Investigating the regulation of multidrug resistance by Cofilin in *Saccharomyces cerevisiae*.

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other university or institute of learning.

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Abbreviations

µg	Microgram
µl	Microliter
ABC	ATP-Binding Cassette
Amp	Ampicillin
Asp	Aspartic Acid
CGY	Campbell Gourlay Yeast
CWI	Cell Wall Integrity
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemilluminescence
ER	Endoplasmic Reticulum
Et al	Et alia
EtBr	Ethidium Bromide
g	Gram
GFP	Green Fluorescent Protein
H_2O	Water
His	Histidine
IT	Itraconazole
kDa	KiloDalton
LiAc	Lithium Acetate
Lys	Lysine
NBD	Nucleotide Binding Domain

M	Molarity (moles per litre)
ml	Millilitre
mM	(Millimolar $^{-3}$ moles per litre)
MDR	Multidrug Resistance
O.D$_{600}$	Optical Density at 600 wavelength
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCG	Plasmid Campbell Gourlay
PCR	Polymerase Chain Reaction
PDR	Pleiotropic Drug Resistance
PE	Phosphatidylethanolamine
PEG	Polyethylene Glycol
PS	Phosphatidylserine
PVDF	Polyvinylidene Fluoride
Rpm	Revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Synthetic Defined
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide
TEMED	Tetramethylethylenediamine
TMD	Transmembrane Domain
Ura	Uracil
V	Volt
VDAC	Voltage-Dependant Anion Channel

v/v	**Volume per volume**
w/v	**Weight per volume**
YPD	**Yeast Extract Peptone Dextrose**

Abstract

The actin cytoskeleton is a dynamic structure which is capable of rapid assembly and disassembly, and has roles in the structural stability and migration of the cell. Cofilin 1 (Cof1) is an intracellular protein which depolymerises F-actin whilst inhibiting the polymerisation of G-actin, and as such dissembling the actin cytoskeleton, in a pH dependant way. Cofilin 1 has also been linked to the function and regulation of mitochondria in *Saccharomyces cerevisiae.* Mutations in the *COF1* gene have been connected to drug resistance to numerous antifungals in *Saccharomyces cerevisiae.* One such mutant is the *cof1-6* strain whose mutations are present outside of the charge - dependant PtdIns(4,5)P$_2$ binding site of Cofilin 1, and has been linked to the up-regulation of several ABC transporters that have roles in drug resistance. We investigate the role of genes involved in lipid biosynthesis and the role of the mitochondrial outer membrane protein Porin 1 in *cof1-6* linked drug resistance. We show that the *Cof1-6* mediated drug resistance is PKC-regulated MAPK pathway independent. We further observe an increase in the expression of the sphingoid long chain base efflux pump Rsb1 in the *cof1-6* strain. This up-regulation of Rsb1 is pH-independent but Porin dependent. Previous literature has demonstrated a link between peroxisomal and mitochondrial function. Our work suggests that peroxisome function does not regulate drug resistance whereas mitochondrial function is a key mediator in the resistance phenotype of the *cof1-6* strain. Furthermore, we demonstrate that *cof1-6* linked drug resistance is very complex with the ABC transporter Pdr5 involved in resistance to antifungals which operate through different modes of action. We hope that this work will further understanding into drug resistance against common antifungal drugs.

Introduction

1. Drug Resistance

Drug resistance is a phenomenon that occurs when microorganisms or cancer cells have evolved in such a way that medications they used to be sensitive to are no longer effective. This presents a problem when pathogenic organisms gain resistance to drugs that are used to treat them, even more so when those resistant pathogens spread amongst a population. Drug resistance can develop due to spontaneous mutations but can also occur due to the wrong treatments being prescribed or prescribed courses of treatments not being completed. An estimated one million deaths per year occur due to fungal infections, mostly in immune-compromised individuals.

1.1 The ABC Transporter Superfamily

ATP-binding cassette transporters, or ABC transporters as they are more commonly known, are members of one of the largest superfamilies of proteins. These proteins are conserved in a range of organisms from single-celled to multi-celled organisms and are broken up into 7 different subfamilies. These subgroups are referred to as, ABCA, ABCB, ABCD, ABCE, ABCF, and ABCG (Prasad, Khandelwal, and Banerjee 2016). The classes of ABC transporters are dependent on the number of α-helices that span the cell membrane, usually varying between 6 and 10 (Wilkens, 2015). ABC transporters commonly comprise multiple domains , at least one of which is a transmembrane subunit, and another is an ATP binding subunit, giving the name to the family (Jungwirth & Kuchler, 2006). Again, as their name indicates, they are involved in the transport of substrates across various cell membranes.

They can also be divided into different groups based on their function in eukaryotes, prokaryotes, or both. These groups are importers, exporters, and a third group that are not involved in transportation directly which have roles in the repair of DNA and translation (Davidson *et al*, 2008). Importers can be broken in to two further groups depending on their mechanism and structure. Importers are found in bacteria whilst eukaryotes, on the most part, only feature exporters (Davidson *et al*, 2008). Functionally, most ABC transporters pump substrate against the gradient, and as such, require ATP to work, and almost all only work in one direction (Vasiliou *et al*, 2009). As such, the transmembrane domains act like gates and change conformation (Wilkens, 2015).

1.2 Structure of ABC transporters

Structurally, ABC transporters have two nucleotide binding domains (NBDs), named NBD1 and NBD2, and two transmembrane domains (TMDs) named TMD1 and TMD2 (Wilkens, 2015). Whilst bacteria have ABC transporters with subunits made up of individual polypeptide chains, eukaryote ABC transporters usually have one polypeptide which contains all the transmembrane and nucleotide binding domains (Wilkens, 2015). The NBD region of the transporters is highly conserved as it contains the ATP binding region, a hallmark of the ABC transporters (Jungwirth & Kuchler, 2006). As such the NBD region is conserved throughout almost all ABC transporters in bacterial, fungal and mammalian cells, and is a feature which allows ABCs to be identified (Biemans-Oldehinkel *et al*, 2006). Between bacterial and eukaryotic cells, similarity between sequences can be as high as 30-50%, with the 3-fold structure and energy coupling mechanism being conserved (Sharma & Rose, 1995).
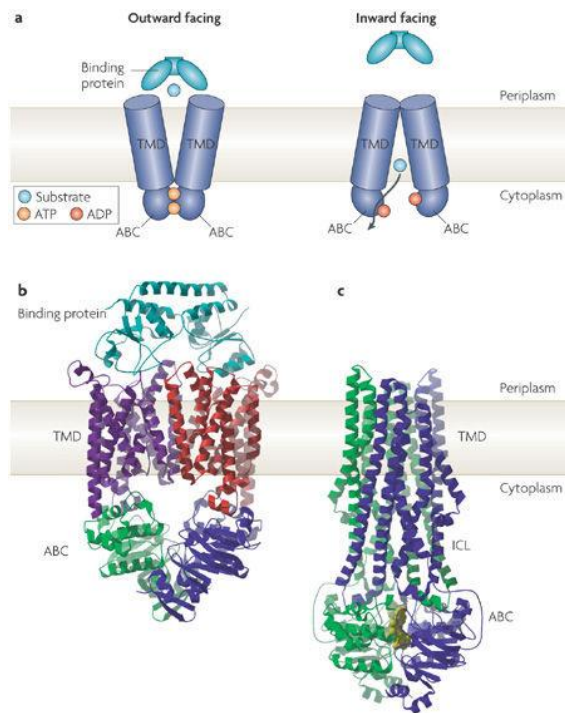
Figure 1.1 A simple schematic diagram of an ABC transporter. The top image is a simple depiction of how the ABC

transporter functions, whilst the bottom image shows the α-helices spanning the cell membrane (Rees *et al*, 2009).

1.3 ABC transporters in lipid transport

A major role of ABC transporters in cells is the movement of lipids and their precursors

(Prasad, Khandelwal, and Banerjee 2016). Lipid transport is essential and defects found in

its mechanism can cause cell dysfunction and lead to disease (Prasad, Khandelwal, and

Banerjee 2016). Within mouse systems the ABC transporter Mdr1 has the ability to move

fluorescent lipid analogues between the leaflets of the cell membrane, whereas Mdr2 is

involved in the movement of certain phospholipids into bile (Zhou *et al*, 1999; Oude Elferink

et al, 1995). Within human systems Mdr1 has a very similar role to Mdr1 found in mice.

These MDR (multidrug resistance) genes have links to multidrug resistance in tumour cells

(Wang *et al*, 2017, 2015). Vaidyanathan et al. showed that MDR1 P-glycoprotein levels

correlated with resistance to paclitaxel and olaparib in resistant ovarian cancer cells, and

when treated with a P-glycoprotein inhibitor, the resistance was reversible (Vaidyanathan *et al*, 2016).

Yeast cells utilise a similiar system of ABC transporters to import and export lipids. In *Saccharomyces cerevisiae*, Pdr5 is a phospholipid transporter, whilst Pdr11 imports sterols across the lipid membrane (Schulz & Prinz, 2007). These PDR (pleiotropic drug resistance) ABC transporters are linked to their MDR counterparts in mammalian cells, and are also seen to convey drug resistance to the cell (Jungwirth & Kuchler, 2006). This drug resistance phenotype is often a result of overexpression of the PDR transcription factors that in turn result in the overexpression of these efflux pumps (Mahé *et al*, 1996). In *S. cerevisiae*, the main transcription factors responsible are Pdr1 and Pdr3, which in turn can activate Pdr5p as well as other ABC transporters involved in resistance such as Snq2p, which also has roles in quorum sensing, and Yor1p, responsible for oligomycin resistance (Hlaváček *et al*, 2009; Katzmann *et al*, 1995). These zinc finger transcription factors bind to Pdr1/Pdr3 Response Elements (PDREs) on the promotor regions of target genes in order to express them (Shahi & Moye-Rowley, 2009). The Pdr1 and Pdr3 transcription factors also upregulate proteins not related to drug resistance, such as Rsb1, a sphingoid long chain base transporter in the case of *PDR1*, and proteins that are involved in DNA damage repair in the case of *PDR3* (Ikeda *et al*, 2008; Katzmann *et al*, 1994)*.

