

### 2.1.4 Plasmid preparation and long-term storage

Well isolated single DH5α *E. coli* colonies were inoculated into 5 mL of LB medium containing 100 µg/mL of ampicillin. Bacterial cultures were grown overnight at 37 °C, with orbital shaking at 200 rpm. When required, glycerol stocks were made for long-term storage, with 500 µL of the broth culture along with 500 µL 30 % (v/v) glycerol and stored at -80 °C. The remaining culture was centrifuged (2000-4000 *g*, 10 min) and DNA was extracted from the bacterial pellets per the manufacturer's protocols in Macherey-Nagel

Nucleospin Plasmid kit (Düren, Germany). In short, bacteria pellets were first lysed under alkaline conditions before the bacterial chromosomal DNA was co-precipitated with insoluble complexes (salt, detergent, and protein). The solution was then neutralised and adjusted to high-salt conditions. Lysates were then cleared by centrifugation and double-stranded plasmid DNA products were adsorbed to the silica columns. DNA products were then washed with ethanol-containing solution and finally eluted in low-salt Tris-EDTA (TE) buffer (45 mM Tris, 1 mM EDTA, pH 8.0).

### 2.1.5 Bacmid DNA preparation

Single transformant colonies from the X-Gal agar selective plates were inoculated into 5 ml of Luria-Bertani (LB) media containing appropriate antibiotics. The bacterial cultures (5 mL) were grown overnight at 37 °C, with orbital shaking (180-220 rpm), following which bacmid DNA was isolated. Briefly, cells were pelleted by centrifugation (3000 *g*, 5 mins) and then re-suspended in 300 µL of solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Following which solution II (300 µL) (0.2 N NaOH, 1 % (w/v) SDS;) was added and gently mixed then incubated at room temperature for 5 minutes. 300 µL of 3 M potassium acetate, pH 5.5 was added afterwards and mixed gently which caused a thick white precipitate of protein and *E. coli* genomic DNA to form while incubated on ice for 5 to 10 minutes. The solution was then centrifuged for 10 minutes at 14,000 *g* following which the transfer the supernatant was transferred to a micro-centrifuge tube containing isopropanol (600 µL). The tubes were inverted a few times to mix and placed on ice for 5-10 minutes. The sample was further centrifuged for 15 minutes at 14,000 *g* at room temperature discarding the supernatant. The pellet which was washed with 70 % (v/v) ethanol by inverting the tube several times and then centrifuging again. Bacmid DNA as being much bigger (> 135 kb) was size is stored at 4 °C in TE buffer to avoid freeze thaw cycle which would promote fragmentation of the product.

### 2.1.6 DNA sequencing

Once the sizes of the DNA products were confirmed using restriction digests, the constructs were sent to Source BioScience Life Sciences (Nottingham, UK)

for DNA sequencing. The gene-specific sequencing primers that were used for the sequencing purpose are listed in Table 2.2 below. DNA Chromatograms from the sequencing data were analysed using Chromas Lite along with sequence data were aligned with the predicted sequences using the BLAST local alignment tool (Altschul et al., 1990).

**Table 2.2: List of non-standard ABCG2 sequencing primers.** The nucleotide positions of which the primers (blue arrows) bind to in the ABCG2 sequence (thick bold line), and the direction for which they provide sequence data, are indicated.

| Primers | Sequences | |
|---|---|---|
| SeqR1 | 5′-TCGTGGTGCTCCATTTAT-3′ |  |
| SeqF0 | 5′-GAGTGGCTTTCTACCTTGTC-3′ |  |
| SeqF2 | 5′-GCAGGGACGAACATTC-3′ |  |
| Seq482 | 5′-AACTCTTTGTGGTAGA-3′ |  |

### 2.1.7 Analysis of Recombinant Bacmid DNA using PCR

Following the Invitrogen Bac-to-Bac® Baculovirus Expression System manual, the resulting recombinant bacmids that were isolated were further analysed via PCR using a thermocycler (SensoQuest, Göttingen, Germany) amplification employing pairs of the following primers:

M13F (5′-GTTTTCCCAGTCACGAC-3′), M13R (5′-CAGGAAACAGCTATGAC-3′), SeqR1 and Seq482 (Table 2.2).

The reaction mixture (50 µL) typically consisted of 100 ng DNA template, 2 units of GoTaq DNA polymerase, 1.5 µL of 1.5 mM $MgCl_2$, 10 µM of forward and reverse primers each and 1 µL of 1 mM dNTP mix all in appropriate buffer conditions. The reaction mix was made up to the final volume using nuclease-free water.

The general program used for PCR was an initial denaturation step of 3 minutes at 93 °C, followed by 35 cycles of amplification using the following steps; separation of the strands for 45 seconds at 94 °C; annealing of the primers for 45 seconds at 55 °C; and extension for 2.5 minutes (in case of M13F/R 5 minutes) at 72 °C. After all the cycles, there was then a final extension step at 72 °C for 4 minutes (7 minutes for M13F/R). The samples were then analysed by agarose gel electrophoresis (Section 2.4.1).

## 2.2 Tissue culture

### 2.2.1 Cell lines and reagents

Sf9 (*Spodoptera frugiperda*) cells which are a clonal isolate of Sf21 cells (IPLB-SF21-AE) (Vaughn et al., 1977) were used as the tool for expression of recombinant proteins. They were chosen primarily due to their ability to produce recombinant protein using baculovirus at a much larger-scale expression system compared to mammalian expression system. One of their preferable growth characteristics is the ability to grow in the absence of serum, and cultured attached or in suspension.

All reagents were obtained from Sigma-Aldrich unless stated otherwise. Insect cell culture media Insect-Xpress was purchased from Lonza (Wokingham, UK). Foetal calf serum (FCS) was obtained from Thermo Fisher scientific (Loughborough, UK).

### 2.2.2 Cell passage maintenance and storage

Sf9 cells were grown in Insect-Xpress (Protein-free Insect Cell Medium with L-glutamine) supplemented with 10 % (v/v) FCS and 2 % (v/v) Pen-Strep (100 units/mL penicillin and 100 µg/mL streptomycin), in either tissue culture coated plastic flasks (T25 flasks) or borosilicate round bottom flasks incubated at 27 °C in air. Sf9 cells have a short doubling time (18-24 hours) but suffer from a long lag phase if they are seeded at densities below 1 x $10^6$/mL. Hence, routinely cells were diluted from dense cultures at 6-8 x $10^6$/mL into fresh media at 1 x $10^6$/mL. Suspension cultures were maintained in flasks with at least 70 % of the volume of the flask for air.

### 2.2.3 Generation of baculovirus and expression of ABCG2

#### 2.2.3.1 Cell seeding and transfection with recombinant bacmid DNA

For transfection purpose Sf9 cells were counted and seeded in Xpress media (containing 2 % (v/v) penicillin/streptomycin; P/S), at 1 x $10^6$ cells/ 2 mL into 6 well tissue culture plates (Greiner, Cellstar) and then left to adhere for 1 hour in the incubator at 27 ºC. The isolated bacmid DNA (5 µL; typically, 1 µg) and the transfection reagent Escort IV (Sigma L-3287) (5 µL) were each diluted in

500 µL Xpress media (serum and P/S free). The diluted DNA and the transfection reagents were mixed together and left for 15-45 mins at room temperature. The media from each well were removed by aspiration and the cells are washed with 1 mL of Xpress media (serum-and P/S free). The transfection mix was added to the cells without disturbance of the cell layer and incubated for 5 hours at 27 ºC. Following this, the transfection mix was removed and replaced with supplemented Xpress media (containing 2 % (v/v) FCS and 2 % (v/v) PS), 3 mL. This was then incubated at 27 ºC for 3-6 days, while observing the cells periodically from 72 h onwards, to examine whether there were increases in cell size or alterations to cell morphology, which are indicative of baculoviral production.

#### 2.2.3.2 Generation of Initial P1 viral stock

Typically, changes in morphology were observed 5-6 days after the bacmid DNA transfection. At this stage the culture media (a source of recombinant virus known as a "P1" stock was aspirated under sterile conditions and centrifuged (500 *g,* 5 min) to remove any suspended cells and the supernatant P1 virus was stored at 4 ºC in the dark. Any remaining insect cells were harvested for analysis of protein expression.

#### 2.2.3.3 Generation of BIIC stock

The initial P1 viral stock is a low volume (< 2 mL) and low titre virus (i.e. contains insufficient viral particles for protein expression). To produce a viral stock suitable for protein expression, baculovirus infected insect cells (BIIC) stocks were produced as described (Wasilko et al., 2009). Briefly, 50 mL Sf9 cells at $0.5x10^6$ cells/mL (in media containing 2 % (v/v) PS) were infected with 500-1000 µL of P1 viral stock. The cell size was monitored on a cell size counter, Moxi Z Mini Automated Cell Counter Kit (Orflo, USA). at 24 and ~36-42 h in comparison to uninfected. When the cell diameter was observed to have increased from 15-16 µm to around 18-19 µm cells were deemed to be virus rich (Jarvis, 2009) and thus highly infectious for protein expression. At this stage, cells were harvested at 750 *g* for 5 mins and then re-suspended in 2.5 mL of Xpress media supplemented with 10 % (v/v) DMSO and 2 % (v/v) P/S. Aliquots of 100-200 µL in cryo-vials were frozen slowly to -80 °C using

an isopropanol-filled controlled freezing chamber.

### 2.2.4 Infection of Sf9 cells for the Expression of ABCG2

Following optimisation (described in the results) protein expression was typically carried with Sf9 cells seeded at $1x10^6$ cells/ml and infected with BIIC stocks after 6-7 hours. Infection are done at 100 µL of BIIC per 150 mL of cell culture. The infection was monitored over the course of 72 hours until the cells are centrifuged (3000 *g*, 15 mins) for cell lysis and membrane preparation.

## 2.3 Protein Expression, Isolation and Purification

### 2.3.1 Membrane preparation

Following infection of the Sf9 cells for typically 72 hours, they were centrifuged at 3000 *g* for 15 mins. The pellets were re-suspended in approximately 6× the volume of the pellet weight (1 mass, 1 volume) in PBS (8.1 mM $Na_2HPO_4$, 1.9 mM $KH_2PO_4$, 140 mM NaCl, 2.7 mM KCl) and washed, followed by again centrifuging it at 3000 *g* for 15 mins. The pellets were then re-suspended at 5× the pellet mass in MIB1 (10 mM Tris pH 7.4, 250 mM sucrose, 0.2 mM $CaCl_2$) containing protease inhibitors (protease inhibitor cocktail III, Merck Millipore, Germany). The insect cells were then lysed in cycle of two passages by decompression (1000 psi) in a pre-cooled nitrogen cavitation chamber (Parr Instruments). The lysate was then centrifuged at 500-1000 *g* at 4 °C for 15 minutes, to spin down cellular debris. The resulting pellet (P1) was re-suspended in the same volume as before in MIB1, whilst the supernatant (S1) was centrifuged at 100,000 *g* at 4 °C for 45 minutes to pellet the membranes. The resulting pellet (P2) was re-suspended in MIB4 (10 mM Tris pH 7.4, 250 mM sucrose) or High salt wash buffer (4 mM EDTA, 500 mM NaCl, 20 mM Tris, pH 8.0) with 2× protease inhibitors by repeated shearing through 19-gauge, then 27-gauge needle at least 15 times. The membranes that have been re-suspended in high salt buffer were re-centrifuged (30 minutes, 100,000 *g*, 4 °C) and then re-suspended in appropriate volumes of solubilisation buffer (4 mM EDTA, 500 mM NaCl, 20 mM Tris, 10 % (v/v) glycerol, pH 8.0) with 2× protease inhibitors following with shearing 20 times. Membrane aliquots were stored at -80°C.

### 2.3.2 Styrene maleic acid (SMA) preparation

SMA 2000 (Cray Valley, USA) requires hydrolysis from an acid anhydride to a di-carboxylic acid in order to have membrane protein solubilising function. It was prepared as described in the protocol by Lee et al., (2016) in the Kerr Lab and also supplied by Dr. Alice Rothnie.

### 2.3.3 Membrane solubilisation

#### 2.3.3.1 Styrene maleic acid solubilisation of ABCG2

Aliquots of crude Sf9 membrane preparations (P2) were centrifuged at 100,000 *g* for 20 mins before re-suspending at 30 mg of wet membrane per mL of SMA buffer (20 mM Tris pH 8, 500 mM NaCl, pH 8.0) (Gulati et al., 2014). Half the volume of the buffer that is required was used to re-suspend the pellet, while the other half was used to re-suspend the styrene maleic acid to give a final concentration of 4 % (w/v). The two solutions are mixed (to give a final concentration of SMA of 2 % (w/v)) and incubated at room temperature for 2 hours with agitation. The solubilised protein was then collected by centrifugation of the suspension at 100,000 *g* at 4 °C for 30 minutes, the protein containing supernatant was retained. Pellets (Insoluble protein) were needed for analysis and were re-suspended in 1 % (w/v) SDS.

#### 2.3.3.2 Detergent solubilisation of ABCG2

Detergent solubilisation was done as per Telbisz et al., (2013). Detergents were made up in solubilisation buffer at 4 % (w/v) (SDS, DDM). 200 µL (4 mg) of *E. coli* total lipid extract (Avanti, Polar lipids) were evaporated under nitrogen gas and dissolved in required volume of 4 % (w/v) detergent solution, with bath sonication for 15-20 minutes. Sf9 cell membrane preparations (P2) that were previously re-suspended in solubilisation buffer (4 mM EDTA, 500 mM NaCl, 20 mM Tris, 10% (v/v) glycerol, pH 8.0) were then homogenously mixed with the detergent-lipid mixture using 27-gauge needle. Following this, to make up the final concentration of the solution, 3 mg/mL protein, 1 % (w/v) detergent and 0.5 % (v/v) lipids additional solubilisation buffer was added. Solubilisation was performed for 90 minutes with agitation, at 4 °C for DDM

and room temperature for SDS. Solubilized membrane proteins were cleared by ultracentrifugation at 100,000 *g* at 4 °C for 30 minutes.

### 2.3.4 ABCG2 protein purification

#### 2.3.4.1 Nickel /Cobalt resin-$His_6$/Strep-$His_{12}$ tagged-ABCG2 purification

SMA or detergent solubilised membranes were incubated overnight at 4 °C with $Ni^+$-NTA™ and HisPur Cobalt™ agarose (Thermo Fisher Scientific, Loughborough, UK) using 100 µL resin/ml solubilised membrane in an end to end rotatory manner. The sample was transferred into a gravity flow column (Biorad), washed 5 times with 2 bed volumes (bv) of SMA buffer, supplemented with 5 mM imidazole (SMA solubilised) or Wash buffer (5 mM EDTA, 500 mM NaCl, 20 mM Tris, 0.1 % (w/v) DDM, 10 % (v/v) glycerol, pH 8.0) supplemented with 5 mM imidazole (detergent solubilised). Following the washes, proteins were eluted 3 times with 1/2 bv of SMA buffer supplemented with 200 mM imidazole (SMA solubilised) or Elution buffer (5 mM EDTA, 500 mM NaCl, 20 mM Tris, 0.1 % (w/v) DDM, 10 % (v/v) glycerol, pH 8.0) supplemented with 200 mM imidazole (detergent solubilised). Samples of various stages of the purification were analysed by SDS-PAGE and visualised by silver staining (Pierce).