1.4 The yeast cell wall

The *Saccharomyces cerevisiae* cell wall is a structure that surrounds the cell and is around 70-100nm thick (Klis *et al*, 2002). The cell wall fulfils many essential functions within a yeast

cell, namely providing protection against physical stress, maintaining osmotic conditions within the cell, maintaining the shape of the cell and acting as a supporting structure for cellular proteins (Orlean, 2012; Kollár *et al*, 1997). In *Saccharomyces cerevisiae,* the shape of the cell wall is crucial for forming the bud, and subsequently cell division (Cabib *et al*, 2001; Lesage & Bussey, 2006). As such, the cell wall structure is highly regulated, with specific proteins and lipids which differentiate depending on the extracellular and intracellular conditions, which thickness increasing in the presence of varying pH, temperature, and oxygen levels in vivo, as well as the composition of the growth media in vitro (Aguilar-Uscanga & François, 2003).

Other factors that can affect the composition of the cell wall are the stage of growth in which the cell is in, mutations in genes responsible for wall formation and drug treatment (Lesage & Bussey, 2006; Dijkgraaf *et al*, 2002; Dallies *et al*, 1998). The cell wall of S. cerevisiae can make up almost 25% of the cells dry mass with around 1200 different genes having an involvement, of this, around 50-60% of the dry mass is due to the inner layer of the wall (Klis *et al*, 2002; de Groot *et al*, 2001). Cells in the logarithmic phase are more porous with soluble glycoproteins of up to 400kDa being able to pass through the growing cell wall. One of the properties of the cell wall is its hydrophobic ability due to the numerous phosphodiester bridges that the surface proteins contain that allow the cell wall to be hydrophobic at the pH range found inside the body.

Broadly, the components of the fungal cell wall can be split into 3 groups. The first is chitin or chitosan, the second is glucan and the third is a huge array of proteins which can be specific to individual yeasts or shared amongst the whole family (Kollár *et al*, 1997). Chitin is synthesized in the plasma membrane of the cell and is either made up of polymers of N-

Acetylgucosamine linked in a β1-4 structure (Lenardon *et al*, 2010). Glucans can be either α or β linked with β glucans adding rigidity to the fungal cell wall, as well as making up the major scaffold, whilst α glucans act as part of the cell matrix (Synytsya & Novak, 2014). The 1-3 β-glucan molecules bind through hydrogen bonds which results in a highly elasticated and flexible structure (Miyoshi *et al*, 2004). The wall is split into an inner and outer section, with the inner section being composed mostly of β 1,3-glucan and chitin, whereas the outer wall is comprised mostly of mannoproteins which are highly glycosylated, limiting the walls exposure to degrading enzymes (Klis *et al*, 2002).

The proteins present in the cell wall often contain mannose, and are responsible for adding rigidity as well as synthesis of the cell wall (Kollár *et al*, 1997). Proteins that are attached to the cell wall can be further broken down into 3 subgroups based on their function within the cell (Orlean, 2012). The first and second group contain glycosylated proteins, with the first comprising of proteins that can aid in the building of the wall such as hydrolases, and the second being proteins which bind the cell wall components together like agglutinins (Teparić & Mrša, 2013). The third and final group are proteins which pass through the membrane once and have serine and threonine rich extracellular regions as well as short intracellular domains (Lesage & Bussey, 2006; Gentzsch & Tanner, 1997). Members of the third group of associated proteins include proteins that act as sensors of cell stress such as Mtl1 which monitors oxidative stress during glucose starvation (Klis *et al*, 2006).

One of the major components of the lipid bilayer is sphingomyelin which is made up of ceramide components, whose synthesis is discussed below. Ceramide has a role in differentiation as well as proliferation and a role in programmed cell death (Thon *et al*, 2005).

1.5 Responses to cell wall Stress

MAPK signalling regulates a varying amount of processes within the cell, ranging from proliferation and apoptosis to gene expression (Levin, 2005). The MAPK pathway that is involved in the regulation of the cell wall stability and most studied is the Cell Wall Integrity (CWI) pathway, activated by PKC (Levin, 2005) . As well as regulating the cells response to cell wall stress such as cell wall damage by antifungal agents, this pathway regulates mating and response to high osmolality amongst other roles. In a linear kinase cascade, Pkc1 firstly phosphorylates Bck1 at several sites between its catalytic and regulatory domains, namely Ser939, Thr1119 and Ser1134, activating it (Levin *et al*, 1994). Bck1 goes on to phosphorylate Mkk1/2 which subsequently goes on to activate Slt2 (Irie *et al*, 1993). This cascade allows small signals on the cell surface to be amplified as long as the threshold of the cell surface receptor is reached. Both Mkk1/2 and Slt2 both reside in the cytoplasm during a stress response, whereas Pkc1 is localised at the cell membrane (Kamada *et al*, 1995). In response to a cell wall stress detected by Rho1 and Rho2 GTPases on the cell surface, this pathway is activated (Santos *et al*, 2003). When Slt2 is phosphorylated, it goes on the activate Plm1 and Swi4 which are transcription factors that upregulate genes involved in cell wall synthesis, including *GSC2* which codes for β1,3-glucan synthase, which in turn is involved in the formation of β1,6-glucan (Douglas *et al*, 1994).

1.6 The cell wall as a target of antifungal drugs

Due to its important function in the maintenance and survival of the cell, the cell wall is an attractive therapeutic target against pathogenic fungi, targeting different components of

the wall, and its synthesis. The main target for a lot of azole drugs is the cytochrome P450 lanosterol C14α-demethylase which is a component in the pathway responsible for ergosterol synthesis (Yoshida, 1988). These drugs are successful in reducing the amount of ergosterol in the lipid membrane which makes the membrane weaker and more prone to antifungal drug action. Ergosterol is produced by removing two of the methyl groups from lanosterol and thus ergosterol is a smaller molecule that its predecessor (Nes, 2011). C14α-demethylase is the enzyme responsible for this step and drugs like itraconazole and clotrimazole inhibit this enzyme preventing ergosterol formation (De Backer *et al*, 2001). A reduction of ergosterol results in the increased permeability of the cell wall, resulting in magnesium potassium diffusing out of the cell (Ellis, 2002). Azole drugs indirectly target the cell wall, and are usually thought to be fungistatic because they limit ability to make membrane and/or produce toxic sterol intermediates. An example of these toxic intermediates are 14α-methylated sterols which result in an increase in the fluidity of the membrane and increased permeability to other drugs (Sorgo *et al*, 2011; Abe *et al*, 2009). However lack of ergosterol synthesis will also affect cell wall building/assembly as it is an essential component of the cell wall (Lesage & Bussey, 2006).

Other drugs like Amphotericin B, a member of the polyene class of drugs, binds to the ergosterol directly, forming pores in the membrane which lead to the leakage of monovalent ions such as potassium, sodium and chloride ions (Mesa-Arango *et al*, 2012). Unlike the azole class of drugs however, Amphotericin B is not fungal specific and will bind to other members of the sterol class of molecules including cholesterol, found in mammalian cells (Paquet *et al*, 2002). As a result of this non specificity, Amphotericin B is toxic in humans and results in nephrotoxicity, fever and chills, as well as nausea and headaches at doses higher than 1.0mg/kilo/day (Hamill, 2013).
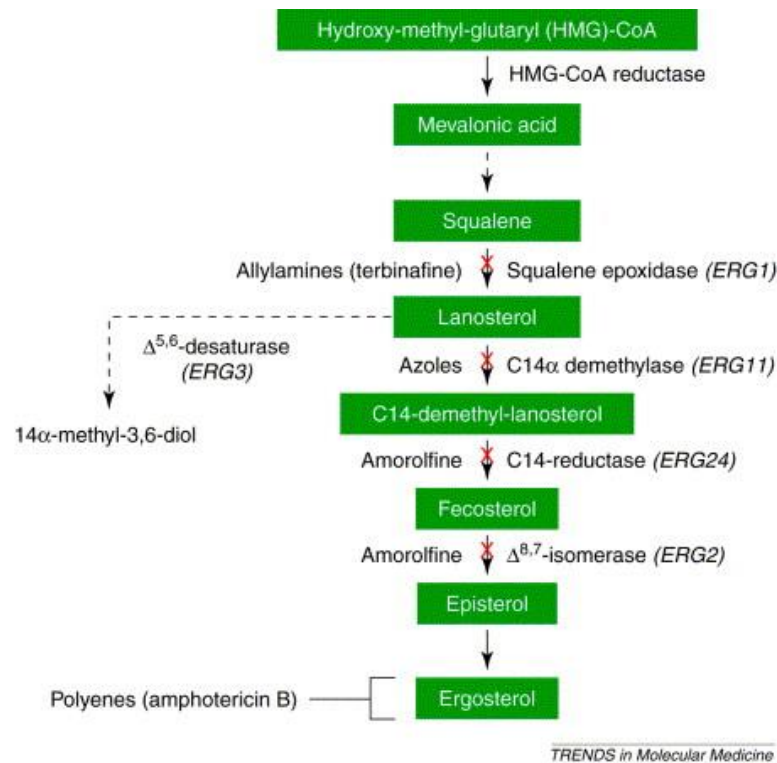
Figure 1.2 *De nova* production of ergosterol in *S. cerevisiae* and potential drug targets. This is the mechanism by which ergosterol is produced in yeast, labelled with the targets for many major classes of antifungals (Lupetti *et al*, 2002).

1.7 Role of lipids in *Saccharomyces cerevisiae*

Lipids have many important roles in the function of the cell and are integral as a source of both structure and energy within cells. They are a major component of cell membranes but also act with signalling pathways as both primary and secondary messengers (Nakamura *et al*, 1995; Ohanian *et al*, 1998). Lipids such as diacylglycerol act as secondary messengers which are associated to the cell membrane and can diffuse into the cell in response to primary messengers. Due to their hydrophilic properties, lipids easily form structures such as vesicles, liposomes and cell membranes both intracellularly and extracellularly.

Examples of lipids important to the cell membrane structure in *Saccharomyces cerevisiae* are ergosterol and sphingolipids.

1.7.1 Ergosterol Biosynthesis in *Saccharomyces cerevisiae*

Ergosterol is a member of the subgroup of steroids known as sterol alcohols or sterols. It has many similar properties and functions as cholesterol does is mammalian cells, although ergosterol requires more energy expenditure to produce (Nes, 2011; Slominski *et al*, 2005). Ergosterol in a major component in the fungal lipid membrane. It is fungal equivalent to cholesterol in humans and a target for the group of antifungal agents known as azoles (Lupetti *et al*, 2002). Ergosterol is not universally present in all fungi and in some, such as *S. cerevisiae*, some components in the pathway are not essential to viability like the other steps are. It is also not present in mammalian cells and, as such, is a good pharmacological target, as the drugs will not cause damage to host cells whilst targeting the pathogenic fungi (Ghannoum & Rice, 1999). As such the ergosterol biosynthesis pathway is targeted by a lot of antifungal drugs.

Ergosterol is the final product in a long process which included many intermediates (Dupont *et al*, 2012). A simplified process is shown in, along with associated drug targets, in **Figure 1.2** and occurs at the rough ER of the cell. From the ER, it needs to be transported to the plasma membrane. This can occur either by contact sites between the ER and other organelles or through the use of transport proteins called oxysterol-binding protein (OSBP)-related proteins (ORPs) (Michel & Kornmann, 2012; Stefan *et al*, 2011).
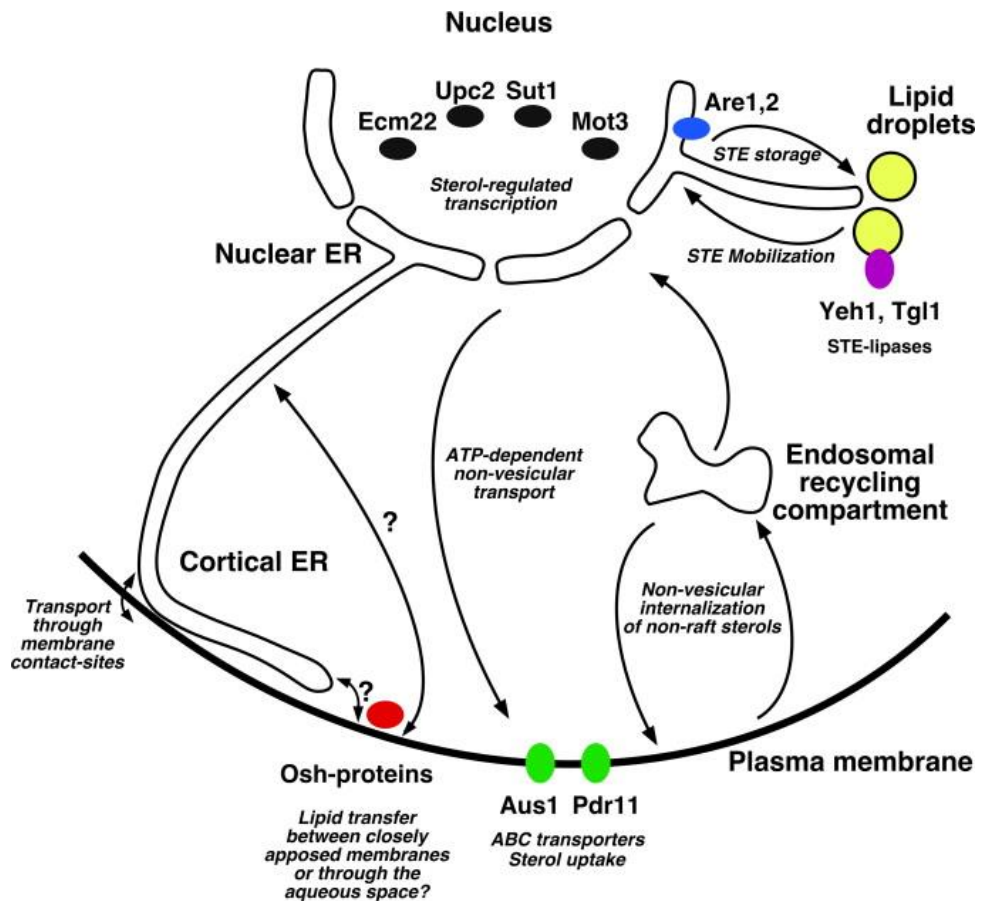
Figure 1.3 Ergosterol transport in *S. cerevisiae*. This is an overview of the key transport mechanisms that are involved in the movement of ergosterol around the cell (Jacquier & Schneiter, 2012).

1.7.2 Sphingolipid Biosynthesis in *Saccharomyces cerevisiae*

Sphingolipids are lipids which have a similar structure to that of phospholipids in that they both are made up of a polar head group and two non-polar tails (Gault *et al*, 2010). However, with the exception of sphingomyelins, these two membrane molecules are individual classes. Sphingolipids have a core made of sphingosine which is a long chain amino alcohol which contains an eighteen carbon amino alcohol backbone(Gault *et al*, 2010). The basic sphingolipid structure undergoes post translational modification in the ER,

and then to the Golgi apparatus to produce the vast family of sphingolipids which are found in cells. All sphingolipids share a common synthesis and catabolism pathway.

Sphingolipid metabolism starts with the production of the sphingoid bases which make up the backbone of the sphingolipid itself. These are produced through palmitoyl-coenzyme A and serine condensing together to form 3-ketosphinganine, through the catalytic action of serine palmitoyltransferase (Mandon *et al*, 1992). This initial reaction occurs in the endoplasmic reticulum's cytosolic side. Once 3-ketosphinganine has been produced, it is then reduced to sphinganine by the enzyme 3-ketosphinganine reductase (Pinto *et al*, 1992). The next step in the process is the acylation of the sphinganine to form dihydroceramide through a process involving six ceramide synthase enzymes in mammals, and sphinganine N-acyl transferases in yeast, which involves attaching different length fatty acid chains (Sugimoto *et al*, 2004). This dihydroceramide undergoes a process of unsaturation by dihydroceramide desaturase 1 to form ceramide which is the direct precursor to sphingosine. Sphingosine is formed from ceramide by ceramidase however its conversion is reversible by the action of the enzyme ceramide synthase (Gault *et al*, 2010). To convert sphingosine back into ceramide requires the addition of fatty acyl-coA, resulting in ceramide and CoA-SH. Ceramide can also be formed by the hydrolysis of sphingomyelin by sphingomyelinase, as opposed to the de nova pathway which is as described above.

The membranes in which certain sphingolipids are found differs based on their size and side chains. For example, dihydrosphingosine and sphingosine, normally accumulate in organelles which have an acidic pH as their free amino group is open to ionisation, whereas ceramide is found more in cell membranes as it flips within membranes rapidly and is localised often to membrane where it was synthesised. This property of ceramide is

important as an important step in its modifications requires enzymes localised in the membrane it was created in (Gault *et al*, 2010).

Movement of the newly formed ceramide to the Golgi apparatus is done through the use of vesicles or the ceramide transfer protein CERT which is localised in the cytosol and moves the ceramide from the ER to the Golgi apparatus where it can then be modified post translationally to form either sphingomyelins or glycosphingolipids (Hannun & Obeid, 2008). The CERTs are directed to the Golgi apparatus through the presence of a pleckstrin homology domain of around 120 amino acids on the N-terminus which recognises and moves towards the PI4P of the Golgi (Raya *et al*, 1999). The primary residue found on the C1 hydroxy group on the sphingolipids determines their class, and this is determined by three distinct pathways that runs through the ER and Golgi apparatus organelles (Gault *et al*, 2010).
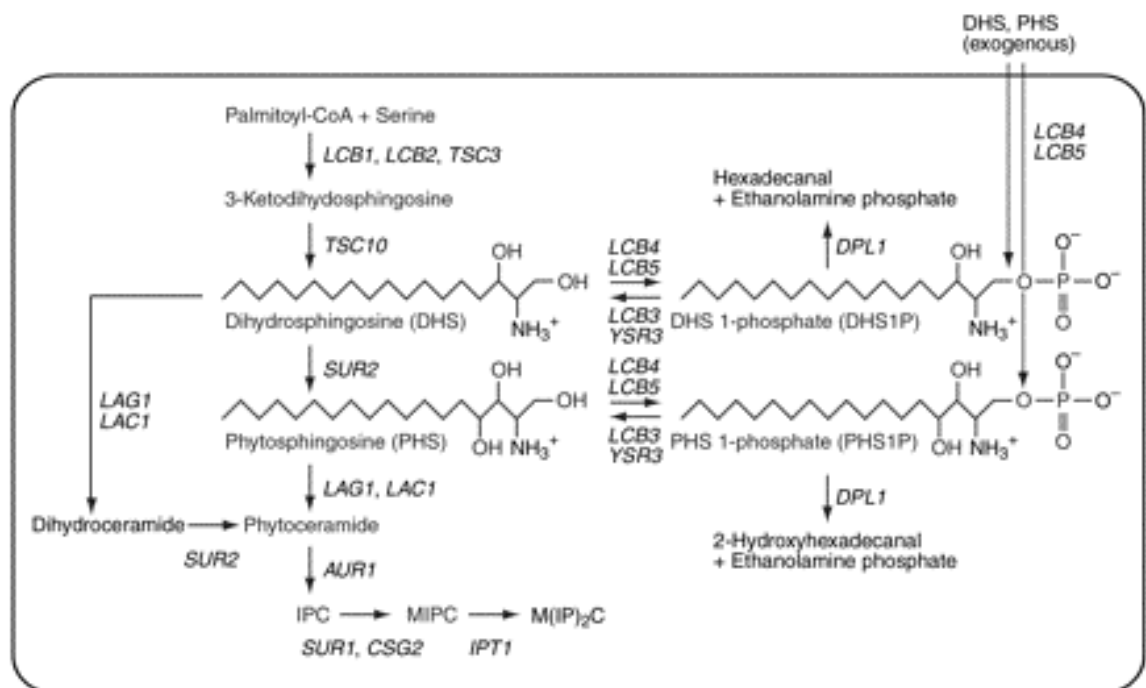
Figure 1.4 *De nova* production of sphingolipids in *S. cerevisiae*. This is the mechanism by which sphingolipids, a major component of the cell wall, are made in yeast (Kihara & Igarashi, 2002b).

Rsb1 is a transporter of the sphingoid bases precursors that make up these sphingolipids (Kihara & Igarashi, 2002a). The sphingoid bases also act as signalling molecules, for example they can signal though Pkh kinases to induce endocytosis in yeast cells (Friant *et al*, 2001).

The regulation of sphingolipids and their precursors has been linked to drug resistance in cancer cells. In particular, ceramide accumulation has been seen to be increased in various resistant cancers, leading to the overproduction of glucosylceramide (GlcCer) (Gouazé *et al*, 2005). This in turn leads to leads to an increase in GlcCer synthase (GCS) activity and expression. GCS has been seen to lead in an increase of expression of Mdr1 which can be reduced by using a GCS inhibitor in breast cancer cells (Gouazé *et al*, 2005).

1.8 Function of Mitochondria and their role in metabolism

The mitochondria are double membraned organelles that are the site of ATP production within almost all eukaryotic cells. They also have a major role in metabolism within the cell, including phospholipid synthesis, through the conversion of phosphatidylserine (PS) to phosphatidylethanolamine (PE), as well as activation of apoptosis and cell death (Kojima *et al*, 2016; Gulshan *et al*, 2008). They have also been shown to be involved in steroid synthesis and the regulation of the metabolism of the wider cell (McBride, Neuspiel, and Wasiak 2006; Rossier 2006).