#### 2.3.4.2 Strep-tactin resin-Strep/Strep-$His_{12}$ tagged-ABCG2 purification

For the Strep-tagged ABCG2, SMA or detergent solubilised membranes were incubated overnight at 4 °C with Strep-tactin resins (Strep-Tactin Superflow®, IBA) using 100 µL resin/ml solubilised membrane in an end to end rotatory manner. The sample was transferred into a gravity flow column (Biorad), washed 2 times with 2 bed volumes (bv) of Wash buffer (100 mM Tris, 10 % (v/v) glycerol, 0.1 % (w/v) DDM, 150 mM NaCl, 1 mM EDTA, pH 8.0) followed by elution 3 times with 1/2 bv of Elution buffer (100 mM Tris, 10 % (v/v) glycerol, 0.1 % (w/v) DDM, 150 mM NaCl, 1 mM EDTA, pH 8.0) supplemented with 3 mM desthiobiotin. After the purification, the Strep-tactin column was washed 3 times with 1 bv of wash buffer (without 0.1 % (w/v) DDM) and regenerated by washing the column 2 times with 1 bv of 1× Buffer R (regeneration buffer, IBA) containing HABA (2-[4'-hydroxy-

benzeneazo]benzoicacid). The HABA displaces desthiobiotin (from the elution buffer) at the biotin-binding site and regenerate of immobilized Strep-tactin for the next round of purification. The columns were stored at 4 °C in 1× R-buffer until next use. Samples of various stages of the purification were analysed by SDS-PAGE and visualised by silver staining (Pierce).

## 2.4 Analytical Methods

### 2.4.1 DNA Analysis

#### 2.4.1.1 Agarose Gel Electrophoresis

Agarose gels used for DNA analysis were prepared with 0.8 % (w/v) agarose in TPE buffer (90 mM Tris-phosphate, 1 mM EDTA, pH 8) containing 0.01 % (v/v) ethidium bromide (Thermo Fisher Scientific, Loughborough, UK). Samples were mixed with DNA 6× loading dye (0.25 % (w/w) bromophenol blue, 40 % (w/v) sucrose) in a 5:1 ratio and resolved on the agarose gel in TPE buffer. Electrophoresis was done at 80-120V then visualised using a 312 nm ultraviolet trans-illuminator and compared against 1kb molecular weight marker loaded alongside the DNA samples.

#### 2.4.1.2 DNA quantification

The concentration and purity of DNA products were determined and assessed using Nanodrop 2000 (Thermo Fisher Scientific). The concentration of DNA was determined using the knowledge that 50 μg/mL double stranded DNA has an absorbance of 1.0 at 260 nm ($A_{260}$). The purity of the DNA was assessed using the $A_{260}/A_{280}$ ratio, where the $A_{280}$ measures the protein content within the samples (due to absorbance by phenylalanine and tyrosine aromatic rings).

### 2.4.2 Protein Analysis

#### 2.4.2.1 Protein assay using Modified Lowry method

The protein concentrations were determined with a modified Lowry assay method (Lowry et al., 1951), according to the manufacturer's instructions (BioRad DC, BioRad, Hercules, USA). The provided kit contains Reagent A (alkaline copper tartrate solution) and Reagent B (dilute Folin reagent). Protein concentrations were calculated by comparing against a standard curve

produced using 0.2 mg/mL to 2 mg/mL of BSA (bovine serum albumin; from Sigma-Aldrich). Absorbance was measured via spectrophotometry on a plate reader (Multi-Skan FC, Thermo Fisher, UK) at 650 nm.

#### 2.4.2.2 SDS Polyacrylamide Gel Electrophoresis

For the analysis of protein expression and purification, sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis was performed (Laemmli, 1970). First, protein samples were denatured by incubation in protein-loading buffer (50 mM Tris base (pH 6.8), 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.005 % (v/v) bromophenol blue, 1.25 % (v/v) β-mercaptoethanol). Acrylamide gels were prepared using 8 % (w/v) acrylamide resolving gels in separating buffer (375 mM Tris base, pH 8.8, 0.1 % (w/v) SDS) and 4 % (w/v) acrylamide stacking gels in stacking gel buffer (125 mM Tris base, pH 6.8, 0.1 % (w/v) SDS). Samples were loaded (typically 20 μg of proteins) and resolved alongside molecular weight markers (SeeBlue® Plus2 Pre-stained Protein Standard, NEB) in protein running buffer (50 mM Tris Base (pH 6.8), 0.192 M glycine, 0.1% (w/v) SDS) for about 1hr 30 mins at a constant current of 40 mA per gel.

#### 2.4.2.3 Gel staining methods

Visualization of separated protein following the electrophoresis was achieved by Instant Blue® stain (Expedeon). The gel was placed in the Instant Blue® stain solution with gentle mixing on a rocking platform at room temperature for 1 hour. Following this the gel was de-stained with ultrapure double distilled water until the background became clear and the bands became prominently visible.

For staining gel with high sensitivity and low background Silver staining technique was employed using the Pierce® Silver Stain Kit (Thermo Fisher Scientific) following the manufactures instructions. Briefly, gels were first washed in ultrapure double distilled water for 10 minutes then fixed in Fixer solution (30 % (v/v) ethanol, 10 % (v/v) acetic acid solution at ratio 6:3:1 water: ethanol: acetic acid) for 15 minutes. After fixing, the gel was washed once with 10 % (v/v) ethanol and once with distilled water for 10 min each. The gel was then sensitized with Sensitizer Working Solution (50 μL

Sensitizer with 25 mL water) and incubated for exactly 1 minute, following by washed with two changes of ultrapure double distilled water for 1 minute each. The gels were then stained with Stain Working Solution (0.5 mL of Enhancer with 25 mL Stain) and incubated for 30 minutes. Before developing, the gels were washed two times with distilled water for 20 s. The bands were developed with Developer Working Solution (0.5 mL of Enhancer with 25 mL Developer). Once the bands started to appear between 2 and 3 minutes and became clearly visible, development was stopped with Stop solution (5 % (v/v) acetic acid). The gels were then washed once with ultrapure double distilled water before imaging them.

#### 2.4.2.4 Western blot

Proteins were transferred from the gel onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, UK) using a western transfer tank filled with western transfer buffer (25 mM Tris, 192 mM glycine, supplemented with 20 % (v/v) methanol). Transfer was usually performed at a constant current (320 mA), for at least 1hr 30 min at 4 °C. Transfer was verified by staining the membrane with Ponceau S stain (Thermo Fisher Scientific). Following removal of Ponceau S by washing gently with distilled water, nitrocellulose membranes were incubated in a blocking solution of PBS/Tween (0.1 % (v/v) Tween, pH 7.4) supplemented with 5 % (w/v) non-fat milk (Marvel™), incubated for 1 hr at room temperature. Subsequently, blots were incubated with primary anti-mouse monoclonal antibody BXP-21, anti-ABCG2 (Millipore) in blocking solution (1:5000 dilution) for 1 h at room temperature or overnight at 4 °C. Blots were then washed four times for 5 min with PBST to remove unbound primary antibody before they were incubated with secondary antibody, polyclonal rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP), from Dako (Glostrup, Denmark), in blocking solution (1:2000 dilution) for 1 h at room temperature. The washing steps were repeated again to remove any unbound secondary antibody.

In case of rabbit polyclonal Anti-strep tag II antibody HRP (LifeSpan Bioscience, UK) and mouse monoclonal Anti-His HRP antibody (R&D system, Minneapolis USA) after an hour incubation the membranes are washed

in PBST following chemiluminescent detection. For the detection of the protein SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used per the manufacturer's instructions. Blots were incubated in 1:1 ratio of Peroxide Solution and Luminol/Enhancer Solution for 1 min then wrapped in cling film followed by exposed onto X-ray films for various intervals (30 s to 2 min) before they were developed in the dark room.

# Chapter 3: Results

## 3.1 Expression of ABCG2

### 3.1.1 Expression of ABCG2 in Sf9 cells

In order to expresses ABC transporter proteins on a much larger scale for structural and functional studies, heterologous systems are often widely used. In this study, high expressing insect cell lines (Sf9) have been used to produce membranes containing ABCG2. Wild type Sf9 membranes (WT-Sf9) and baculovirus infected Sf9 membranes expressing ABCG2 (ABCG2-Sf9) have been utilised throughout all the experiments. ABCG2 expressing cells (ABCG2-Sf9) were lysed to get the crude membrane preparation samples. These samples were used to confirm the presence and estimated expression levels of ABCG2 using methods such as SDS-PAGE and western blot. The Figure 3.1, A shows the ABCG2 transporter is present in the membrane preparation sample (P2), following insect cell membranes preparation.

ABCG2 is represented by the bands near the 64 kDa marker. Among all the membrane preparation samples, bands were expected within the lysate, S1 (first supernatant, obtained after centrifuging the lysate) and P2 (resulting second pellet, obtained after centrifuging the S1 fraction), however not in P1 (resuting first pellet, obtained after centrifuging the lysate) and S2 (second supernatant, obtained after centrifuging the S1 fraction) as this should contain large debris only: protein was therefore lost here. Unidentified bands seen at ~60kDa within lysate, P1 and P2 represent degraded ABCG2 due to insufficient protease inhibition. The ABCG2 bands at about 65 kDa above is composed of two bands one 1 kDa bigger than the other presumably representing the core glycosylation that is in insect cell line.

### 3.1.2 Expression of ABCG2 in Mammalian and Sf9 cells

Initially, it was of interest to determine the expression level of ABCG2, in insect and mammalian cells (HEK293T cells transiently transfected with GFP-tagged ABCG2 constructs). Parallel samples of the membrane preparation from both the cell-lines were subsequently immunoblotted with BXP-21 (anti-ABCG2) and stained with Instant blue®.

The membrane preparation P2 samples (20 μg of protein) were resolved in 8 % (w/v) polyacrylamide gel. The immunoblot established that ABCG2 expressed in insect cell line are several folds higher than that expressed in mammalian cells. Apart from that, the ABCG2-Sf9 exhibit a single immune-reactive band at around 65 kDa. In case of mammalian cells ABCG2, there are two bands one around 100 kDa and the other ~80 kDa representing the two sub-population of ABCG2 having the GFP-tagged glycosylated and non-glycosylated protein (Figure 3.1, B). To check equal protein loading, P2 samples (20 μg of protein) were resolved in 8 % (w/v) polyacrylamide gel and then stained with Instant blue alongside samples expressing no ABCG2 WT-Sf9 (Figure 3.1, C).

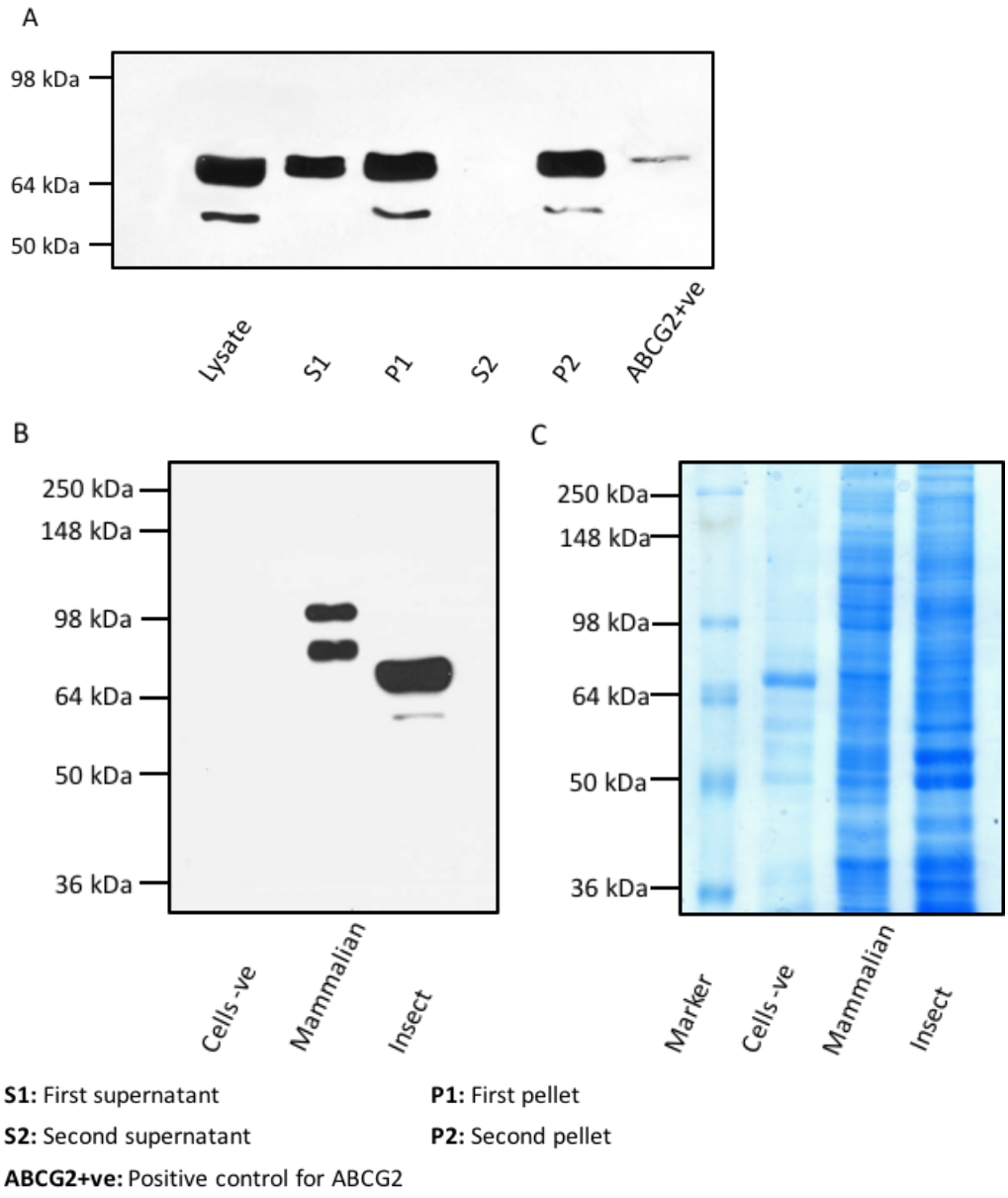



**Figure 3.1 Expression of ABCG2 in insect and mammalian cells:(A) Fractionation and Membrane preparation of Sf9 insect membranes expressing ABCG2**. Membrane preparation samples: Lysate, S1, P1, S2 and P2 of 20 µg each along with the molecular weight markers were resolved in 8 % (w/v) acrylamide. Electro-transfer to nitrocellulose membrane was probed using mouse BXP-21 antibody (1:5000) and a peroxidase-conjugated rabbit anti-mouse secondary antibody (1:2000). Following which membrane incubated in chemiluminescent reagent and then exposed for 90 second to X-ray film to allow detection. **(B) ABCG2 expression comparison in both Sf9 and Mammalian Cell Immunoblot analysis.** Cell P2 samples (20 µg protein) were prepared post membrane preparation, and were immunoblotted with BXP-21 antibodies. ABCG2-Sf9 exhibit a single immune-reactive band at around 65 kDa, whereas ABCG2-mammalian shows two immune-reactive band at around 100 kDa and ~80 kDa representing the two sub-population of ABCG2 having the GFP-tagged and non GFP-tagged protein. **(C) Gel staining analysis using Instant Blue®.** Cell P2 samples (20 µg protein) as done for the western blot immune-staining were also stained using Instant Blue to show equal protein loading. Equal protein loading can be observed when comparing the ABCG2 expressed in both insect and mammalian cell. In case, of negative control less total protein is only lysed WT-Sf9 has been used showing no expression of ABCG2 also shown in the Immunoblot. The band at around the 64 kDa marker in the negative control lane is BSA (molecular weight = 66.5 kDa)

## 3.2 $His_6$ ABCG2 solubilisation from Sf9 cell

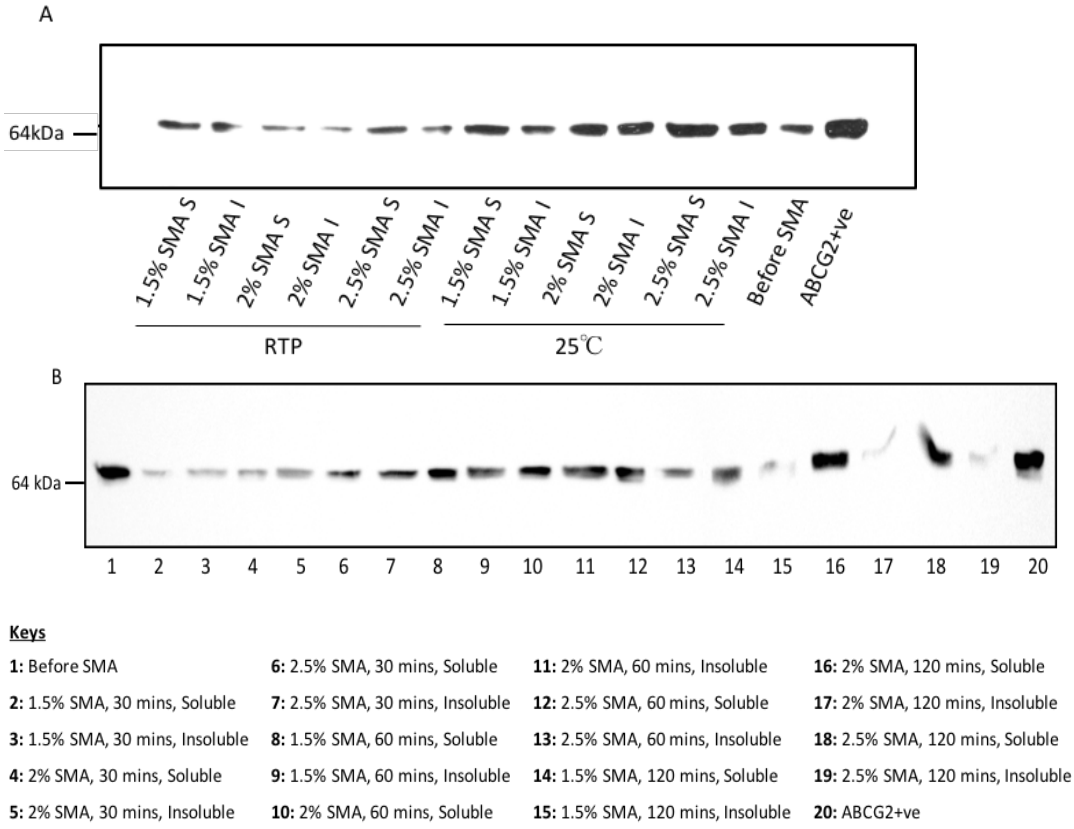
### 3.2.1 Solubilisation of ABCG2 using Styrene maleic acid (SMA)

In order to obtain sizable amount of protein, large scale cell culture (300 mL) were infected with BIIC stocks of historical construct $His_6$-ABCG2 (from I. D. Kerr). Infection was done at 150 μL of BIIC stock/ 100 mL of $0.5x10^6$ cells/ml. Typically from a single infection of 300 mL of $0.5x10^6$ cells/ml, 0.60 g or 600 mg of wet membrane pellets were obtained. To purify membrane proteins, it is necessary to first solubilise them i.e. extract them from the membrane. This extraction process is typically achieved using micelle-forming detergents, but in this research a relatively new approach of detergent-free novel, nanodisc forming membrane protein solubilisation technique, utilising SMA was used (Introduction, 1.4.2). Sf9 membranes were solubilized by incubation with SMA and the insoluble and soluble fractions separated by high- speed centrifugation. The insoluble fraction was re-suspended, and samples analysed by Western blot. The immunoblotted gel (Figure 3.2, A and B) indicates the overall protein solubilisation in comparison with the insoluble fractions i.e. the pellet obtained after centrifugation.

To optimize the solubilisation efficiency of SMA, membrane fraction P2 was solubilised using different percentage of SMA (w/v), 1.5 %, 2 %, 2.5 %. To check how temperature might affect the overall solubilisation process, samples were incubated at 25 °C and room temperature (18 °C). The samples were then analysed by immunoblotting after SDS-PAGE separation (Figure 3.2, A). The analysis indicates that ABCG2 recovery was more at 25 °C then room temperature as visibly thicker bands are observed in both the soluble and the insoluble fractions (the reason for this increase in recovery is not clear). Comparing the percentage of SMA used for solubilising, 1.5 % (w/v) in both room temperature and 25 °C solubilised less ABCG2, as bands in both the soluble and insoluble fractions are visibly of similar densities. However, not much difference was observed between the 2 % (w/v) and 2.5 % (w/v) of SMA used. In other words, 2 % (w/v) SMA does sufficient solubilisation and does not require the addition of any extra amount.

#### 3.2.1.1 Optimization of solubilisation using different percentage of SMA at 25 °C with varied incubation time

Following the results in Figure 3.2, A it was necessary to see how the incubation time effects the overall solubilisation efficiency. The temperature was kept 25 °C as this seems to help the overall ABCG2 recovery. Again, different percentage of SMA (w/v), 1.5 %, 2 %, 2.5 % was employed with variation in the incubated time by 30 minutes, 60 minutes and 120 minutes. Samples were then centrifuged to obtain the soluble and insoluble fraction, and analysed using immunoblot. Figure 3.2, B illustrates the result of the analysis where it was clearly observed that the solubilisation for 120 minutes had the most effect as the soluble fraction compared to the insoluble had more ABCG2 i.e. much thicker band; whereas for 30 minutes and 60 minutes the soluble and insoluble fraction were similar. The 2 % (w/v) and 2.5 % (w/v) of SMA had similar effects, which is most prominently observed in case of the 120 minutes' incubation.




**Keys**

| | | | |
|---|---|---|---|
| **1:** Before SMA | **6:** 2.5% SMA, 30 mins, Soluble | **11:** 2% SMA, 60 mins, Insoluble | **16:** 2% SMA, 120 mins, Soluble |
| **2:** 1.5% SMA, 30 mins, Soluble | **7:** 2.5% SMA, 30 mins, Insoluble | **12:** 2.5% SMA, 60 mins, Soluble | **17:** 2% SMA, 120 mins, Insoluble |
| **3:** 1.5% SMA, 30 mins, Insoluble | **8:** 1.5% SMA, 60 mins, Soluble | **13:** 2.5% SMA, 60 mins, Insoluble | **18:** 2.5% SMA, 120 mins, Soluble |
| **4:** 2% SMA, 30 mins, Soluble | **9:** 1.5% SMA, 60 mins, Insoluble | **14:** 1.5% SMA, 120 mins, Soluble | **19:** 2.5% SMA, 120 mins, Insoluble |
| **5:** 2% SMA, 30 mins, Insoluble | **10:** 2% SMA, 60 mins, Soluble | **15:** 1.5% SMA, 120 mins, Insoluble | **20:** ABCG2+ve |

**Figure 3.2 $His_6$ ABCG2 solubilisation from Sf9 membranes with SMA:(A) Solubilisation efficiency using different percentage of SMA at room temperature and 25 °C.** Membrane samples P2 were solubilised on 1.5 %, 2 % and 2.5 % of SMA and incubated at room temperature (18 °C) and 25 °C. The solubilisation mixture was then centrifuged to obtain the soluble and insoluble fraction, resolved in 8 % (w/v) acrylamide and immunoblotted probing using mouse BXP-21 antibody. Recovery of ABCG2 was observed to be more efficient at 25 °C in comparison to room temperature as more ABCG2 is seen in both the soluble and insoluble fractions. **(B) Solubilisation efficiency using different percentage of SMA at 25 °C with varied incubation time.** Cell P2 samples were again solubilised with different percentage of SMA and incubated at different times 30 minutes, 60 minutes and 120 minutes. All the incubation was done at 25 °C. The soluble and insoluble fraction were then analysed, which showed that the longer incubation (120 minutes) was more efficient in comparison to the shorter incubation time as more ABCG2 was present in the soluble fraction than the insoluble. Insoluble fractions were re-suspended in a 2 % (w/v) SDS solution in SMA buffer. Either 20 µL or 15 µL of protein sample was loaded on a SDS-PAGE gel. 'I' represents insoluble and 'S' represents soluble fractions respectively.

## 3.3 $His_6$ ABCG2 purification from Sf9 cells

### 3.3.1 His6 ABCG2 cannot be purified from SMA solubilised Sf9 membranes using Ni-NTA purification

The SMA solubilised ABCG2 proteins were purified by utilizing the expressed hexa-histidine tag's affinity for nickel ions, using immobilised metal affinity chromatography (IMAC). Solubilised protein sample (4 mL, from 120mg membranes) was mixed overnight in with Ni-NTA™ resin (400 µL), loaded onto a gravity filtration column. Samples were washed once in SMA buffer followed by elution with 200 mM imidazole, in 200 µL volumes (1/2 bed volumes).

#### 3.3.1.2 Immunoblot

An anti-ABCG2 immunoblot, (Figure 3.3, A) indicates that ABCG2 is present in the soluble, insoluble and flow through fractions but are not present in the wash and elution fractions. This showed that the protein comes off in the flow thorough and does not bind to the Ni-NTA resins. Purification was repeated several times with different batches of membranes prepared at different dates along with different batches of SMA (Batch 1: prepared in our lab, Batch 2: provided by Alice Rothnie, personal communication) yet similar results were obtained. In order to confirm the binding affinity, an anti-His HRP conjugated antibody was also employed showing that the same samples have an active anti-His epitope (Figure 3.3, B). Similar results of no bands being present in the wash and elutions fractions as the Anti-ABCG2 blot was observed. Bands were expected in the flow through and soluble fractions as the BXP21 antibody immunoblot showed the presence of ABCG2. Remarkably there was no immune-reactive band present with anti-His antibody in the soluble, flow through and wash fractions (compared with the red dashed boxes in the same region between the two blots). As the insoluble fraction band is near the 64 kDa molecular weight marker, it showed that the $His_6$ tag was not proteolytically degraded rather the SMA somehow masks and creates a barrier around the $His_6$ tag thus does not allow the tagged protein to bind to the nickel resin (further enlightened in Discussion 4.1).

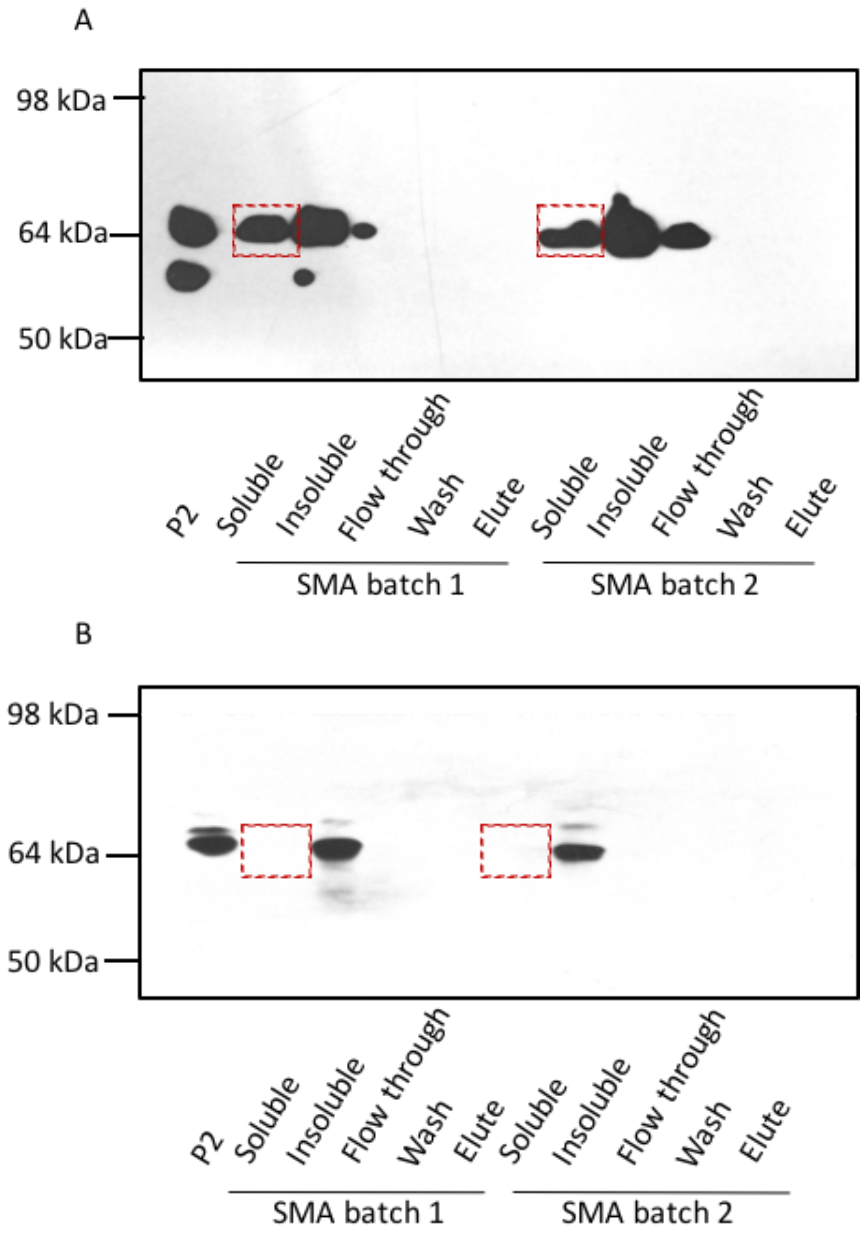



**Figure 3.3: Purification of His6 ABCG2 with Ni-NTA resins. (A) $His_6$ ABCG2 Ni-NTA purification immunoblot analysis using BXP-21**. Solubilised protein sample (4 mL, from 120mg membranes) was mixed overnight in with Ni-NTA resin (400 µL), loaded onto a gravity filtration column. The purification samples were then resolved in 8% (w/v) acrylamide and immunoblotted probing using mouse BXP-21 antibody. Immuno-reactive bands were observed in the soluble, insoluble, flow through fractions indication the presence of ABCG2. No ABCG2 is observed in the wash and the strip fraction, indicating that the protein has come off in the flow through. **(B) $His_6$ ABCG2 Ni-NTA purification immunoblot analysis using Anti-His HRP.** Same purification samples where immunoblotted using Anti-His HRP conjugated antibody. Immuno-reactive bands were observed in the P2 and insoluble fractions indication the presence of the active $His_6$-tag. Bands were expected in the soluble and flow through fractions as ABCG2 is present shown in **(A)**. Interestingly, this shows that the $His_6$-tag is masked by the SMALPs and as there are no free $His_6$-tag present to bind to the Nickel resins thus no protein has been purified.

## 3.4 Strategy for generation of Strep-tagged and StrepHis$_{13}$-tagged ABCG2

### 3.4.1 Plasmid construct used for this study

During the course of this study though, it was found that the 6×His tagged ABCG2 protein was difficult to purify as the N-terminal His$_6$ tag is masked by SMA, thus a much stronger affinity based tag Strep tag was designed to give better binding to the resins and purification of the protein.