There are many diseases which have been linked to mitochondrial function, of which many are endocrine diseases such as diabetes mellitus (Larsson *et al*, 2000). Mitochondria also have a separate genome to the cell, and although they still have most of their key proteins coded for by the nuclear genome, they are also able to encode a few essential respiratory

proteins required for function independently. Mutations in this mitochondria DNA can let rise to diseases of its own such as Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes (MELAS) and Leigh syndrome (Baertling *et al*, 2014; El-Hattab *et al*, 2015).

Mitochondria have been seen to play a key role in apoptosis and mitochondrial damage is seen to have an effect on ageing (Wang & Youle, 2009). Caspases are proteases that cleave many cellular proteins resulting in cell apoptosis and are key proteins in the apoptosis process (Salvesen & Dixit, 1997). Proteases such as Smac and Omi that usually reside within the mitochondria, and whose primary role is to perform functions required for cell growth, are also activators of caspase activity (Vande Walle *et al*, 2008).

Most of the functions of the mitochondria are reliant on the proton gradient generated by respiration within the cristae of the mitochondria. This proton gradient is required for ATP synthesis as well as the movement of calcium ions and the entry of proteins in the mitochondria (Duchen & Szabadkai, 2010).

The entry and exit of these calcium ions and other small molecules occurs through pores in the mitochondrial outer membrane called porins that regulate the membranes permeability (Weeber *et al*, 2002). These porins, also known as voltage-dependant anion channels (VDACs), are highly conserved structures made up of beta sheets, forming a beta barrel structure (Sampson *et al*, 1996). Porin 1, the most abundant porin in the mitochondrial membrane is formed of 19 of these beta-sheets with both the N- and C-terminus facing the mitochondrial intermembrane space (Tomasello *et al*, 2013). As well as being the most abundant, porin 1 is also the main calcium ion channel as well as being

responsible for the transport of ATP and other ions across the mitochondrial membrane (Tomasello *et al*, 2013; Chu *et al*, 2014).

Cells missing their mitochondrial DNA have been seen to upregulate Pdr5, establishing a link between mitochondria and drug resistance (Moye-Rowley, 2005). Moye-Rowley hypothesised that the upregulation of Pdr5 could be a compensation mechanism to export toxic intermediates that accumulate in the inactive mitochondria but the exact mechanism is yet to be determined (Moye-Rowley, 2005).

1.9 Drug resistance in *Saccharomyces cerevisiae*

As mentioned previously, multidrug resistance in *Saccharomyces cerevisiae* resistance is linked to overexpression of certain ABC transporters such as Pdr5, as well as Snq2 and Yor1 (Nourani *et al*, 1997). Pdr5 is a member of the ABCG subclass of transporters and in knockout studies, the PDR5 gene knockouts resulted in increased sensitivity to an array of drugs (Paumi *et al*, 2009; Gulshan & Moye-Rowley, 2007).

These proteins have roles in lipid transport but they also are transporters of many antifungal agents that are in use today. The two predominant transcription factors for the PDR genes are Pdr1 and Pdr3 which bind to PDRE sites on their target gene. The number of PDREs on a gene correlates strongly to how sensitive they are to the levels of transcription factor in the cell. In the case of PDR5 which is the most abundant PDR gene within *S. cerevisiae*, it has three PDRE sites and so is highly sensitive (Katzmann *et al*, 1996). As well as *PDR1* and *PDR3*, the PSD1 gene which encodes phosphatidylserine decarboxylase has been linked to the upregulation of the protein Pdr5 (Clancey *et al*, 1993).

As well as Pdr5 which is specific for itraconazole, amphotericin B and cycloheximide, there is other ABC transporters which are specific for other drug substrates (Gulshan & Moye-Rowley, 2007). Pdr15 is a Pdr5 homologue and is specific to similar classes of drugs, whereas Yor1 of the ABCC family, bestows oligomycin resistance on a cell (Katzmann *et al*, 1995).

1.10 Drug resistance in *Candida albicans*

Drug resistance in pathogenic yeast is an ever-growing problem. *Candida albicans* is a pathogenic yeast which also displays acquired drug resistance, whilst being more resistant to metabolic inhibitors than the yeast *S. cerevisiae*. *Candida* have their own genes that convey resistance such as CDR1, *Candida* Drug Resistance 1, which is functionally equivalent to PDR5 found in *S. cerevisiae* as discovered by Prasad et al. by transforming CDR1 in to a PDR5 knockout. Addition of this CDR1 gene results in drug resistance to cycloheximide amongst others (Prasad *et al*, 1995). Whilst CDR1 is functionally equivalent and encodes for a similar ABC transporter, the drugs that it gains resistance to is altered (Prasad *et al*, 1995).

Work underdone by Nourani et al. showed there to be a link between PDR5 and HXT11 drug resistance due to fact they are both regulated by the transcription factors Pdr1p and Pdr3p. Deletions of the HXT11 and HXT9 genes results in drug resistance, whereas Hxt11p overproduction results in increased sensitivity to the drugs cycloheximide and 4-Nitroquinoline 1-oxide (Nourani *et al*, 1997).

1.11 The role of cofilin in the regulation of actin

Cofilin-1 is a 20 kDa protein involved in the depolymerisation of actin filaments in the cytoskeleton. It binds to the F-actin when it is bound to ADP, destabilising actin filaments and promoting their disassembly. This helps to create a pool of free actin monomers that can be used to create new actin filaments. As such, cofilin is not only seen to have a role in the depolymerisation of actin, but a role in the polymerisation as well. The role of cofilin in either assembly or disassembly of actin filaments is dependent on its concentration relative to the actin subunits, with the bias towards disassembly at low concentrations and assembly at high concentrations (Bamburg & Bernstein, 2010). Cofilin can be activated by actin interacting protein-1, Aip1, and also the cyclase associated protein-1, CAP1. The negative regulation of cofilin occurs through phosphorylation, with the protein being inactivated of phosphorylated at the terminal serine-3 residue.

Cofilin has been shown interact with VDAC (Porin) in mammalian cells. When HL-60 promyelocytic leukaemia cells were treated with the protein kinase inhibitor staurosporine to induce apoptosis, cofilin was seen to translocate to the mitochondria (Chua *et al*, 2003). It is hypothesised that cofilin has a role in the regulation of apoptosis through interaction VDAC (Leadsham *et al*, 2010). Cofilin has also been shown to play a role in drug resistance in cancer cells, with the phosphorylation of cofilin-1 being linked to HDAC inhibitor resistance in hepatocellular carcinoma cells (Liao et al. 2016). Phosphorylation of cofilin by ERK1/2 prevented interaction with the pro-apoptotic signal Bcl-2-associated X protein (BAX), preventing its translocation to the mitochondria (Liao et al. 2016).

Saccharomyces cerevisiae is an ideal model organism for studying drug resistance due to their fast doubling time and being easy to genetically manipulate. The genome of *S.*

cerevisae has been completely sequenced and is available on the *Saccharomyces* genome database (SGD) (www.yeastgenome.org/), along with information about individual genes and their interactions. In addition, *S. cerevisae* has a large number of genes that are conserved in humans, two of which are the voltage-dependant anion channels, Porin 1 and Porin 2. Porin 1 is a homologue of the mammalian protein VDAC1 and is non-essential in *S. cerevisae*, allowing it to be knocked out without being lethal to the cell.

1.12 Aims of this study

One minor component of the lipid membrane is the secondary signalling molecule phosphatidylinositol 4, 5-bisphosphate ($PI_{45}P_2$) which is a substrate for several proteins involved in signalling including the protein cofilin-1. Previous work by Kent Fungal Group, under the supervision of Campbell Gourlay, have shown changes in the charges on the surface of Cofilin-1 to have a role in drug resistance, through interaction with the mitochondria.

A number *cof-1* mutants display increased levels of respiration and peroxisomal fatty acid β-oxidation, as well as the upregulation of numerous ABC and lipid transport transporters, included those known to have a role in drug resistance, such as PDR5, PDR10 and PDR15 (**Figure 1.5**). One such mutation is the *cof1-6* mutation, in which residues at Asp18 and Lys20 have been changed to lysine (**Figure 1.6**) (Kotiadis *et al*).
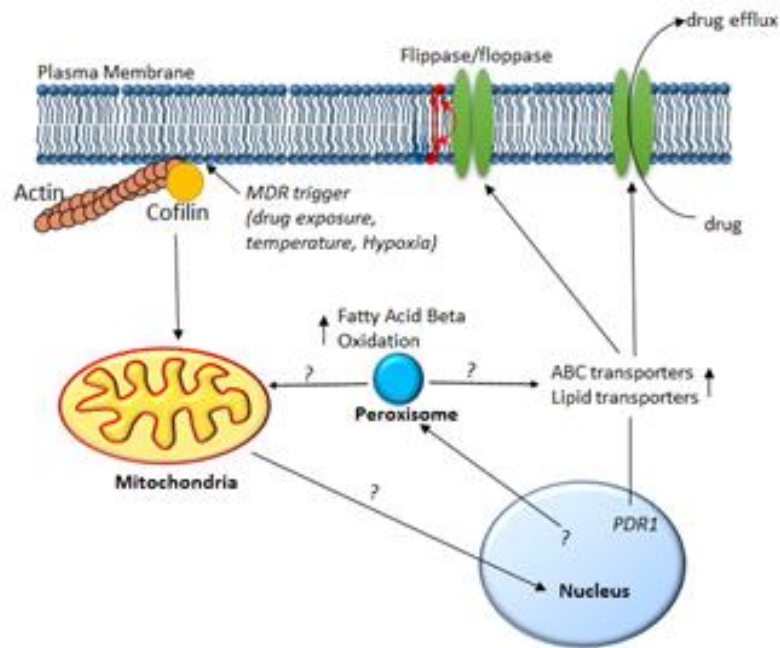
Figure 1.5 Pathways upregulated in the *cof1-6* cofilin mutant. The *cof1-6* mutant displayed elevated levels of respiration, β-fatty acid oxidation, as well as the upregulation of numerous ABC and lipid transport transporters, included many pleiotropic drug transporters seen to have a role in drug resistance

In this study, we aim to determine the involvement of porin, the mitochondrial membrane pore, in the drug resistance seen in the *S. cerevisiae* mutant *cof1-6*. We also look to discover which, if any, of Pdr5, an ABC transporter, Rsb1 a lipid transporter, or Pox1, involved in Beta-oxidation within the peroxisome, are involved in drug resistance within the *cof1-6* mutant. We hoped that these investigations would expand our understanding of the control of drug resistance in yeast cells, with broader implications in other eukaryotic systems.
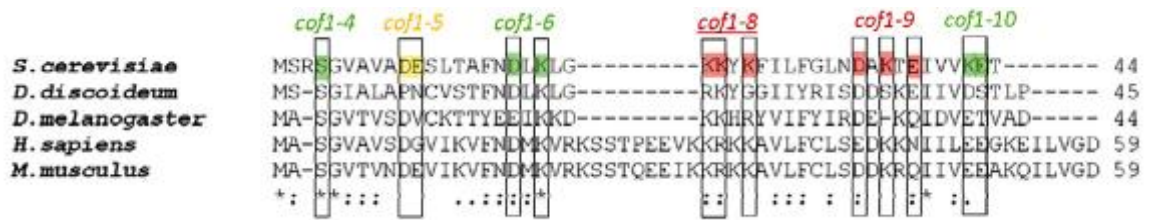
Figure 1.6 Mutations in cofilin in *S. cerevisiae* compared to other organisms. Here we look at the *cof1-6* mutant in

S. cerevisiae which has been linked to drug resistance.

Materials and Methods

2.1 Growth Media

All media and agar was sterilised for 15 minutes at 121°C in an autoclave. All water used in the making of media, agar and buffers was sterile MilliQ water.