The plasmid pFastBac_HTC_ABCG2 was chosen as the backbone for all DNA construct engineering in this study. The vector typically, contains an N-terminal Hexa-Histidine (6×His) tag fused N-terminally to ABCG2 for purification of recombinant fusion proteins using metal-chelating resin and a TEV protease cleavage site for removal of the His tag subsequent protein purification (Polayes et al., 1996). C-terminally tagging of ABCG2 was previously reported to be detrimental to ABCG2 localisation and purification (Haider et al., 2011). The vector was modified to contain two different constructs one a Strep tag instead of a His$_6$ tag, and two a double tag of Strep tag and His$_{13}$ tag. A longer sequence of His tag was used to mainly increase the length of the tag so that it does not get buried inside the SMALP particles (Further explained in Discussion 4.1) and also so that different affinity resins for binding can be used due to the presence of two different tags.

### 3.4.2 Strep-tagged ABCG2 and Strep-His$_{13}$-tagged ABCG2

The overall strategy for construction of Strep-ABCG2 and His$_{13}$-Strep-ABCG2 is illustrated in Figure 3.4, A and B. Briefly, it involves the annealing of primer pairs encoding the desired tags, and the ligation of these products into existing pFastBac based expression vectors. For the generation of Strep-tagged ABCG2 the entry vector was the pFastBac_HTC_ABCG2_His$_6$ while for the His$_{13}$-Strept-tagged ABCG2 was the pFastBac_HTC_ABCG2_His$_{12}$ (Haider et al., 2011) provided by Dr. Ian Kerr (University of Nottingham).

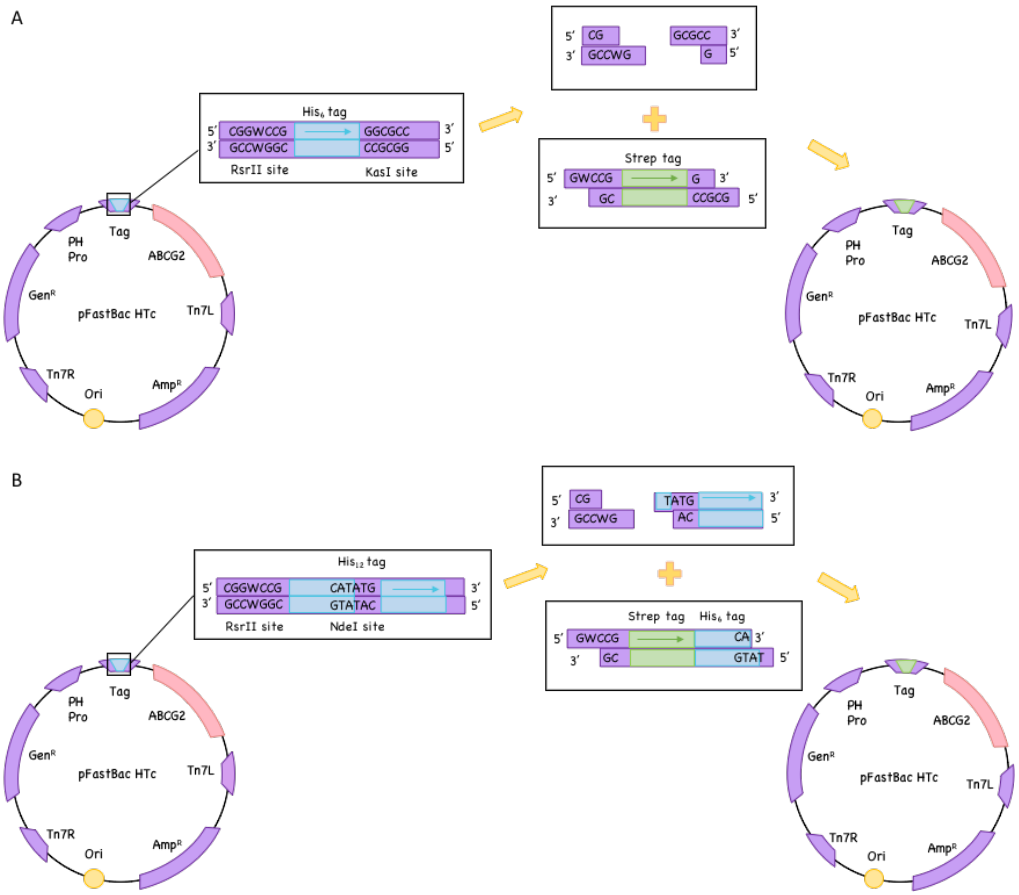



**Figure 3.4 Strategy for generation of Strep-tagged and StrepHis$_{13}$-tagged ABCG2:(A) Schematic illustration of the strategy to change from His$_6$ tag to a Strep tag.** The starting templates were digested with *RsrII* and *KasI.* The desired vector was purified and ligated to the annealed oligonucleotide containing the sequence coding for the Streptavidin protein and half of both the *RsrII* and *KasI* restriction sites. As the annealed oligonucleotide ligates with the digested vector, thus the His$_6$ tag is subsequently replaced by a Strep tag. **(B) Schematic illustration of the strategy to change from His$_{12}$ tag to a Strep-His$_{13}$ tag.** The starting templates were digested with *RsrII* and *NdeI.* The double digest removes the N-terminal His$_6$ tag from the His$_6$-tagged construct and His$_6$ of the His$_{12}$ from the His$_{12}$ tag The desired vector was purified and ligated to the annealed oligonucleotide containing the sequence coding for the Streptavidin protein followed by a His$_6$ and half of both the *RsrII* and *NdeI* restriction sites. As the annealed oligonucleotide ligates with the digested vector, thus the vector additionally get a Strep tag followed by its slightly modified His$_{13}$ tag.

### 3.4.3 Molecular cloning

The pFastBac_HTc_ABCG2_$His_6$/$His_{12}$ plasmids (200-500 ng) were digested with *RsrII* and *KasI*/*NdeI*, respectively and analysed on 0.7 % (w/v) agarose gel. Undigested vector, single digest as well as double digest were run parallel to check the restriction digest activity (Figure 3.5, A, B and C). Double-digested plasmid DNA was extracted as described in the methods.

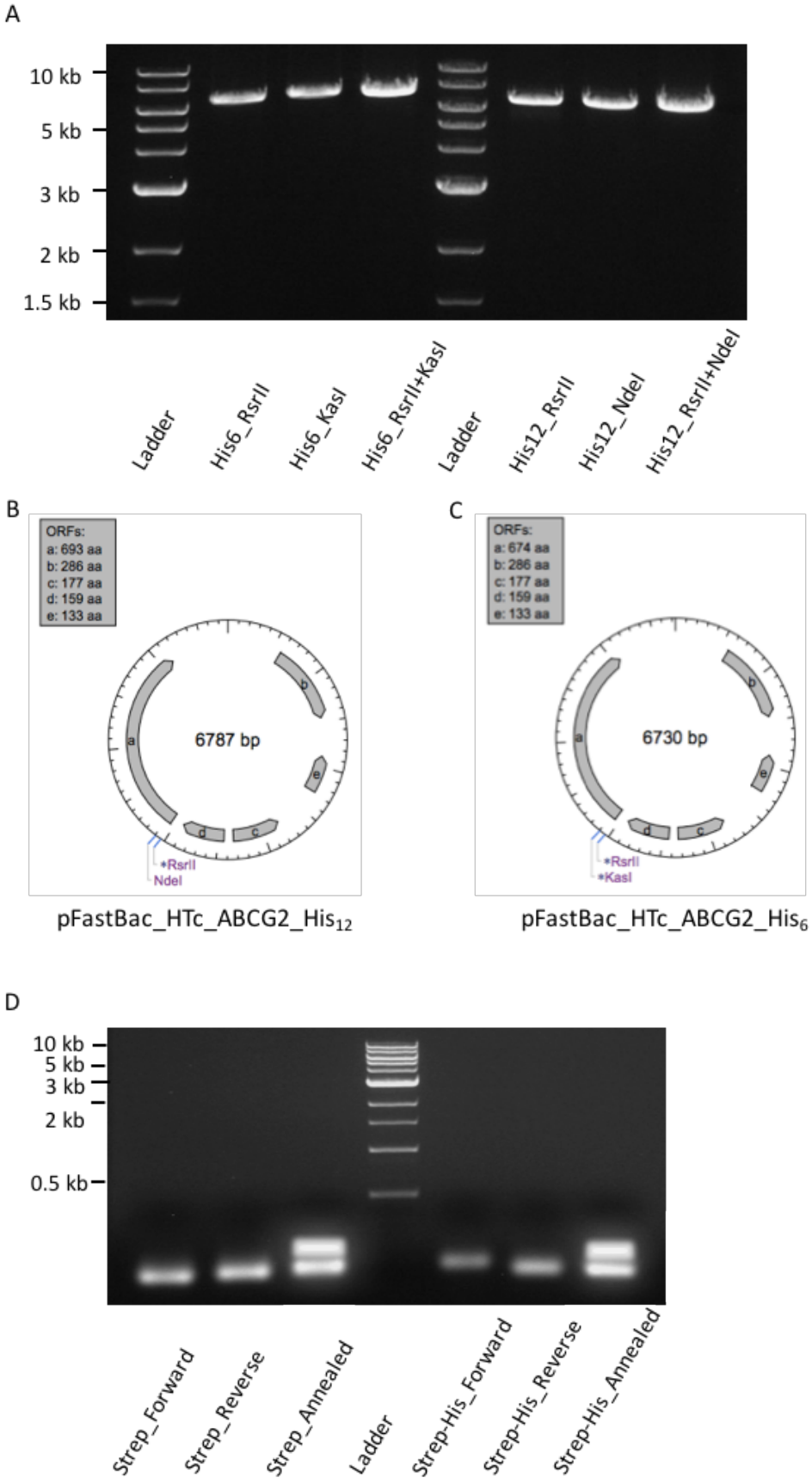
The forward and reverse oligomers (10 mM, 5 µL) for both Strep and Strep-$His_6$ tag were annealed together (Methods 2.1.2.2) and a sample of the annealing reaction was analysed on a 1 % (w/v) agarose gel shown in Figure 3.5, D, with an easily observed presence of a higher molecular weight product in the annealing reaction.

### 3.4.4 Diagnostic digest for the confirmation of Strep, Srep-His13-tagged ABCG2 presence

Following ligation of the annealed double-stranded oligonucleotides with the gel extracted linear plasmid, DNA was transformed into DH5α competent cells and plasmid DNA was then isolated from single colonies prior to confirmatory restriction digest with restriction enzyme *BstBI* and *HindIII* to confirm correct band sizes before the samples were sent for DNA sequencing. Figure 3.5, E below shows the gel electrophoresis from the resultant restriction digest, confirming that the bands generated were of the right size of approximately 5000 and 2000 base pairs (bps).

### 3.4.5 Sequencing of the Strep, Srep-His13 constructs of ABCG2

The restriction digest analysis verified the incorporation of Strep-tag in place of $His_6$ tag and Strep-$His_{13}$ tag into the pFastBac_HTC_ABCG2 plasmid, but to ensure no secondary mutations were incorporated constructs were entirely sequenced as described in (Methods 2.1.6). DNA sequencing chromatograms (generated using Chromas), were first used to confirm the reading frame and signal quality of the data then analysed with NCBI: BLAST, to align the predicted and observed sequence data. The sequencing data demonstrates the correct insertion of the tag into the plasmid (Figure 3.5, F and G).


A
10 kb
5 kb
3 kb
2 kb
1.5 kb
Ladder
His6_RsrII
His6_KasI
His6_RsrII+KasI
Ladder
His12_RsrII
His12_NdeI
His12_RsrII+NdeI
B
ORFs:
a: 693 aa
b: 286 aa
c: 177 aa
d: 159 aa
e: 133 aa
6787 bp
*RsrII
NdeI
pFastBac_HTc_ABCG2_His12
C
ORFs:
a: 674 aa
b: 286 aa
c: 177 aa
d: 159 aa
e: 133 aa
6730 bp
*RsrII
*KasI
pFastBac_HTc_ABCG2_His6
D
10 kb
5 kb
3 kb
2 kb
0.5 kb
Strep_Forward
Strep_Reverse
Strep_Annealed
Ladder
Strep-His_Forward
Strep-His_Reverse
Strep-His_Annealed

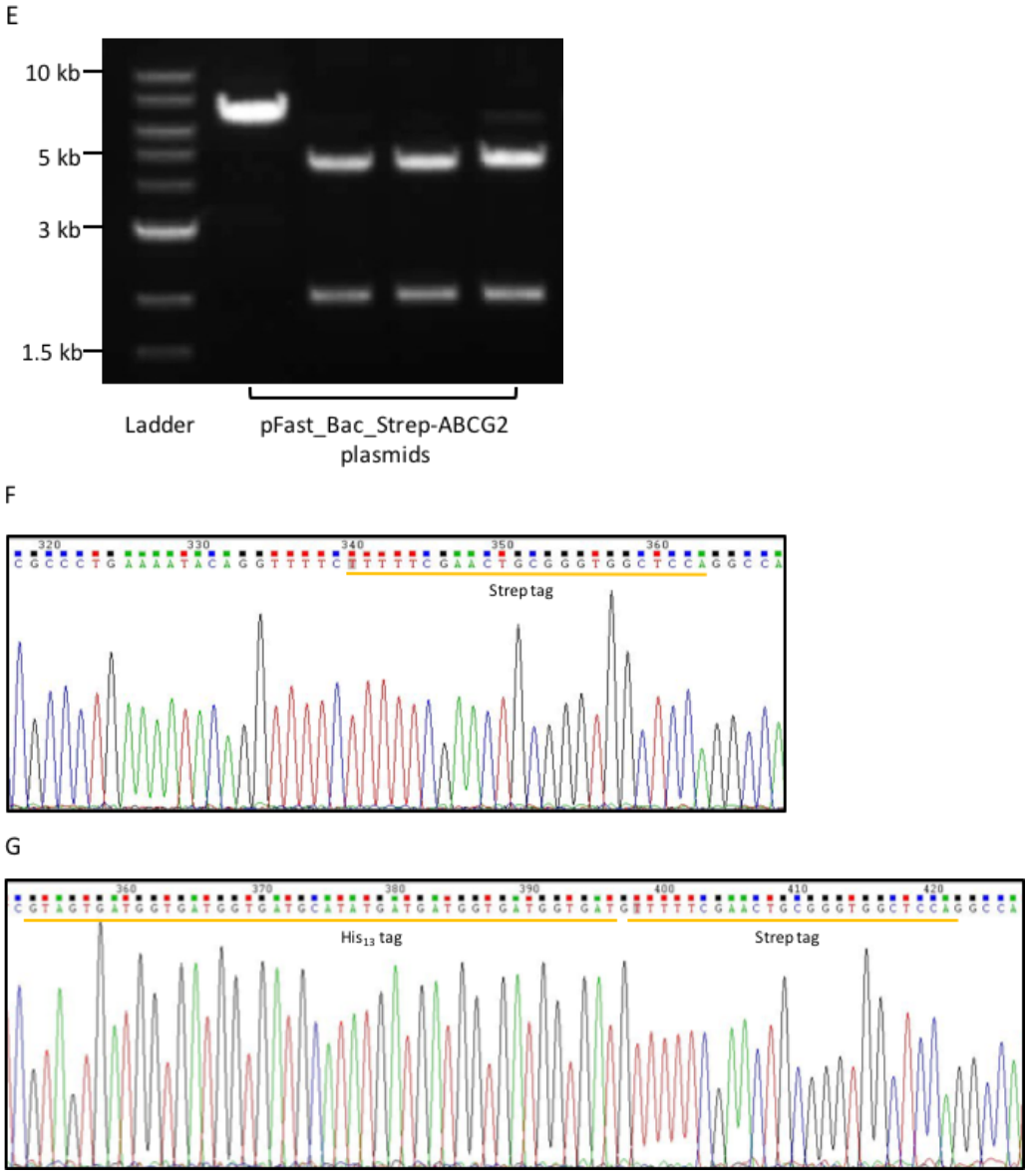



**Figure 3.5 Generation of Strep-tagged and StrepHis$_{13}$-tagged ABCG2: (A) Restriction digest of plasmids.** 200-500 ng of the pFastBac_HTC_ABCG2 His$_6$/His$_{12}$, plasmids were digested with *RsrII* and *KasI/NdeI*, and analysed on a 0.7 % (w/v) agarose gel, carried out by Deb Briggs. **(B &C) Restriction maps of pFastBac_HTC_ABCG2 His$_{6/}$His$_{12}$.** Schematic representation of restriction maps for both the constructs digested with the enzymes. **(D) Primer annealing.** The forward and reverse oligomers (10 mM, 5 µL) for both Strep and Strep-His$_6$ tag were annealed together was analysed on a 1 % (w/v) agarose gel, running alongside the single-stranded oligonucleotides. **(E) Diagnostic digest.** The restriction digest analysis to verify the incorporation of Strep-tag in place of His$_6$ tag and Strep-His$_{13}$ tag into the pFastBac_HTC_ABCG2 plasmid. If the ligation is successful, the plasmid gains a *BstBI* site which is thus used as a diagnosis to whether the ligation has worked. **(F & G) Sequencing chromatograms confirming the generation of pFastBac_HTC_ABCG2_Strep and pFastBac_HTC_ABCG2_ His$_{13}$-Strep**. A Seq R1 primer covered the cDNA which shows a specific sequence at the N-terminus of pFastBac_HTC_ABCG2; the Strep tag and His$_{13}$ obtained in frame, leading to the tags confirming the incorporation to ABCG2. 1kb (kilo bases) DNA Ladder was loaded alongside the samples.

## 3.5 Generation of Recombinant baculovirus for Strep-tagged and StrepHis$_{13}$-tagged ABCG2 expression

### 3.5.1 An overview of the generation of Bacmid DNA

The pFastBac_HTC_ABCG2_Strep and pFastBac_HTC_ABCG2_ His$_{13}$-Strep generated were used to transform competent DH10Bac *E.coli* cells to generate the corresponding recombinant bacmid DNA. DH10Bac cells contain a baculovirus genome (bacmid), and site specific transposition between this bacmid and the pFastBac_HTC_ABCG2 vector occurs at the mini attTn7 site resulting in the disruption of the *LacZ* gene enabling the transformants to be identified as white colonies in the presence of a chromogenic substrate such as X-gal and the inducer IPTG, Figure 3.6.

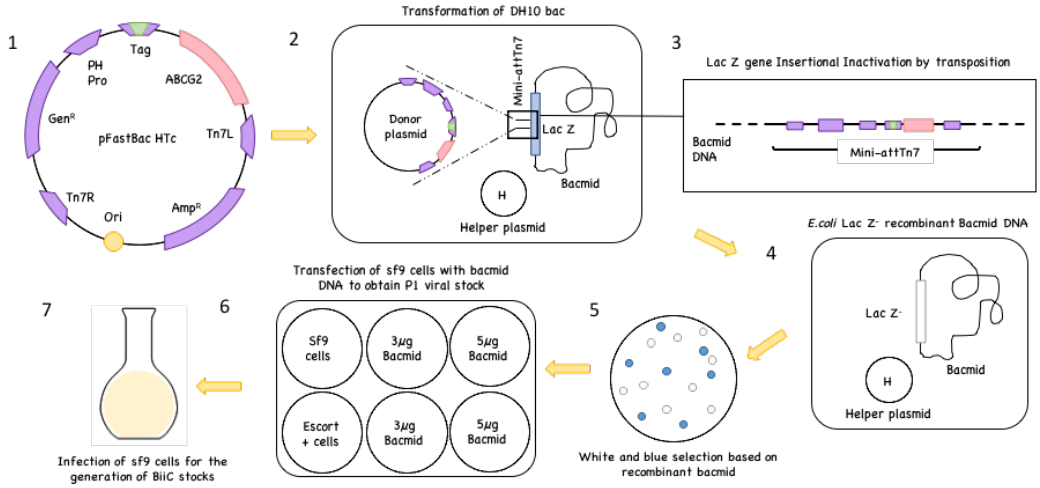



**Figure 3.6: Generation of recombinant viruses from pFastBac_HTC_ABCG2_Strep and pFastBac_HTC_ABCG2_ His$_{13}$-Strep.** Using the Bac-to-Bac Baculovirus Expression system (1) the gene of interest is first cloned into a suitable vector (in our case pFastBac_HTC_ABCG2) containing a polyhedrin promoter and a mini-Tn7 element. (2) The recombinant donor plasmid is then transformed into a competent *E.coli* cell (DH10Bac) which contains a bacmid with a mini-*att*Tn7 target site and a helper plasmid. (3) The mini-Tn7 element on the recombinant donor plasmid facilitates transposition of the gene of interest (GOI) into the target site on the bacmid along with transposition proteins provided by the helper plasmid, resulting in insertion of the GOI into the bacmid DNA (4). *E.coli* cell (DH10Bac) containing the recombinant bacmid DNA causes the disruption of the *LacZ* gene thus when spread plated onto the media containing X-gal and the inducer IPTG, colonies appear white (5). (6) Following this recombinant bacmid DNA is purified and transfected into insect cells (Sf9) to produce a P1 virus stock. (7) The P1 viral stock is tested by performing a mini-infection of Sf9 insect cells with the recombinant viruses to produce BIIC stocks. (adapted and modified from Bac-to- Bac® Baculovirus Expression System manual).

### 3.5.2 Confirmation of recombinant Bacmid DNA

Following the guidance suggested by the Invitrogen Bac-to-Bac® manual, DH10Bac cells were transformed with the pFastBac_HTC_ABCG2 vectors. Well-isolated colonies that were collected from the spread plate were re-streaked onto the LB-Agar plates supplemented with X-Gal and IPTG to confirm their phenotype (Figure 3.7, A). Blue colonies were also selected as control to ensure the fidelity of blue-white screening. The high molecular weight of bacmid DNA (> 100 kbp) precludes the use of conventional mini-prep kits for plasmid DNA which result in shearing of the larger bacmid. Therefore, high molecular weight DNA was prepared from small scale overnight cultures (Methods 2.1.5) using a manual alkaline lysis protocol. The resulting recombinant bacmids were analysed by PCR employing M13F (5'-GTTTTCCCAGTCACGAC-3') and M13R (5'-CAGGAAACAGCTATGAC-3') primers along with ABCG2 primers Seq R1, and Seq 482 (Figure 3.7, B) to confirm that the integration of the ABCG2 had been successful.

### 3.5.3 Transfection of Sf9 cells with the Bacmid DNA

Once the PCR confirmation was done, the bacmid DNA (3 µg or 5 µg) was then used to transfect monolayer Sf9 cells (Methods 2.2.3.2). Over a period of 5-6 days, changes in cell shape, cell size and density of the cells were observed daily under the microscope where the cells became bigger and more rounded while the number of cells decreased in comparison to the control cells. This phenotype is indicative of viral production, and at this stage cells were then harvested and centrifuged with the supernatant being a low titre "P1" viral stock. The remaining cell pellet was lysed by sonication in ice-cold sterile PBS-glycerol (10 % (v/v)) to check for the protein expression (Figure 3.7, C). ABCG2 expression levels can be seen to vary (thicker bands) with the amount of bacmid DNA used for the transfection process. Parallel to this immunoblotting was also done by using anti-strep-HRP conjugate antibody (1:2000) to check the expression of the Strep tag (Figure 3.7, D). Unlike the ABCG2 blot the anti-strep blot only shows bands near the 64 kDa region showing the highest concentration of the protein found due to the antibody's low reactivity in comparison to the BXP-21. It can also be possible that anti-

ABCG2 antibody also detects higher molecular weight species (ABCG2 oligomers not denatured by SDS), but that the anti-strep antibody does not detect these for reasons unexplored.

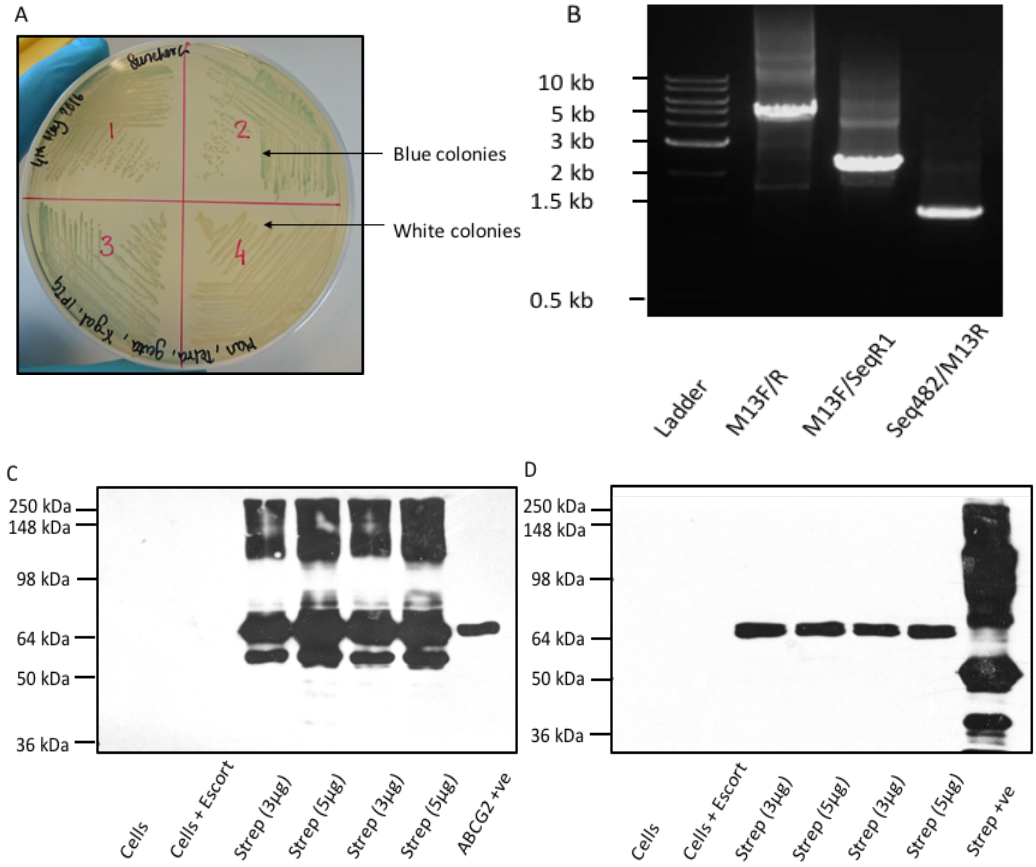



**Figure 3.7 Generation of recombinant viruses for expression of Strep and $His_{13}$-Strep-tagged ABCG2: (A) White and Blue selection of DH10Bac colonies.** Bacmids containing the recombinant gene will give white colonies. The *lac Z* fragment is induced by IPTG, which in normal cases hydrolyse X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and form blue colonies when grown on supplemented media. Due to insertion of the recombinant DNA, it causes an inactivation of the *lac Z* gene thus cannot hydrolyse the X-gal in the media giving colonies their white colour. Only the cells with the bacmid containing the gentamycin resistance ($gen^R$) gene will survive. **(B) PCR confirmation of the recombinant bacmid.** The gel electrophoresis result shows PCR amplification of the recombinant bacmid DNA. The primers M13 forward and reverse bind upstream and downstream of the mini-attTn7 site, a total of 2.3 kb either side of the inserted ABCG2 cDNA. Thus, the M13F/M13R PCR product is 2.3 + 2.0 kb (the size of tagged ABCG2) = 4.3 kbp. Use of ABCG2 specific primers (Seq 482 and Seq R1) results in smaller PCR products of the predicted sizes **(C) Western blot analysis indicating the expression of ABCG2 with BXP-21.** Proteins were loaded onto each lane in the 8 % w/v polyacrylamide gels (reducing conditions). Following electrophoresis and transfer, nitrocellulose membrane was probed using using mouse BXP-21 primary monoclonal antibody. **(D)Western blot analysis indicating the expression of ABCG2 with Anti-strep HRP.** Samples were also probed using the anti-strep-HRP conjugated mouse antibody to check for the expression of the strep-tag. Similar results were obtained for the Strep-$His_{13}$ constructs.

## 3.6 Expression of Strep-tagged and StrepHis$_{13}$-tagged ABCG2

### 3.6.1 Generation of BIIC (Baculovirus Infected Insect Cell) stocks

The P1 viral stocks that were obtained are not sufficient for large scale expression of protein as they are low volume (< 2 ml) and low titre (i.e. probably fewer than 1 x $10^6$ viral particles/ml). To expand the viral stock two alternatives are possible. The first is to amplify the volume and titre of the virus by making a P2 and then a P3 viral stock by two cycles of viral infection and cell lysis (Hitchman et al., 2009). An alternative is to generate "BIIC stocks" of the Strep-tagged and Step-His$_{13}$-tagged ABCG2 constructs. BIIC stocks allows long-term storage of baculovirus virus in conditioned medium thus eliminating the need for virus amplification and re-titering (Wasilko et al., 2009). It requires for the cells to *not be lysed* but to be harvested just prior to lysis, such that upon reintroduction into media, the BIIC cells burst releasing all the viral DNA for the infection of the cell culture and protein expression (Wasilko et al., 2009).

Insect cells are normally around 15-16 μm diameter/radius but when rich in viral particle increase to about 18-19 μm which is deemed to be highly infectious. Typically, a 50 mL culture of Sf9 cells was infected with 0.5 mL of the P1 stocks and after 24-48 hours the expected increase in diameter was observed. Control cells and infected cell diameter were measure using the Moxi Z Mini Automated Cell Counter Kit (Orflo, USA). Figure 3.8, A shows the increase in the cell diameter measure in the control (uninfected cells) and the infected cells.