Liquid cultures of yeast strains were grown in either YPD (1% Yeast extract (Difco), 2% Bactopeptone (Difco) and 2% Dextrose (Fisher Scientific)) or SD media (0.675% Yeast Nitrogen base without amino acids (Formedium), yeast synthetic dropout supplement without specific amino acid (Formedium) added as per the manufacturers' guidelines, and 2% Dextrose (Fisher Scientific)) overnight on a 30°C rotary shaker at 180 rpm unless specified otherwise. *E. coli* were grown in YT media (0.5% Yeast Extract (Difco), 1% Tryptone (Difco), 1% Sodium Chloride (Fisher)) and shaken at 180 rpm overnight at 37°C. Solutions made up by percentage were done so by weight/volume (w/v) unless specified otherwise.

Strains and transformants were also grown on culture plates, made up as the media with the addition of agar (Oxoid) at 4mg/ml. Agar used for spotting assays was autoclaved and cooled down to 50°C, divided into 40ml aliquots and the drug concentrations required were added before pouring.

2.2 Yeast strains and growth conditions

The strains used are listed in **Table 2.1**. Primers used to generate knockout strains and verify correct ablation of the target gene are listed in **Table 2.2**. Plasmids used in this study are listed in **Table 2.3.**

Strain	Genotype	Reference
CGY1260	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, COF1::LEU2*	Kotiadis et al, JCS 2012
CGY1261	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, cof1-6::LEU2, Δpor1*	A kind gift from Prof. Manuela Corte Real (University of Minho, Braga, Portugal)
CGY1264	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, cof1-6::LEU2,*	Kotiadis et al, JCS 2012
CGY1265	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, cof1-6::LEU2, Δpor1*	A kind gift from Prof. Manuela Corte Real (University of Minho, Braga, Portugal)
1252	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, COF1::LEU2, Δrsb1::URA3*	This study
CGY1254	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, COF1::LEU2, Δpox1::URA3*	This study
CGY1255	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, cof1-6::LEU2, Δpox1::URA3*	This study
CGY1257	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, COF1::LEU2, Δpdr5::URA3*	This study
CGY1258	MATα *ura3-52, his3D200, leu2-3,112, lys2-801, cof1-6::LEU2, Δpdr5::URA3*	This study

Table 2.3 Strains used in this study.

Gene	Direction	Primer
RSB1	Forward	TTAGGCAGTCTTTTGCCACAGTTAGAAGCAGCAGCTAACAGTAATT CCCTTTATGGTGGTCAGCTGAAGCTTCGTACGC
	Reverse	AAAGTAAGAATTGAAAAATGCATATGAACGATCAGATTGACATTC AAATTCTACGTATCAGCATAGGCCACTAGTGGATCTG
	Control (Forward)	GATCGGTTGTACCCACGG
PDR5	Forward	AAGAAATTAAAGACCCTTTTAAGTTTTCGTATCCGCTCGTTCGAAA GACTTTAGACAAAACAGCTGAAGCTTCGTACGC
	Reverse	ATGTTTATTAAAAAAGTCCATCTTGGTAAGTTTCTTTTCTTAACCAA ATTCAAAATTCTAGCATAGGCCACTAGTGGATCTG
	Control (Forward)	GTGATTCCGTGGAAAGGTC
POX1	Forward	ACACTTGACACTAATAAGTATCACAGAAAAAAGAAAATATAATA AATTAGTATTGCGATCAGCTGAAGCTTCGTACGC
	Reverse	AGTTGAATTGAAACAAAAGTCGCAAAACAGAGGGTTCGAAGGAA AACAGGAAACCTCTACGCATAGGCCACTAGTGGATCTG
	Control (Forward)	CCCTGACACATTTTAAGCC

Table 1.1 Primers used in this study.

Plasmid	Description	Selective Marker	Supplier
PIKD493	*RSB1-LacZ* plasmid. *LacZ* gene cloned into the XhoI-SmaI site. *RSB1* promoter cloned into XhoI site.	*HIS3*	(Ikeda et al.)
PCG633	Made by Gateway expression system. POR1 was inserted into pAG416 which contains a CEN origin of replication with a GPD promoter.	*URA3*	CWG
pCS276	pRS426-pPRC1-GFP-2xPHOsh2	*URA3*	(Stefan *et al*, 2011)
pRS426GFP-PH(FAPP1)	pRS426GFP-2×PH(PLCδ)	*URA3*	(Stefan *et al*, 2002)

Table 2.3 Plasmids used in this study.

2.3 Antibodies used in this study

In order to detect proteins of interest by western blot, the following primary and secondary antibodies were used.

Primary antibody used for Pdr5 detection: Rabbit anti- Pdr5, a gift from Karl Kuchler, Department of Molecular Genetics, University and Biocenter of Vienna, and was used at a dilution of 1/2000.

Primary antibody used for Slt2 detection: Rabbit Anti-Phosphpo-44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signalling, catalogue number #4370) and was used at a dilution of 1/2000.

Primary antibody used for Porin detection: Mouse anti-VDAC1/Porin (Abcam, catalogue number # 16G9E6BC4) and was used at a dilution of 1/2000.

Primary antibody used for actin detection: Sheep anti-actin**, a** kind gift from Prof. John Cooper (Washington University) and was used at a dilution of 1/2000.

Secondary antibody used for Pdr5 and Slt2 detection: Goat Anti-Rabbit IgG HRP (Sigma, catalogue number #A0545) and was used at a dilution of 1/5000.

Secondary antibody used for Porin detection: Goat Anti-Mouse IgG HRP (Sigma, catalogue number #A4416) and was used at a dilution 1/5000.

Secondary antibody used for actin detection: Donkey Anti-sheep IgG HRP (Sigma, catalogue number A3415) and was used at a dilution of 1/5000.

2.4 Growth Analysis

Strains were grown overnight in 5ml of YPD, shaking in a rotary shaker at 180rpm at 30°C. The absorbance at OD$_{600}$ of the overnight culture was measured in the morning using an Eppendorf Biophometer plus. The overnight cultures were inoculated to a starting OD$_{600}$ of 0.1 in a sterile 24 well plate (Greiner) in technical triplicates. The plate was measured for 24 hours using a BMG LabTech SPECTROstar nano plate reader using the following settings:

Cycle Time	1800 seconds
Flashes Per Well	3
Excitation	600 nm
Shaking Frequency	400 rpm
Shaking Mode	Double Orbital
Additional Shaking Time	30 Seconds before Each cycle
Temperature	30°C
Positioning Delay	0.5 Seconds

Table 2.4 The protocol used to measure growth rate in the SPECTROstar nano plate reader.

2.5 Plasmid purification from *E. coli*

E. coli strains containing plasmids used in this study were grown overnight at 37°C on a rotary shaker, shaking at 180 rpm in YT with ampicillin at 1mg/ml. The QIAprep Spin Miniprep Kit was used to extract and concentrate the desired plasmids as per the manufacturer's instructions.

2. 6 Molecular Biology Methods

2.6.1 Polymerase Chain Reaction

PCR was done in order to amplify linear DNA that was used to knockouts genes in the wild-type and *cof1-6* background. The PCR mix contained Oligonucleotides, nucleotides, a DNA template and Taq polymerase and the reaction was done in a C1000 Thermocycler (Biorad). The Thermocycler used different temperatures for different time periods to initiate the processes of denaturation, elongation and annealing required to make single stranded DNA

fragments. The polymerase worked from the 5' to 3' end of the DNA template, in between where the two primers bound, using the nucleotides to produce the fragments. The fragments act as DNA templates to make more fragments that are copies of the DNA fragment in between the two nucleotides that have bound to the DNA template first used.

A standard 50μl PCR mix used was as follows:

35μl Sterile MilliQ H20

1μl Forward Primer

1μl Reverse Primer

5μl DNA template (20ng/ml)

1μl 100mM dNTPs (INVITROGEN)

5μl 10x Buffer (INVITROGEN)

1.5μl 100mM MgCl2 (INVITROGEN)

0.5μl Taq Polymerase (INVITROGEN)

The program for the C1000 Thermocycler was as follows:

95°C for 5 minutes: Denaturation of template DNA

95°C for 40 seconds: Short Denaturation

56°C for 40 seconds: Annealing ⎤ Repeat for 30 cycles

72°C for 1 minute 40 seconds: Elongation ⎦
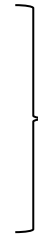
72°C for 15 minutes: Final Elongation

The program for the C1000 Thermocycler for the control PCR was as follows:

95°C for 5 minutes: Denaturation of template DNA

95°C for 40 seconds: Short Denaturation

55°C for 40 seconds: Annealing ⎤ Repeat for 30 cycles

72°C for 40 seconds: Elongation ⎦

72°C for 15 minutes: Final Elongation

2.6.2 Yeast Transformation

Yeast transformations were used to introduce new DNA into parental yeast cells, either to knockout genes or introduce plasmids. A 5ml overnight culture of yeast cell was grown in either YPD or SD media overnight at 30°C shaking in a rotary shaker at 180rpm. 1ml of overnight culture was then taken and added to a 1.5ml sterile Eppendorf tube. Cells were harvested for 1 minute at 10000rpm in a table top centrifuge before being washed in 1ml

of TE (10mM Tris-Cl pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA)). The cells were then washed in 1ml of 0.1M Lithium Acetate (LiOAc) in 1x TE. The cells were next resuspended in 100μl 0.1M LiOAc in 1 xTE. Next, 0.1mg of a 10mg/ml stock of single stranded DNA from heron sperm, that had been boiled at 90°C for 10 minutes and frozen, was added. The solution was mixed gently and either 2μl of plasmid (200ng), or 15μl (1.5mg) of single stranded DNA made by PCR, was added followed by 700μl of 40% Polyethylene Glycol (PEG) in 1x TE. The solution was mixed by rotation at room temperature for 60 minutes before undergoing a heat shock at 42°C for 15 minutes. The cells were then harvested for 1 minute at 10000 rpm using a table top centrifuge and re-suspended in 100μl of sterile MilliQ H_2O. The sample was then plated on the appropriate selective media plate and left for 3 days at 30°C until individual colonies formed. These colonies were then re-streaked onto selective media plates and incubated at 30°C for 2-3 days.