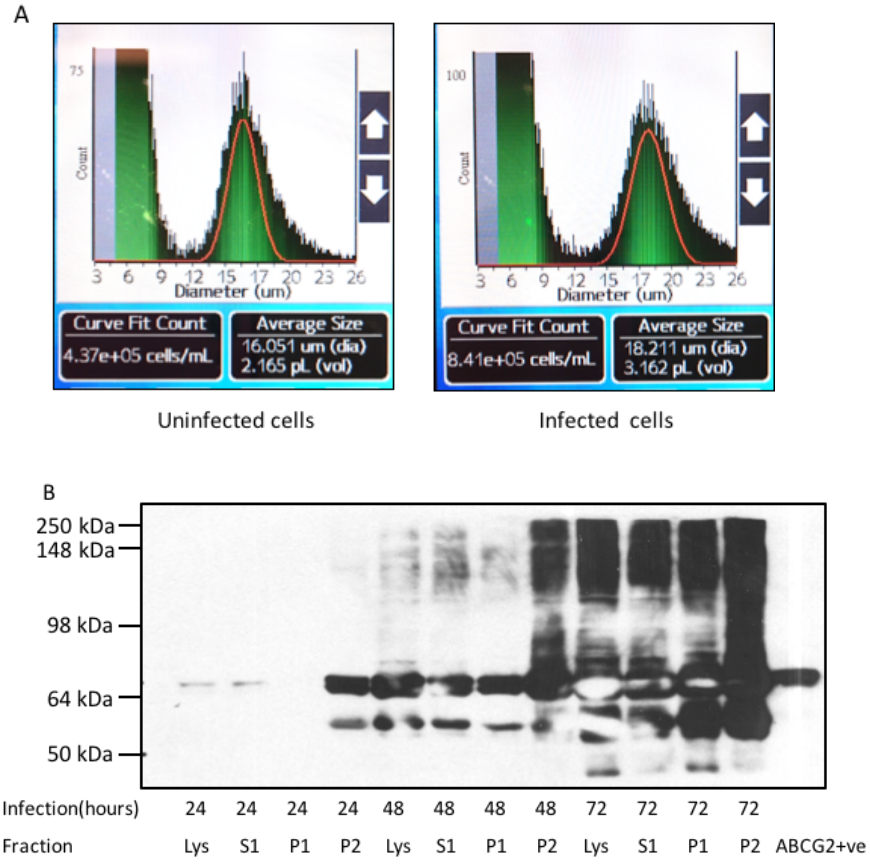
### 3.6.2 Time-course expression of the BIIC stocks

To check the efficiency of the BIIC stocks, optimisation of protein expression is necessary to determine the best point to harvest cells post infection. Cells were seeded at $0.5\times10^6$ in 150 mL and infected with 100 μL of BIIC stock. Starting from $T_0$ (at infection) samples (10 mL) were collected at $T_{24}$, $T_{48}$ and $T_{72}$ and then lysed to carry out small-scale membrane preparation.

The membrane preparation samples of the time-course experiment were resolved on 8 % (w/v) polyacrylamide gels and immunoblotted with anti-ABCG2 BXP-21and anti-strep antibody (Figure 3.8, B, C). The protein expression is low at 24 hours, and increases at both 48 and in particular 72 hours. Protein is found in the membrane fraction as expected, and again as previously observed, SDS-resistant ABCG2 aggregates are detected by BXP-21 but not by anti-Strep antibodies.

### 3.6.3 Comparison in protein expression of the $His_6$, Strep, Strep-$His_{13}$ BIIC stock

Three small-scale (150 mL) infections were performed to check the expression level of the $His_6$, Strep, Strep-$His_{13}$ tagged ABCG2 BIIC stocks. Samples were harvested, lysed and membrane preparation samples were immunoblotted with BXP-21 and stained with Instant Blue. Protein expression level are seen to be much higher in the Strep and Strep-$His_{13}$ constructs compared to the $His_6$ constructs (Figure 3.8, D, E)



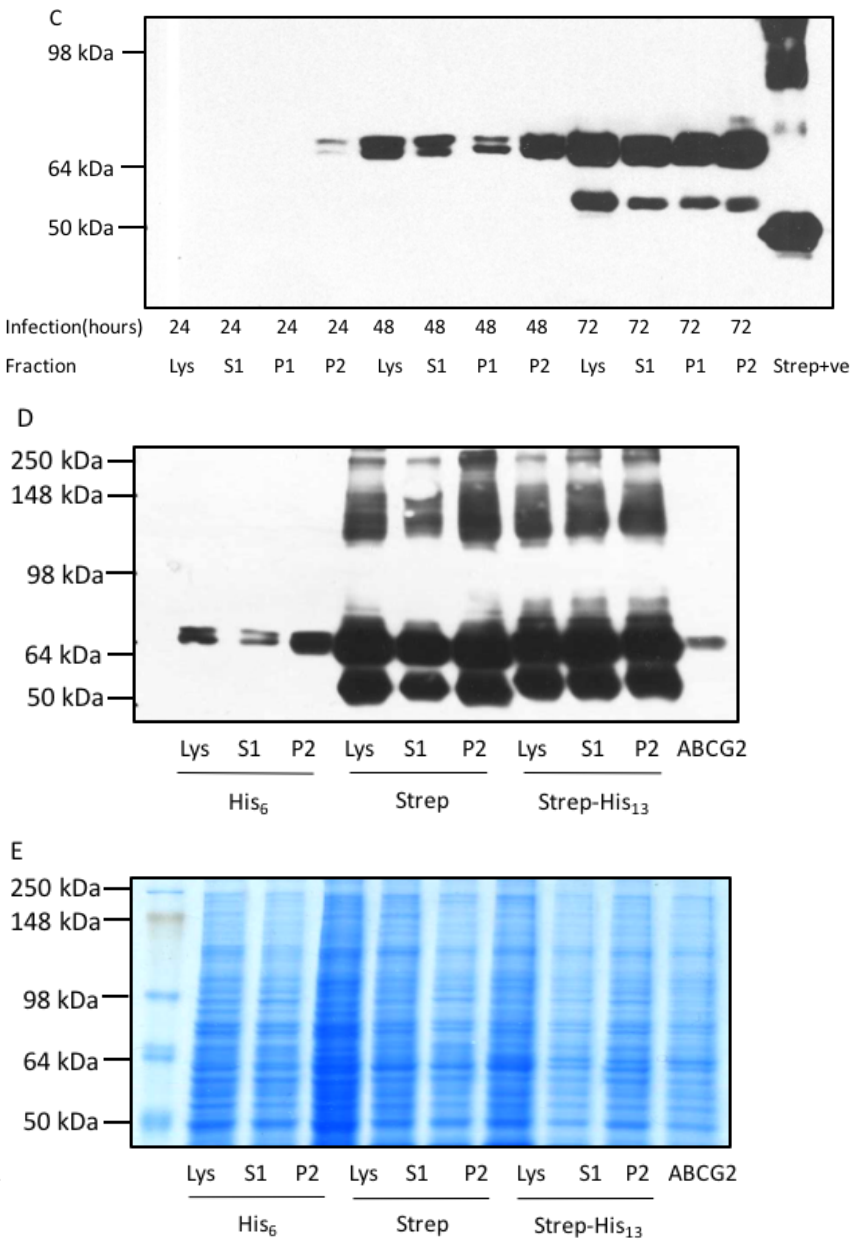



**Figure 3.8 Generation of BIIC stock and optimization of ABCG2 expression: (A) Cell diameter Histogram.** Control cell and Infected cells diameter were measure using the Moxi Z Mini Automated Cell Counter Kit. 75 µL of the cell sample were loaded onto the machine, following which the machine gave the cell diameter reading in µm along with the number of cells/mL. **(B) & (C) Time-course expression of the Strep constructs.** Cells (150 mL) were infected with strep-tagged ABCG2 BIIC stocks. Sample (10 mL) were collected every 24 hours $T_{24}$, $T_{48}$ and $T_{72}$ and lysed to analyse by western blotting and probing with BXP-21 and Anti-strep antibody. Similar results were obtained with the strep-$His_{13}$ constructs. **(D) Western blot analysis of the comparison between the BIIC stocks of $His_6$, Strep, Strep-$His_{13}$ tagged ABCG2 constructs.** Three small-scale expression were performed samples were lysed followed by membrane preparation at 72 hours. Proteins were loaded onto each lane in the 8 % (w/v) polyacrylamide gels (reducing conditions). Following electrophoresis and transfer, nitrocellulose membrane was probed using using mouse BXP-21 primary monoclonal antibody. **(E) Instant Blue analysis of the comparison between the BIIC stocks of $His_6$, Strep, Strep-$His_{13}$ tagged ABCG2 constructs.** Samples were also run in a parallel gel and stained with Instant Blue to confirm approximately equal protein loading.

## 3.7 Solubilisation of Strep-tagged and StrepHis$_{13}$-tagged ABCG2 with SMA

### 3.7.1 Strep-tagged ABCG2 solubilized well with SMA

Once Strep-tagged and Strep-His$_{13}$-tagged ABCG2 were expressed, cells were lysed and membrane preparation was performed. Membrane fractions P2 were solubilised with 2 % (w/v) SMA at 25 °C for 2 hours. The solubilisation fraction were then centrifuged to get the insoluble fraction, which were both resolved alongside in 8 % (w/v) polyacrylamide gels. Gels were stained in Instant blue and Immunoblotted with BXP-21 and anti-strep antibody (Figure 3.7 A, B and C). It was observed that SMA can solubilise Strep-tagged ABCG2, having plenty of the protein in the soluble fraction than the insoluble. When probed with the anti-strep antibody a visible band is observed in the soluble fraction than the insoluble showing that the antibody can bind with exposed tag. Thus, in contrast to the His$_6$ tag (Figure 3.3) the SMA appears not to occlude the affinity tag in the Strep-ABCG2 construct. The same observation was made of the Strep-His construct but the data is not shown.

$$$$$$

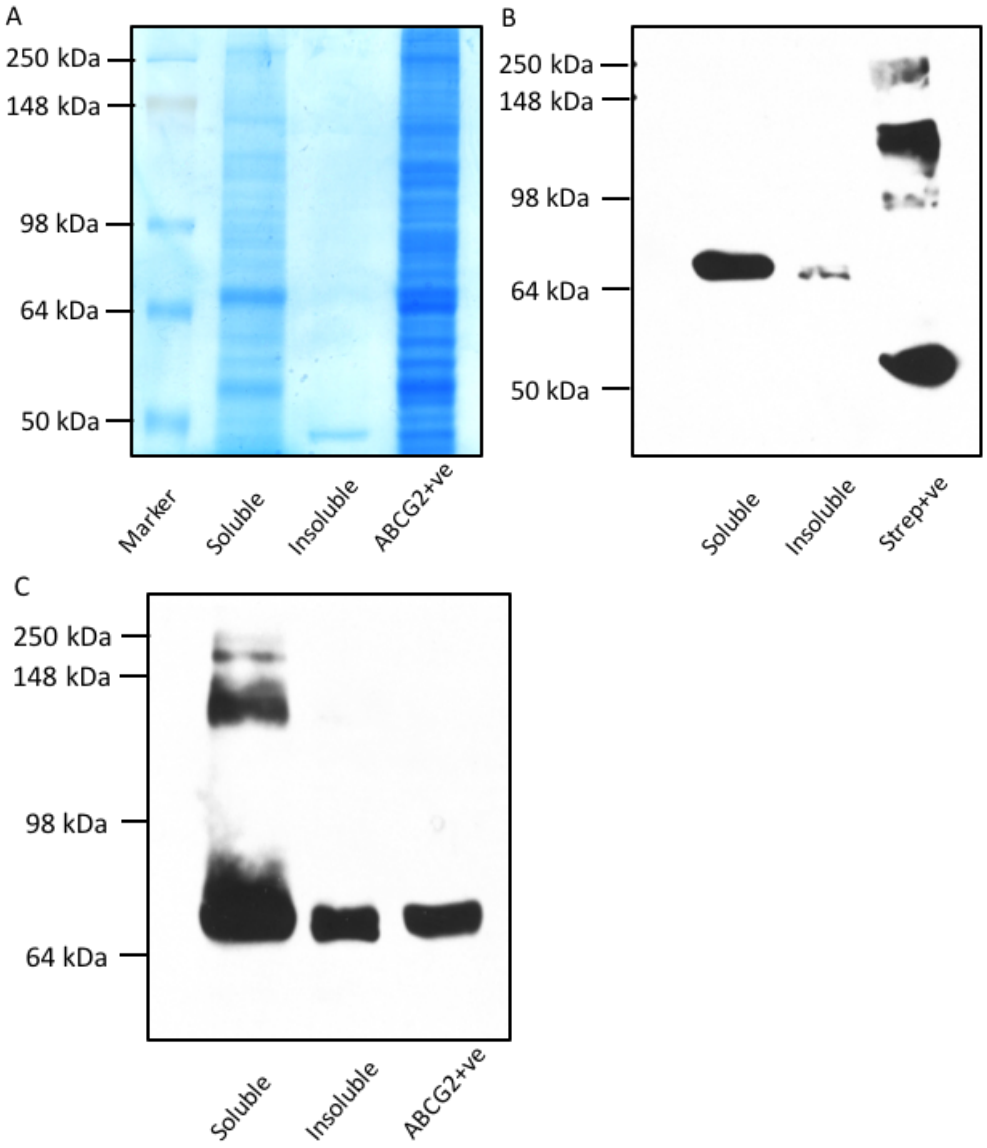



**Figure 3.9 Solubilisation of Strep-tagged and StrepHis$_{13}$-tagged ABCG2 with SMA (A), (B) & (C) SMA solubilised ABCG2.** The protein samples of equivalent protein were loaded and resolved in 8 % (w/v) polyacrylamide gels. Gels were then stained with Instant Blue **(A)**, and immunoblotting with anti-strep antibody **(B)** and BXP-21antibody **(C)**. A stronger signal in observed in the soluble fractions for both the BXP21 and anti-strep antibody blots compared to the insoluble fractions suggesting more protein has been solubilised. Although, the differences in both the blots for the insoluble fraction can also be due to the reactivity of the two different antibodies to their subsequent epitopes. Positive controls were run alongside soluble and insoluble fractions. The band at around the 64 kDa marker in the negative control lane is BSA (molecular weight= 66.5 kDa)

## 3.8 Purification of SMA solubilised Strep-tagged and StrepHis$_{13}$-tagged ABCG2

### 3.8.1 Strep-tagged and Strep-His$_{13}$-tagged ABCG2 cannot be purified from SMA solubilised Sf9 membranes using Streptactin or Ni-NTA purification

Following solubilisation with SMA, Strep-tagged and Strep-His$_{13}$ tagged ABCG2 were subjected to purification using streptactin and nickel resins. Solubilised Strep-tagged and Strep-His$_{13}$ tagged ABCG2 protein sample (2 mL, from 60 mg membranes) was mixed overnight with streptactin or Ni-NTA resin and loaded onto gravity filtration columns. Resins were washed twice in suitable wash buffers (Methods 2.3.3.1 and 2.3.3.2) and then eluted thrice with either 3 mM desthio-biotin (for streptactin resins) or 200 mM imidazole (for Ni-NTA), in 120 µL volumes (1/2 bv).

An anti-ABCG2 immunoblot, (Figure 3.9) indicates that ABCG2 is present in the soluble, flow through and wash fractions but not present in the elutions. Despite the use of two different tags and resins, this again showed that the protein comes off in the flow thorough and wash and does not show any binding to the Ni-NTA and streptactin resin.

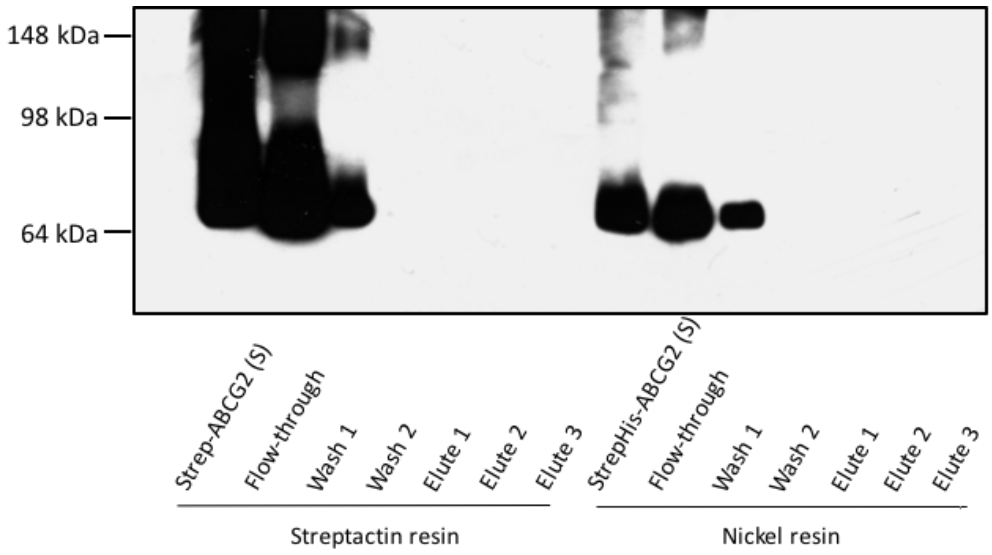


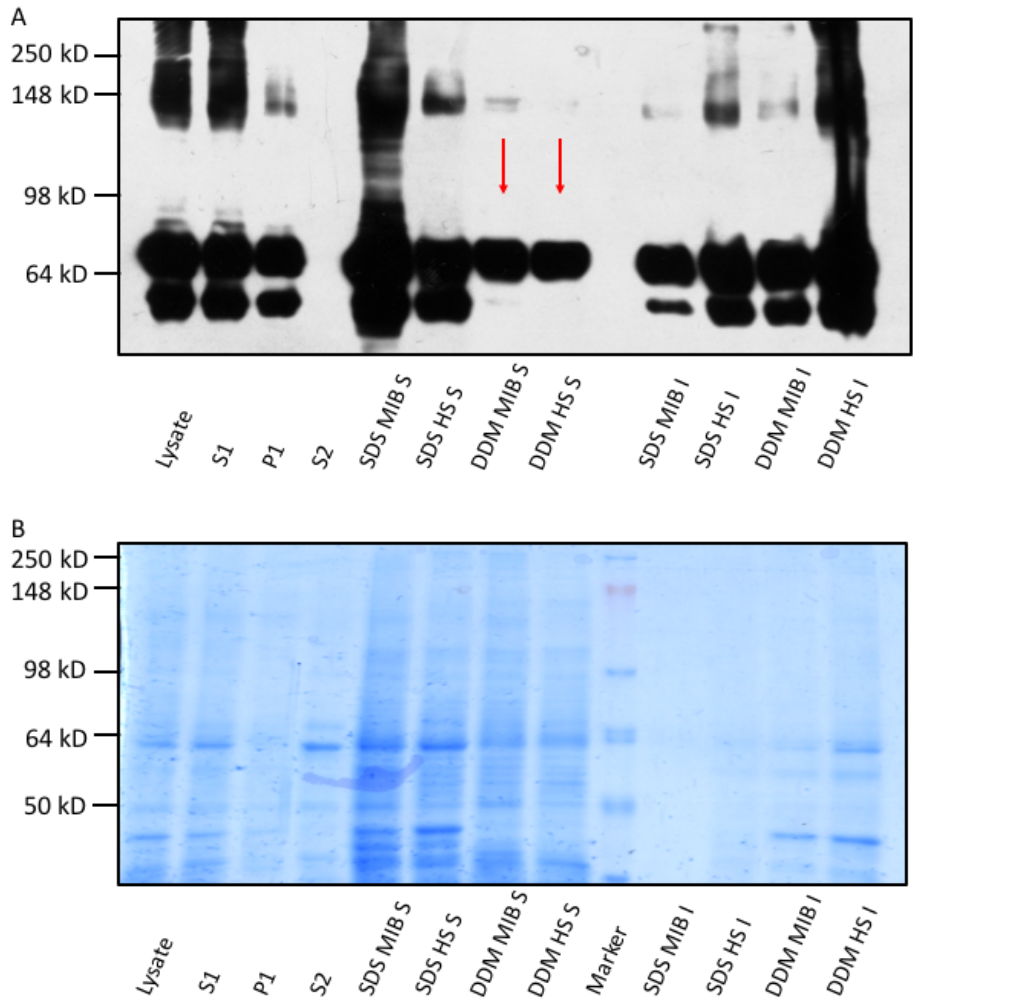

**Figure 3.10: Strep-tagged and StrepHis$_{13}$-tagged ABCG2 cannot be purified from SMA solubilised Sf9 membranes using Streptactin and Ni-NTA purification.** The protein samples were resolved in 8 % (w/v) polyacrylamide gels followed by immunoblotting with BXP-21 antibody. Figure shows the use of two resins Streptactin for Strep-tagged ABCG2 and Nickel resins for the Strep-His13-tagged ABCG2.

## 3.9 Detergent solubilisation of ABCG2

### 3.9.1 Strep-$His_{13}$-tagged ABCG2 can be solubilised using DDM-lipid mixture

Following the SMA solubilised ABCG2 purification problems, the classical approach of solubilisation using detergent was employed (detailed procedure explain in Methods 2.3.2.2). Previous work in the Callaghan lab (McDevitt et al., 2006) had shown that insect cell expressed ABCG2 was refractory to detergent solubilisation except in the expensive fos-choline-16 and fos-choline-14 detergents. More recent papers by the Sarkadi group has indicated that detergent/lipid mixtures in high salt buffers could solubilize ABCG2 from insect cells (Telbisz et al., 2013). Thus, detergent/ lipid mixture solubilisation was done as per Telbisz's methods.

As described in the methods, membranes were prepared by ultra-centrifugation and either re-suspended in a low salt buffer (10 mM Tris pH 7.4, 250 mM sucrose, pH 8.0) or a high salt buffer (4 mM EDTA, 500 mM NaCl, 20 mM Tris, 10 % (v/v) glycerol, pH 8.0). These are referred to hereafter as low salt membranes and high salt membranes. Membranes were then incubated with detergent: lipid mixtures to allow solubilisation for 90 minutes, using SDS as a positive control as this strong ionic detergent should effectively solubilize ABCG2 from membranes regardless of salinity. Insoluble and soluble fractions were resolved and analysed as previously. Figure 3.10, A shows the immunoblot of detergent solubilisation of the low salt membranes and high salt membranes in SDS and DDM. SDS solubilizes the majority of ABCG2 as expected. Promisingly, DDM solubilizes a significant proportion of the protein, with the salt concentration seemingly of no great significance (compared lanes DDM MIB S and DDM HS S). This observation, which was seen reproducibly (Ian Kerr, personal communication) is remarkable as mentioned above the lab hasn't previously been able to solubilize ABCG2 with detergents from insect cells. This is discussed in section 4.2. Figure 3.10, B shows the corresponding Instant blue stained gel, showing the total amount of protein per lane.




**Keys**

**S1:** First supernatant
**P1:** First pellet
**MIB:** Membrane Isolation buffer
**S2:** Second supernatant
**P2:** Second pellet
**HS:** High salt buffer
**S:** soluble
**I:** Insoluble
**SDS:** Sodium dodecyl sulfate
**DDM:** Dodecyl-β-D-Maltoside

**Figure 3.11: Detergent solubilisation of ABCG2. (A) & (B) StrepHis$_{13}$-tagged ABCG2 can be solubilised using DDM-lipid mixture.** The protein samples were resolved in 8 % (w/v) polyacrylamide gels followed by staining with Instant Blue, and immunoblotting with BXP-21 antibody. SDS solubilises the majority of the protein, due to its ionic strength. Whereas DDM promisingly solubilises a significant proportion of ABCG2, regardless of either high salt or low salt buffer used (shown with orange arrow). SDS-Lipid mixture was used as a solubilisation control.

## 3.10 Purification of Detergent-Lipid mixture solubilised StrepHis$_{13}$-tagged ABCG2

### 3.10.1 StrepHis$_{13}$-tagged ABCG2 can be purified from Sf9 membranes using Ni-NTA purification

Detergent solubilised samples were incubated overnight at 4 °C with 600 µL of HisPur™ Cobalt Resin (Thermo scientific) or Ni-NTA™ resins and with gentle agitation. The resin was subsequently washed with 2 bed volumes (1200 µL) of solubilisation buffer supplemented with 5 mM imidazole and eluted with 200 mM imidazole. The subsequent samples were analysed by immunoblotting with BXP-21(Figure 3.11, A & C). Parallel gels were also stained with silver stain (Methods 2.4.2.3) (Figure 3.11, B & D).

Silver stain was employed as it is more sensitive and is able to stain protein at a much lower concentration. The figure A & B shows purification with nickel resin where it is observed clear band of ABCG2 purified in elution 2 and 3 both in the blot and the silver stain. The figure C & D shows purification with cobalt resin where protein come off mostly in the flow through and wash. As for the purity, the elutions consists of some other protein along with ABCG2.

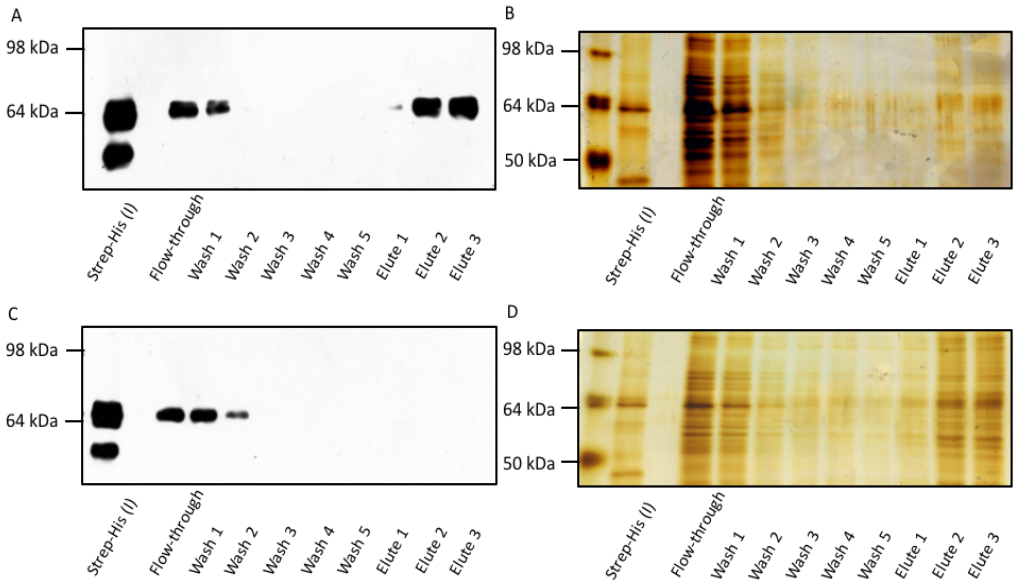



**Figure 3.12: StrepHis$_{13}$-tagged ABCG2 purified by detergent-lipid mixture from Sf9 membranes using Ni-NTA purification.** The protein samples were resolved in 8 % (w/v) polyacrylamide gels followed by staining with Silver stain, and immunoblotting with BXP-21 antibody. (A) & (B) represents purification with nickel resin while (C) & (D) represents purification with cobalt resin.

# Chapter 4: Discussion

In light of the complexities of the lipid bilayer and membrane proteins, solubilisation i.e. extraction from the membrane, purification and further crystallization of membrane proteins, are considered something of a "Holy Grail" for those who work with them. However, achieving such a goal is not always as easy and straightforward as it may seem. The principal difficulty encountered in the study of membrane proteins is obtaining the protein of interest itself. Following which, due to membrane proteins being naturally embedded in a mosaic lipid bilayer, which even in the case of simplest organism is a complex, heterogeneous and dynamic environment limits the use of many standard biophysical techniques to determine structure–function relationships such as NMR and X-ray crystallography. Finally, membrane proteins are not generally soluble in aqueous solution and thus need to reside in surroundings that satisfy their hydrophobicity so that their complex native structure can stay as close to how they are naturally found as possible.
This study aims to shed light towards some of these areas by investigating the solubilisation and purification of one such crucial multidrug resistance membrane protein ABCG2.

## 4.1 SMA does not allow for ABCG2 purification from insect cells

One of the main objectives of this study was to first use a suitable expression system where ABCG2 can be expressed at a very high level. This is where insect cells play a vital role, providing numerous advantages over mammalian cell lines: the main one being the easy manipulation for protein overexpression using well characterized baculovirus infection methods (Jarvis, et al., 2009). Also, they grow in suspension naturally whereas HEK293 need to be adapted to suspension cell culture. ABCG2 was overexpressed in both insect (Sf9, this study) and mammalian cell line (HEK-293, Aaron Horsey, PhD Student) and was perceived the stark difference in their expression levels (Results Figure 3.1 B and C).

In this study $His_6$-tagged ABCG2 (historic construct) was first expressed in Sf9 cells and was successfully solubilised using a relatively new non-detergent SMA mediated nanodisc forming technique called SMALPs (Knowles et al., 2009). It was observed that SMA solubilises proteins rather well as the ABCG2 fractionates obtained in the soluble fraction was much higher than the insoluble (Result Figure 3.2). Although, several studies before have shown to have successfully purified different His-tagged membrane proteins using Ni-NTA resin, expressed mostly in bacterial cell membranes (Introduction 1.4.2). However, purification of the $His_6$-tagged ABCG2 protein from insect cell membrane proved to be problematic when using a Ni-NTA resin. Multiples purification were performed with different batches of membranes prepared at different dates, as well as different batches of SMA. ABCG2 was shown not to bind to the resin and coming off in the flow through. This difficulty in purifying $His_6$-tagged proteins is not unheard of amongst the members of SMA embracing community (personal communication, SMALP conference, January 2016).

To explore this conundrum, anti-His antibodies were employed to check whether the $His_6$-tag was available in the solubilized protein. While the anti-His antibody was able to pick up the $His_6$ present in the flow through and insoluble fractions, while nothing was observed in the soluble (ABCG2-rich) and elutions (Result Figure 3.3). On the contrary when ABCG2 ($His_6$-GFP-tagged) was expressed in mammalian cells (HEK-293), and solubilised with SMA it purified well with both Nickel and Cobalt resins (personal communication, Aaron Horsey, PhD Student). This contrasting result lead us to come with two possible explanations: one the $His_6$-tag could be occluded or masked by the SMA co-polymer due to the anionic character of the polymer interacting with the zwitterionic $His_6$ residues and the tag being masked within the SMALP molecule. Secondly, there is also a potential problem with the overall ionic charge interactions present surrounding the environment of the SMALPs and the $Ni^{2+}$. Experimental attempts based upon this suggestion are discussed below. Notably, an explanation must also be sought for the fact that ABCG2 expressed in mammalian cell-line can be purified while in the insect cell line it cannot.

Looking at the first rationalization, i.e. the $His_6$ tag being obstructed by SMA, there is a possible potential for the SMALPs to envelope the transmembrane region surrounding ABCG2, as when it solubilises membranes it takes along portion of the lipid bilayer with it to stabilize the membrane protein (Dörr, et al., 2016, Postis et al., 2015) by conserving the bilayer organization of the incorporated lipid molecules (Jamshad et al., 2015b; Orwick et al., 2012). But then if that was the case it would do the same for both the Sf9-ABCG2 and Mammalian-ABCG2.

This displays that there may be something crucial about the sequence constructs between the Sf9-ABCG2 and Mammalian-ABCG2 that causes them to behave differently. Looking closely at the ABCG2 constructs of both the Sf9 and mammalian expression systems (Figure 4.1), it can be seen that upstream of the ABCG2 present is the $His_6$-tag in both the mammalian and Sf9 cells but in the mammalian ABCG2 construct there is an extra GFP-tag present (238 amino acid residues, 26.9 kDa). This GFP-tag possibly acts as a linker sequences, thus when SMALPs are formed the long sequence of the GFP extends the $His_6$-tag out of the nano-discs making it available to bind to the $Ni^{2+}$ resins.




**Figure 4.1: The different tags present upstream of ABCG2 in insect (Sf9) and mammalian (HEK-293) cell line.** The ABCG2 constructs (PFastBac_HTc_ABCG2-His6) only has the His6 present giving a length of 6 amino acid residues. Whereas, in the mammalian constructs (p3.1zeo_ABCG2-His6) has both the His6 sequence and the added GFP-tag sequence of 238 amino acid residues in the middle.