2.6.3 Agarose gel electrophoresis

In order to make a 1% agarose gel, 0.5g of agarose (Melford) was dissolved in 50ml of TAE buffer (10M EDTA, 0.04M Tris-acetic acid (v/v)). The agarose was dissolved in TAE by heating, making sure all the agarose was fully dissolved. Ethidium bromide was added at 0.05μl/ml and the gel was poured into casts (Biorad) and left to cool for 30 minutes. The gel was then added to an electrophoresis tank (Biorad) and covered in 1x TAE. The samples (2μl sample, 2μl 6x Loading Dye. 8μl sterile H_2O) were added to the gel, and 6μl of 1Kb+ DNA ladder (ThermoFisher) was added to the first gel well. The ladder was used to determine the correct molecular weight of the sample DNA bands. The gel was then run

for 90V for 30 minutes until the ladder had reached at 75% down the gel. The gel was then visualised in a FLA-5100 gel imaging system (Fujifilm) to see and capture the location of the DNA bands.

2.7 Protein Analytical Methods

2.7.1 Whole Cell Protein extraction

Cells were grown up overnight in YPD or SD media to mid log phase in a $30^{\circ}C$ rotary shaker before protein samples were prepared by harvesting 1 x 10^8 cells and re-suspended in 200µl of lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, 2% β-mercaptoethanol). These samples were then heated for 10 min at $90^{\circ}C$ before 5µl of 4M acetic acid was added and the samples were vortexed for 30 s. They were then incubated at $90^{\circ}C$ for a further 10 min before 50ml of loading buffer (0.25 M Tris-HCl pH 6.8, 50% Glycerol, 0.05% Bromophenolblue) was added. These samples were then either frozen at $-20^{\circ}C$ or used immediately. To use the samples for western blotting, the lysate was cleared through centrifugation and the supernatant loaded into the SDS PAGE gel.

2.7.2 Preparation of polyacrylamide gels.

All the samples were run on a 10% SDS PAGE gel composed of both a stacking (5% Acrylamide, 240nM 0.5 Tris Buffer, 0.07% TEMED, 0.23% APS) segment on top of a resolving segment (10% Acrylamide, 126mM 1.5 Tris Buffer, 0.07% TEMED, 0.12.5% APS). The SDS-PAGE gels were made up in a glass cassette (Biorad) 0.75mm thick. The first layer to be poured was the resolving layer, 6.5cm long from the bottom of the cassette. 1ml of

isopropanol was added to ensure the resolving layer was free from bubbles and level after the gel had been cast. After being left for 20 minutes to set, the stacking segment was added up to the top of the cassette and a comb with the correct number of wells was placed into the top of the cassette. The gel was then left to set for a further 20 minutes.

2.7.3 Protein gel electrophoresis

The samples were taken and 12μl of each was loaded into the acrylamide gel and run for 90V for around 30 minutes attached to a Biorad power supply, until the samples had passed through the stacking gel. The samples were then run at 120V for a further 2 hours until they had neared the bottom of the resolving gel.
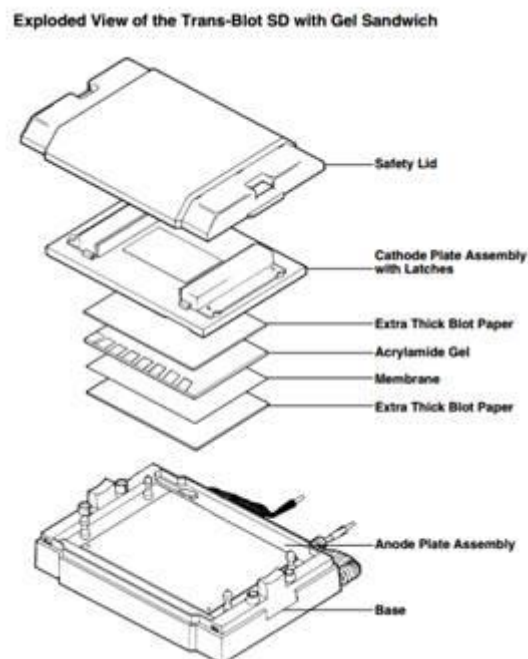

Figure 2.1 Breakdown of the Trans-Blot SD with Gel Sandwich taken from the Trans-Blot SD user manual.

2.7.4 Semi-dry protein transfer

The resulting separated proteins were transferred onto a PVDF membrane through semi dry transfer using a Biorad Trans SD machine. Two pieces of blotting paper of equal size (8 x 9cm) were wetted in transfer buffer (50ml 10x transfer buffer, 100ml methanol, 350ml distilled H_2O) and the PVDF membrane (8x9cm) was wetted in methanol before being soaked in transfer buffer for 10 minutes. The transfer was layered in a 'sandwich' arrangement with a piece of blotting paper placed first followed by the PVDF membrane, the SDS PAGE gel, and finally the second piece of blotting paper, ensuring all air bubbles are rolled out (**Figure 2.1**). The transfer was run at 25V for 30 minutes per gel.

2.7.5 Western Blot detection Using Enhanced Chemiluminescence (ECL)

After semi-dry transfer, the PVDF membrane was blocked in blocking buffer (5% semi skimmed milk powder, 1x Phosphate Buffered Saline +0.2% Tween 20 (PBS/T)) for 45 minutes at room temperature on a rotary shaker. The membrane was then briefly rinsed in PBS/T and then placed in a 50ml Sarstedt tube with 5ml blocking solution and with primary antibody at a 1:2000 dilution. The tube was left to incubate overnight in a 4°C cold room on a roller mixer. Next the membrane was removed and briefly washed in PBS/T twice before being washed for 15 min followed by two 5 min washes in PBS/T. The membrane was next placed in a 50ml Sarstedt tube with 5ml blocking buffer with a horseradish conjugated secondary antibody at a 1:5000 dilution. The tube was left to incubate at room temperature for 30 min on a roller mixer.

After the incubation period was up the membrane was briefly washed twice in PBS/T twice before being washed for 15 min followed by three 5 min washes in PBS/T. The membrane was transferred to PBS. Two ECL solutions were made up as follows:

Solution I (2.5 mM luminol, 0.396 mM coumaric acid, 0.1 M Tris.HCL pH 8.5).

Solution II (6.266 mM hydrogen peroxide, 0.1 M Tris.HCL pH 8.5).

The solutions were mixed at a 1:1 ratio and the membrane was drained of PBS. The mixed solutions were then poured on the membrane and left for exactly one minute before the excess was removed and the membrane was transferred to Saran wrap. The membrane in Saran wrap was transferred to a Syngene G:Box for imaging.

2.8 Beta Galactosidase Assay

1×10^7 cells, equating to an optical density of 0.5, were harvested at 10000rpm for 1 minute. The cells were then washed in 500µl Z Buffer (60mM Na_2PO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol (Sigma Aldrich)). The pellet was then re-suspended in 600µl of Z Buffer and incubated at 28°C for 30 minutes. Next, 150µl of 4mg/ml ONPG solution (Sigma Aldrich) was added and mixed by hand before being incubated at 30°C until the solution developed a yellow colour. The reaction was stopped by the addition of 400µl of 1.5M Na_2CO_3 and centrifuged for 30 seconds at 13000rpm to pellet cells. Finally, 1ml of supernatant was then taken and the optical density measured at 420nm, 550nm and 600nm. In order to normalise the results when biological repeats were done, the measurements at 420nm were divided by the time taken for the colour to develop in each assay. The resulting values were then averaged and plotted on bar charts.

2.9 Spotting Assay

Cells were grown overnight in 5ml of YPD or SD media to mid log phase at 30°C in a rotary shaker before the optical density was recorded. Plates containing the required drug were prepared as follows. YPD agar was autoclaved and cooled down to 50°C, divided into 40ml aliquots and the drug concentrations required were added before pouring. The plates were then allowed to set before being dried to remove all moisture from the agar surface.

Using a metal spotting plate which had been autoclaved at 121°C for 15 minutes and dried in a drying oven overnight, we inoculated the cells to a starting OD_{600} of 0.1 in 550µl of sterile MilliQ H_2O in the first well of the spotting plate. Next, 50µl of liquid from the first well was diluted in 500µl of sterile MilliQ H_2O in the second well. This process was repeated in the third well, with 50µl of liquid being discarded from the third well to make a total volume of 500µl in each well. Serial dilutions, as described above, were done for each of the samples on the spotting plate.

A metal spotting plate stamp was sterilised in 100% ethanol, flamed and let to briefly cool before being placed into the spotting plate. The stamp was then placed on each agar plate, sterilised and left to cool in between each plate. The plates were then left to dry before being incubated at 30°C for 2-3 days. Plates were then scanned using a CanoScan 4400F scanner to gather the images.

2.10 Microscopy

2.10.1 Sample Preparation

GFP-labelled yeast cells were grown in 5 ml SD media overnight at 30°C before being inoculated in the morning for YPD media at 30°C, both on a rotary shaker at 180 rpm. The cells were then left for 24 hours to view the strains at stationary phase. To visualise the cells, a thin layer 1% agarose in water was poured on a sample slide before being dived into 4 even slices. Next, 2µl of sample was placed on each slice and a covered using a 0.4mm cover slip. A drop of immersion oil was added to the surface of the cover slip ready to be visualised.

2.10.2 FM4-64 Staining

Cells were grown to exponential and stationary phase overnight in SD medium at 30°C in 5ml of culture. The strains were then spun down and re-suspended in 500µl of YPD and FM4-64 stain added to a final concentration of 8µM. The strains were then incubated at 30°C in the dark for 30 minutes before being spun down again and washed in 500µl of YPD. They were then spun down again and re-suspended in 1ml of YPD, before being transferred into a culture tube and incubated at 30°C in the dark in a rotary shaker for 30 min. Next, the cells were spun down and re-suspended in 30µl PBS, ready to be visualised.

2.10.3 Visualisation PI3P and PI4P Sensors

To visualise the images an Olympus IX81 microscope was used coupled a Cool Led pE-4000 LED illumination system. The GFP filter used produced excitation at 488(+/-10 nm) and emission at 512

(+/- 10 nm). The RFP filter used produced excitation at 555 (+/- 10 nm) and emission at 584 (+/- 10 nm). The camera used was an Andor Zyla 5.5CMOS camera linked to Micromanager control software. The images were visualised at 100x objective with 20ms exposure in the case of GFP signal, and 50ms in the case of the RFP signal.

Results

3. Drug resistance linked to the Cofilin mutant, *cof1-6*.

Previous research has found links between the function of mitochondria and the drug resistant phenotype shown by non-lethal cofilin-1 mutants. A correlation was found between increased respiration and resistance to a range of drugs. Although the exact mechanism has yet to be determined, research indicated a mediating factor was the charge of the cofilin surface. The multi drug resistance was associated with an upregulation in drug pumps and plasma membrane lipid transporters. Research has also shown that expression of the cofilin-1 mutant *cof1-6* allele, results in the upregulation of genes involved in fatty acid β-oxidation within the peroxisome. As a result, we hypothesise that cofilin links signalling from the plasma membrane to a novel stress response pathway that controls the expression of drug resistance. One possibility is that response to a change in charge in the lipid membrane due to damage by antifungal drugs activates the cofilin sensing mechanism and a drug resistance response. We sought to investigate the role of mitochondria, fatty acid β-oxidation and membrane transporters in cofilin medicated drug resistance. Initially we deleted the mitochondrial outer membrane protein porin, encoded by POR1 as pervious work has suggested a functional link between cofilin and porin.

3.1 Itraconazole resistance in the *cof1-6* mutant

Cells expressing *cof1-6* had much better growth in the presence of Itraconazole when compared to the wild-type (**Figure 3.1**). Knocking out the *POR1* gene in the *cof1-6* background was sufficient to re-sensitize cells to itraconazole treatment. This indicates a role for porin in the control of cofilin mediated drug resistance.
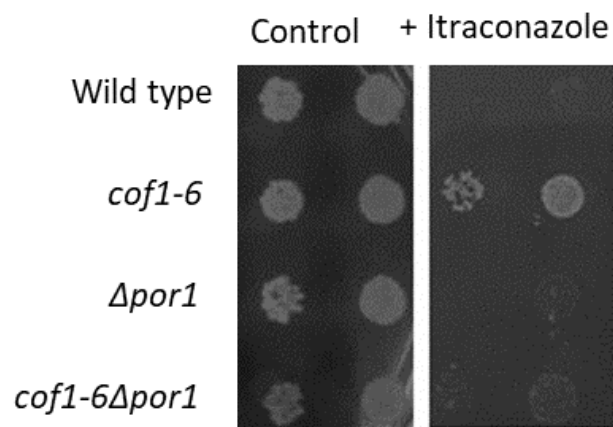

Figure 3.1 *cof1-6* resistance to Itraconazole is dependent upon *POR1*. Strains were grown overnight to mid-log phase in YPD before being plated on either YPD plates (**Left**) or YPD with 4.4mM Itraconazole (**Right**). The strains were plated at optical density of 0.1 and diluted 10-fold in a serial dilution and left to grow for 48 hours.

The *cof1-6* mutant has also been seen to display elevated levels of respiration, fatty acid beta-oxidation, as well as increased ABC and lipid transporters. We set about studying three genes that corresponded to each of these phenotypes. The ABC transporter Pdr5 is the most prevalent drug transporter in the cell and has been seen to be linked to drug resistance. Pox1 is an enzyme required to complete fatty acid beta oxidation within the

peroxisome, converting acyl-CoA into 2,3-dehydroacyl-CoA, and Rsb1 is a sphingoid long chain base transporter.

3.2 Generation of *RSB1, POR1, POX1* and *PDR5* knockouts in the *cof1-6* background

Our published result suggests that respiratory function within the mitochondria and beta oxidation, as well as various ABC and lipid transporters, are increased within the *cof1-6* mutant. To determine if one or more of these phenotypes is involved in drug resistance, we knockout out one gene from each. These genes were the ABC transporter *PDR5*, *POX1* which is involved in beta-oxidation, and *RSB1* a sphingoid long chain base transporter.
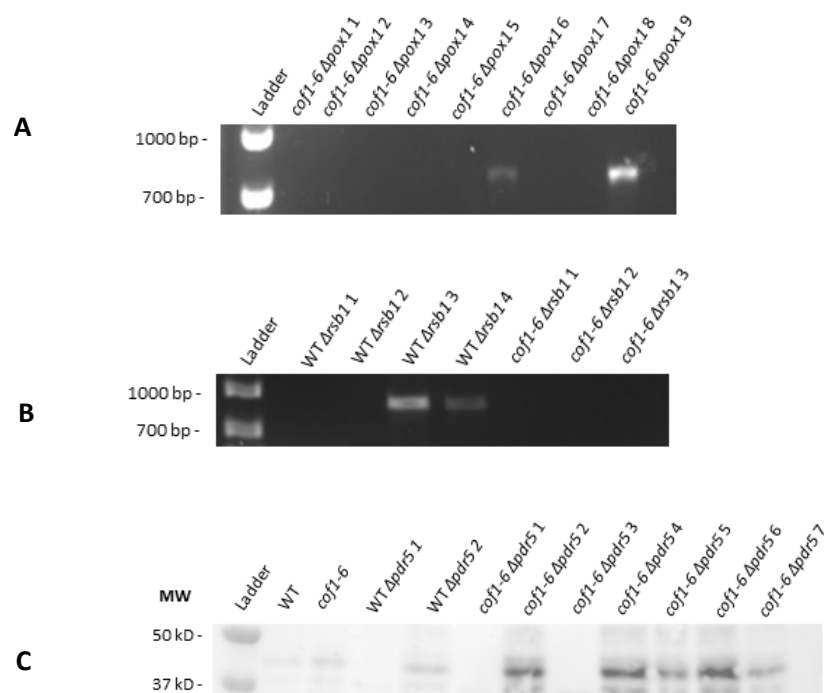
Figure 3.2 Confirmation of knockout clones by PCR or Western Blot. The *Δrsb1* and *Δpox1* strains were confirmed by PCR using control primers and then visualised using gel electrophoresis as seen in image **A** and **B.** The *Δpdr5* strains were confirmed by western blot using a Pdr5 specific rabbit antibody. The labelling indicates the number of the clone being tested. The expected sizes were ~800bp for the *rsb1* and *pox1* knockouts.

We next set about knocking out these genes of interest. To do this we amplified a cassette containing *URA3* flanked by targeting sequences to promote gene deletion by homologous recombination. Linear DNA designed to delete each target gene was amplified by PCR, verified by gel electrophoresis and transformed into both the wild-type and *cof1-6* mutant strains. Colonies arising on selective plates were isolated and tested for correct gene deletion by PCR using a primer located within the selective marker gene and one downstream of the target gene. In the case of the *PDR5* knockout, a western blot using an anti-Pdr5 rabbit polyclonal antibody was carried out to confirm deletion. Out of all of the clones tested we managed to get positive clones for the deletion of *POX1* and *PDR5* in both the wild-type and *cof1-6*. For *RSB1* we only succeeded in acquiring knockouts in the *cof1-6* background. These confirmed knockouts were taken forward for further testing. *POR1* had already been deleted in wild type and *cof1-6* cells and were a kind gift from the lab of Prof Manuela Corte Real (University of Minho, Braga, Portugal).

3.3 Elevated Rsb1 expression in *Cof1-6* dependant on Por1

The Rsb1 lipid transporter protein is crucial in the movement of sphingoid long chain bases from the cytoplasmic to the extracytoplasmic side of the lipid cell membrane. Previous microarray data from our group suggested that Rsb1 expression is increased upon expression of the *cof1-6* allele (unpublished observations). To confirm that Rsb1 is upregulated wild type and *cof1-6* expressing strains, were transformed with a plasmid containing the *RSB1* promoter coupled to a *LacZ* gene. *LacZ* codes for beta galactosidase which cleaves lactose into glucose and galactose. As part of the assay, ONPG is used, which when beta galactosidase is present, is cleaved into galactose and ortho-nitrophenol. Ortho-

nitrophenol has a yellow colour which can be measured at 420nm using a spectrophotometer. As a positive control, an *NRG1* knockout strain was used as it Nrg1 is a repressor of Rsb1 expression. The results were normalised by the time taken for the colour to develop as explained in the methods section
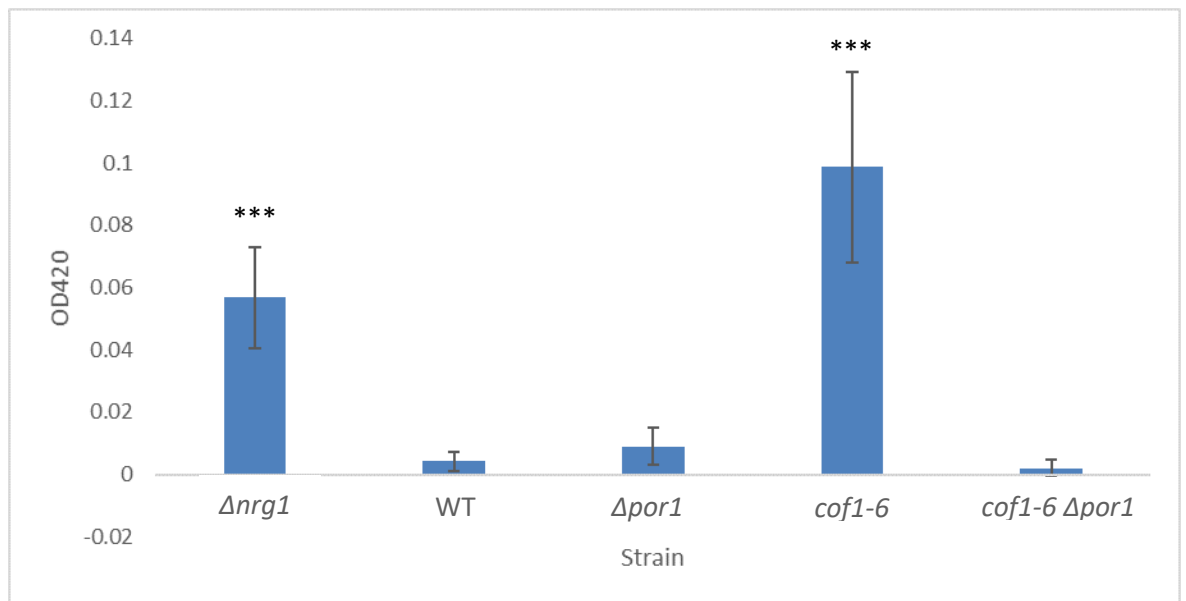

Figure 3.3 Beta-Galactosidase assay to determine Rsb1 expression. The wild-type, Δpor1, *cof1-6* and *cof1-6 Δpor1* strains were transformed with a plasmid containing the Rsb1 promoter linked to the LacZ gene. Deletion of *NRG1* is known to lead to an increase in Rsb1 promoter activity and so was used as a positive control. Results were normalised by assay run time and analysed using a T-test comparing each strain to the wild-type *** corresponds to a P value < 0.0001 when compared to the wild-type.