When it comes to ABC transporters, several previous papers have shown functional purification from either insect (Gulati et al., 2014, Dezi et al., 2010, Pozza et al., 2006), mammalian (Loo et al., 1995) or yeast (Jacobs et al., 2011) using either detergent or SMA solubilisation. However, few papers cite the use

of a $His_6$-tag, with an apparent preference for a longer deca- (Loo et al., 1995), or dodeca-tags (Pollock et al., 2014) instead. According to Dörr et al., (2016) the physicochemical properties of SMA (anionic character of the polymer), allows it to form several electrostatic interactions are possible with the His-tag. This happens due to the binding of the phenyl groups of SMA to His tags, and occupying of the free coordination sites of immobilized $Ni^{2+}$ by the carboxylates in SMA.

On a personal communication with a group facing similar concerns regarding $His_6$-tagged ABC transporters (David Carrier, University of Leeds), it seems likely that an increased number of residues and them existing in an extended conformation, improves the likelihood of successful binding of the protein, overcoming any SMA interfering actions. With this in mind, it seemed that modifications to the tag, such as increasing the length of the histidine tag, or engineering a new type of tag onto the protein and changing the purification matrix entirely would be the best way to proceed.

Thus, in this study the $His_6$-tag was engineered to change into the Strep tag and Strep-$His_{13}$ tag. Strep tag was chosen, as the tag has a high binding affinity for the streptactin resins (8 amino acids residues, $K_D$ = 1 μM; IBA, 2011). Along with this combinatorial tagging strategy was also utilized by engineering Strep-$His_{13}$ tag where this much longer tag, should potentially act as the linker not getting buried within the SMALPs conferring a greater flexibility of the tag.

However, again ABCG2 was shown not to bind to the resin and coming off in the flow through. So, one possibility for the failure of SMA-solubilised protein to be purified by IMAC in insect cells but not mammalian cells is that the GFP present in the mammalian cell expression systems keeps the His tag exposed. Time to express a GFP-ABCG2 in insect cells was not available. However, a further possibility is that the insect cell membrane is a contributor to the problem. At physiological pH, SMA copolymers carry multiple negative charges due to the partial deprotonation of maleic acid groups (Ferry et al., 1951, Garrett et al., 1951). Biological membranes (Sf9 specifically) is rich in anionic lipids, such as phosphatidyl-glycerol (PG), phosphatidic acid and phosphatidylinositol (PI) (Marheineke et al., 1998). Although, when compared to mammalian cell membranes, insect cell membranes do have some

differences such as they have a rather low quantity of cholesterol and no phosphatidylserine (PS) in their plasma membrane, whereas the PI content is comparatively much higher than mammalian. The very low content of sterols as compared to phospholipids (a ratio of 0.04; for mammalian and yeast plasma membranes, for insect it is > 0.5) (Opekarová and Tanner 2003). However, the main differences between Sf9 and mammalian membrane composition is the presence of cholesterol. Cholesterol is an amphipathic molecule, which has been said to decreases surface charge in the physiological environment (Magarkar et al., 2014).

This presence of higher anionic lipids and lower cholesterol in Sf9 membranes might lead to electrostatic repulsive during membrane solubilisation with SMA. Such electrostatic interactions would be particularly relevant when the polymer interacts with the membrane surface and inserts into the membrane. During the SMALP conference, January 2016, it was suggested that using amino acid Arginine which has a guanidine side chain, that is positively charged in neutral, acidic, and even most basic environments could help increase the overall charge density surrounding the environment of the SMALPs and resins by contributing some positive charges. Personal communication during the UK ABC meeting, June 2016, it was also suggested that increasing the amount of salt (NaCl) in the buffers can also facilitate the process.

Due to time constraints, it was not possible to explore these parameters fully enough for their inclusion in the results but some preliminary attempts were made. Thus, to check the solubilisation efficiency for effective binding of the protein to the resins, solubilisation was performed with SMA buffer supplemented with high salt (NaCl, 500 mM, 1 M) and/or arginine. This was done on the basis of increasing the charge density around the SMALPs environment so that the tag can interact with the resins. High salt up to 1 M was added and as usual procedure of solubilisation was performed (Methods 2.3.1). Figure 4.2, A shows the solubilisation of the Strep-tagged ABCG2 while B shows the solubilisation of the Strep-$His_{13}$-tagged ABCG2. In both cases, it was observed that the normal NaCl concentration (150 mM) was not very efficient in solubilising, whereas 500 mM of NaCl facilitated

solubilisation i.e. having ABCG2 in the soluble fraction than the insoluble. Having arginine (100 mM) on the other hand did not make much difference. Several optimisation with high salt buffers were performed and purification was once again done under the high salt conditions, however the protein would come out in the flow through and washes not binding to the resins. Regardless, of the tag being exposed or not (Results Figure 3.10).

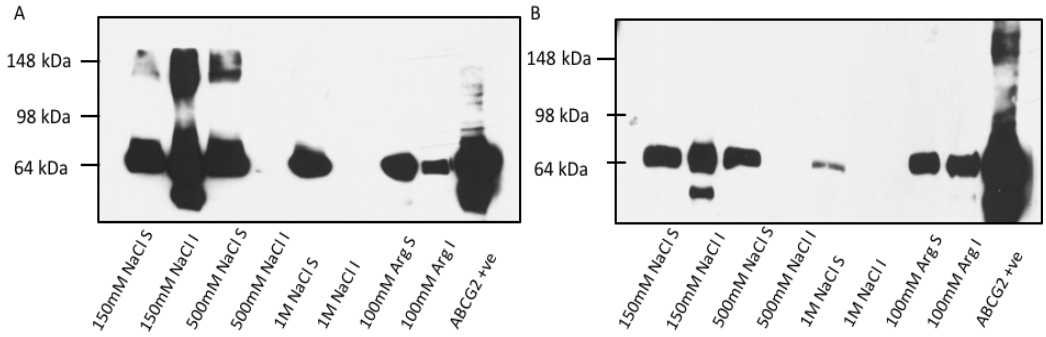



**Figure 4.2: Solubilisation efficiency of SMA with high salt and arginine (A) & (B).** The protein samples were resolved in 8 % (w/v) polyacrylamide gels followed by staining with Instant Blue, and immunoblotting with BXP-21 antibody. Positive controls were run alongside soluble and insoluble fractions. (A) represents Strep-tagged ABCG2 while (B) represents Strep-$His_{13}$-tagged ABCG2.

## 4.2 Detergents in the presence of high salt do allow for the purification of ABCG2 from insect cells

Subsequent the unsuccessful purification of ABCG2 solubilized with SMA, an alternative and classical approach of solubilisation using detergent was considered. ABCG2 and detergent solubilisation have quite a long conflicting history considering how several groups reported with variable results. On one hand Sarkadi's group (Telbisz et al., 2013) have shown to successfully solubilize and purify ABCG2 using detergents like DDM. However, the Callaghan's group, Di Pietro's group (Dezi et al., 2010) and our own group have had problems both solubilising and purifying ABCG2 using detergents indicating its difficulty (McDevitt et al., 2006, McDevitt et al., 2009). Other than the zwitterionic detergents such as Fos-choline-16 (McDevitt et al., 2006) which is cost prohibitive other detergents did not seem to work very well.

Thus, given their success, we decided to pursue the exact protocol given by Telbisz et al 2013 of detergent-lipid mixture solubilisation. DDM, a mild detergent (Seddon et al., 2014), was used for the membrane solubilisation, so

as to preserve the homodimer and allowed the purification of a functional protein. Following solubilisation trails, purification trails were done using high-salt wash buffer (Results Figure 3.12). Detergent-lipid mixture solubilisation was performed several times, at different days with different batches of membranes and ABCG2 expressed with different tags. Solubilisation was performed both in the absence and present of *E. coli* total lipid extract to see how it affects the overall performance. Although, not largely enough but the addition of lipids did help in the process of detergent solubilisation while supplementing the buffers with high salt (NaCl) seemed to be less important (Result Figure 3.11).

## 4.3 Is the membrane composition the key reason why the SMA purification did not work but detergent purification did?

Our final rationalization was the membrane composition, which is undoubtedly one of the most important criteria when it comes to membrane proteins as they are structurally embedded within the bilayer. One of the key difference between the lipid bilayer composition between mammalian and insect cells as mentioned above is the amount of cholesterol present. According to Gimpl et al., (1995) it has been said that the cholesterol levels in insect membranes are 10-20 folds lower compared to mammalian membranes. When it comes to membrane fluidity, cholesterol is a key membrane regulator. Addition of cholesterol disrupts interaction between tightly packed fatty acid chains thereby increasing fluidity and reducing integrity (Berg et al., 2012). During protein extraction process, maximal disruption of bilayer integrity is required (Duquesne et al., 2010), and it is believed that the presence of cholesterol aid this process, suggesting why solubilisation from cholesterol-poor Sf9 membranes is challenging. When it comes to ABCG2, cholesterol plays a vital role both physiologically and functionally. Cholesterol is said to be an essential activator, selectively increasing both ATP hydrolysis and substrate transport activities (Telbisz et al., 2007, Pál et al., 2007), however it has not been determined yet how this mechanism works (Gál et al., 2015).

Looking at the mechanism of action for solubilisation of membranes using both detergent (DDM) and SMA; detergents, solubilise bilayers completely, generally forming micelles instead of nanodiscs (Introduction 1.4.1) and would do this regardless of what type of lipids make up the bilayer itself. SMA on the other hand inserts into the hydrophobic core of the membrane solubilizing the bilayer and simultaneously formation of nanodiscs i.e. SMALPs (Introduction 1.4.2).

It is due to their mechanism of action, their solubilisation is greatly affected and subsequently so is their purification. It has been noted that lipid packing and bilayer thickness, i.e. tightly packed and thick membranes effects the penetration of SMA into the hydrophobic core of the lipid bilayer (Dörr et al., 2016) subsequently the solubilisation. Thus, if the membranes are thicker the free energy cost of breaking up the bilayer is larger, while if more tightly packed the hydrophobic groups of the polymer that will have to insert in between the hydrophobic chains of the lipids to form the nanodiscs, will have more difficulty. Also, since insect cells over-express ABCG2 compared to mammalian cells this might cause the bilayer to be more rigid as the lipid: protein ratio is low. Since more proteins are expressed by insect cell, and the membrane being more rigid than the mammalian, when SMA form SMALPs of about ~10 nm (Dörr et al., 2016) in diameter, the membrane proteins are tightly squeezed together, which possibly can put them in a confirmation where the tag is pushed inside. Scheidelaar et al. (2015) showed that membranes with lipids in a fluid phase are more efficiently solubilized, thus once again proving the importance of membrane fluidity when it comes to solubilisation and purification using SMA.

# Final remark and Future work

At this point data regarding ABCG2 structure and function is still very limited. There are several things about this transporter that we do not know about such as, exactly how does ATP hydrolysis takes place? What are the drug binding sites? How does site-directed mutagenesis affect its structure and function? And most of all where does research of ABCG2 purification lead us to?

The challenge in working with membrane protein like ABCG2 is obtaining the purified protein itself, as previous research has shown that, other than the expensive detergents like fos-choline 14 and 16 (McDevitt et al., 2006) guaranteed purification of ABCG2 has been a topic of contradiction. The detergent free alternative SMA, is still new in the community of membrane protein biologist thus the amount of data associated with studies on the SMA extracted protein (and the relative ease of use of the technique) encourage the continuation of attempts to use it. Even though work is still ongoing to understand more about the properties of this polymer and how is behaves differently with different membrane proteins, clearly optimisation is still required to achieve the best results. Detergent-lipid mixture solubilisation on the other hand opens a new avenue, knowing how notoriously ABCG2 behaves with detergents (Telbisz et al., 2013, Dezi et al., 2010, McDevitt et al., 2006), the results obtained in this research can prove to be quite promising.

Several research on ABCG2 has been performed, ranging from pharmacological studies (Clark et al., 2006), understanding ATP hydrolysis (McDevitt et al., 2008), EM studies (Gulati et al., 2014, McDevitt et al., 2006), mutational studies (Haider et al., 2015), and oligomerization studies (Wong et al., 2015). However, the most important piece of the puzzle which is a reliable crystal structure data or a homology model data is still not available. As unlike ABCB1. ABCG2 has very low sequence similarities in comparison to the X-ray crystal structures present like Sav1866. Recently, the crystal structural data of another ABCG family member, ABCG5/G8 has been published (Yeuan Lee et al., 2016). This opened a new door to understanding a bit more about the structural information of the G family transporters.

One other avenue to be understood is exploring how ABCG2 oligomerization takes place. Wong et al., (2015) has established the fact that ABCG2 is most likely to adopt a tetrameric organization in the plasma membrane, but several questions are still unanswered, such as why and how does the tetramers form? If dimeric ABCG2 is the functional unit than what is the importance does tetrameric ABCG2 play? McDevitt et al., (2008) on $ABCG2^{R482G}$ isoform looked into drug binding and ATP hydrolysis. However, due to lack of information on the ABCG2 structure, understanding the basis of oligomerization studies and its link to ATP hydrolysis is still uncertain. on the other hand, site-directed mutagenesis can also be performed in the TM5-TM6 region which has been proposed to have motifs for homo-dimerization (Mo et al., 2012), to see how this might affect the overall process. In other words, purification of the protein (both wide type and mutants) can let us analyze the regions part by part required for the protein-protein interactions between the formation of oligomers rather than see the picture as a whole.

When it comes to pharmacology studies of ABCG2, up until now one study by Clark et al., (2006) highlighted on substrate binding affinity in ABCG2 in a completion binding experiments, as previously discussed. Several mutational studies have also been performed, among which one work by Haider et al., (2015) showed the disruption in glycosylation and reduced trafficking due to a mutation of I573A site. Currently, in the Kerr lab research is ongoing on several ABCG2 mutants. Thus, the next step will be gain information regarding expression and localization to investigates these mutants and achieve drug accumulation and efflux assay. Having purified ABCG2 mutants (expressed by insect cells) is essential to address biophysical alteration with respect to substrate binding assays in different mutants which can be investigated through microscale thermophoresis (MST).

When it comes to ATP hydrolysis mechanism, the ATP switch model has been based upon the work of Higgins and Linton (2004), to describe ABC exporters functional mechanism. However, even though there are several crystal structures of ABC exporters, the crystal structure of Sav1866 (Dawson and Locker; 2006, 2007) has been seen as the most accurate and relatable with the transport model proposed. However, ABCG2 is unlike other ABC transporters,

in a sense that it is a reverse half-transporter and its sequence homology is very low. Thus, purification of the protein will allow to start studying the ATP hydrolysis, not just by mutation studies but also by substrate transport functional assays to trace the transport cycle step by step to obtain a more precise transport model specific for ABCG2.

For all the required process, above, stabilization and reconstitution methods is essential. As the extraction of these proteins is difficult and tricky, most of the studies done so far are performed by overexpressing the protein of interest in a heterologous host and preparing vesicles from the host membranes to characterize the protein. However, use of these membrane vesicles could sometimes provide misleading results, as the activity or transport seen for a protein could be a result of some other intrinsic proteins of the host organism. In such cases, reconstitution of the protein into artificial membrane systems provides reliable information about the protein. Different methods are available for efficient reconstitution and solubilization of membrane proteins by using appropriate detergents (Seddon, Curnow et al. 2004, Zehnpfennig, Urbatsch et al. 2009). Stabilization and reconstitution can also be utilized and looked into by freeze drying ABCG2 with disaccharides, which has been previously done on purified P-gp (Heikal et al., 2009).

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