The results of the beta-galactosidase assay showed a clear increase in *RSB1* promoter activity in *cof1-6* mutant cells in comparison to the wild type (P value = <0.0001). This in in line with our unpublished the microarray data and may indicate the involvement of Rsb1 and the transport of sphingoid long bases in cofilin mediated drug resistance. The *Δpor1* strain showed no significant difference in expression levels in comparison to the wild type

with a P value = 0.1149. There was also no significant difference in the expression levels between the wild type and *cof1-6 Δpor1* mutant (P value = 0.2288) showing that the Rsb1 elevation seen in the *cof1-6* mutant is dependent on the presence of porin.

Rsb1 levels have been shown to be responsive to changes in the charge of the plasma membrane. We hypothesised that commonly used antifungals may alter plasma membrane charge and activate Rsb1 expression through activating the *RIM101* pathway. To investigate whether drug exposure is sufficient to activate Rsb1 expression we exposed cells transformed with the RBS2-lacZ reporter to different concentrations of the drugs Amphotericin B and Itraconazole (**Figure 3.4**).
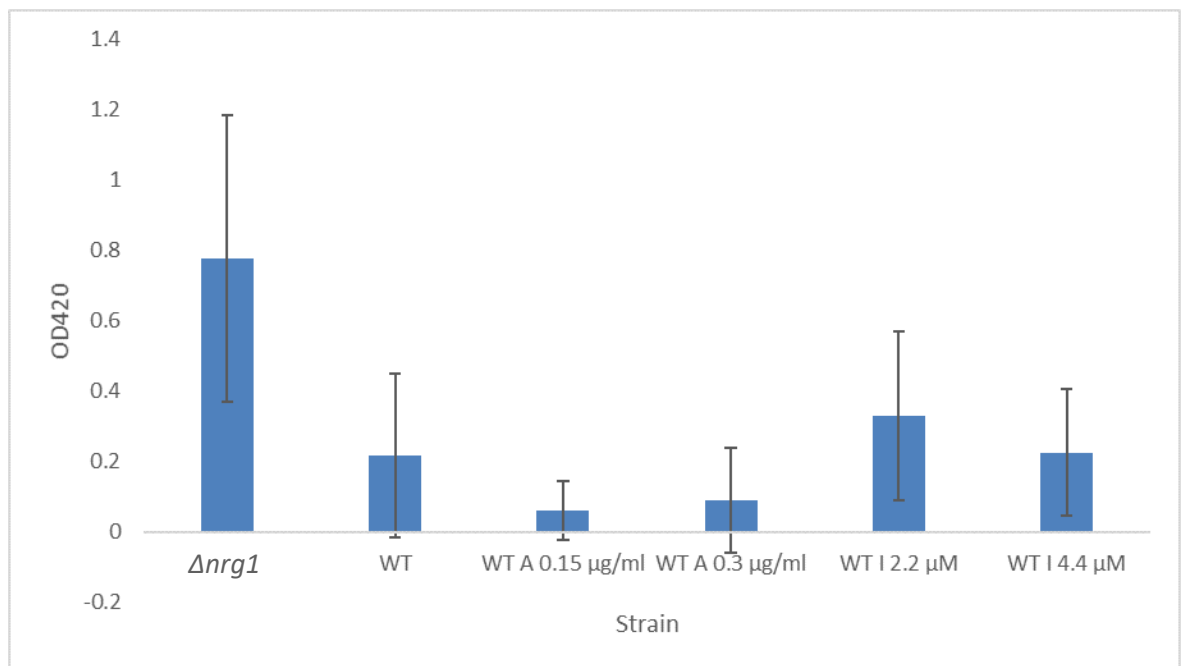
Figure 3.4 Beta-Galactosidase Assay showing the effect of Amphoterecin B and Itraconazole on Rsb1 promoter activity. Overnight cultures of the strains were inoculated for four hours in either amphotericin B at 0.15μg/ml or 0.3 μg/ml, or itraconazole at either 2.2μM or 4.4 μM. A beta galactosidase assay was then done as described in the materials and methods section. There was no significant difference in expression between the wild-type control and the treated strains, showing Rsb1 expression is not induced through antifungal exposure. There was also no significant difference between the *NRG1* positive control and the wild-type with a P value = 0.1069.

The results showed no significant difference in Rsb1 expression between the wild-type control and the treated wild-type strains treated with amphotericin B at 0.15 μg/ml (P value = 0.3366) or 0.3 μg/ml (P value = 0.471). There was also no significant change in Rsb1 expression between the wild-type control and wild-type treated with itraconazole at 2.2μM (P value = 0.5938) and 4.4μM (P value = 0.9603). This indicated that elevated Rsb1 expression in the *cof1-6* is not a result of antifungal induced membrane damage.
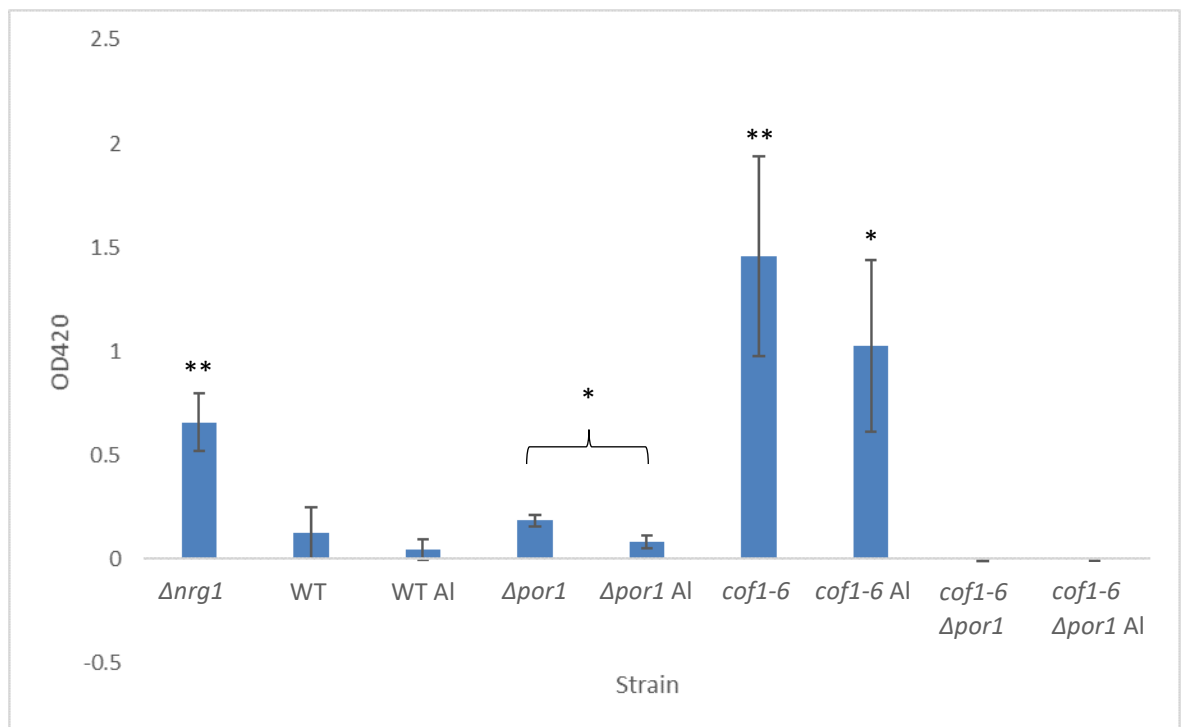
Figure 3.5 Beta-Galactosidase Assay showing the effect of alkaline pH on Rsb1 expression. Strains were either inoculated for 4 hours from an overnight culture in YPD or YPD with 100mM Tris Buffer at pH8. These strains were then used for a beta-galactosidase assay as described in the materials and methods section. * corresponds to a P value = < 0.05, ** corresponds to a P value = < 0.01. *Δnrg1*, *cof1-6,* and *cof1-6* Al were significant compared to the wild-type, whilst *Δpor1* was significant compared to *Δpor1* Al.

Alkaline exposure can be detected by the Rim21 sensor on the cell membrane, activating the *RIM101* pathway and inducing genes involved in cell wall synthesis such as Rsb1. Rim101 indirectly activates Rsb1 expression by repressing Nrg1 which in turn represses Rsb1 (Ikeda et al. 2008). The determine if Rsb1 promoter activity expression would differ in the wild-type and *cof1-6* mutant in response to an alkaline pH exposure, a beta galactosidase assay was conducted after growing the cells into early log phase in YPD with 100mM Tris buffer at a pH of 8. The results showed the same trend found in the untreated strains, with the *cof1-6* mutant showing elevated levels of Rsb1 expression, significantly more than the wild-type. However, there was no significant difference between the expression levels in the wild-type, porin deletion and *cof1-6* porin deletion strains. If Rsb1 promoter activity was induced by alkaline pH exposure, it would have been expected that the wild type would have shown raised levels of Rsb1 when exposed to alkaline conditions but this was not the case (P value = 0.3709). When compared to the untreated strains, the wild-type, *cof1-6* and cod1-6 *Δpor1* strains grown in alkaline conditions no significant difference in Rsb1 promoter activity under the conditions used (P value = > . The *Δpor1* deletion grown in alkaline conditions showed significantly reduced Rsb1 activity although this does not appear to affect drug resistance (P value = 0.0122). This may be explained by alkaline conditions reducing growth.
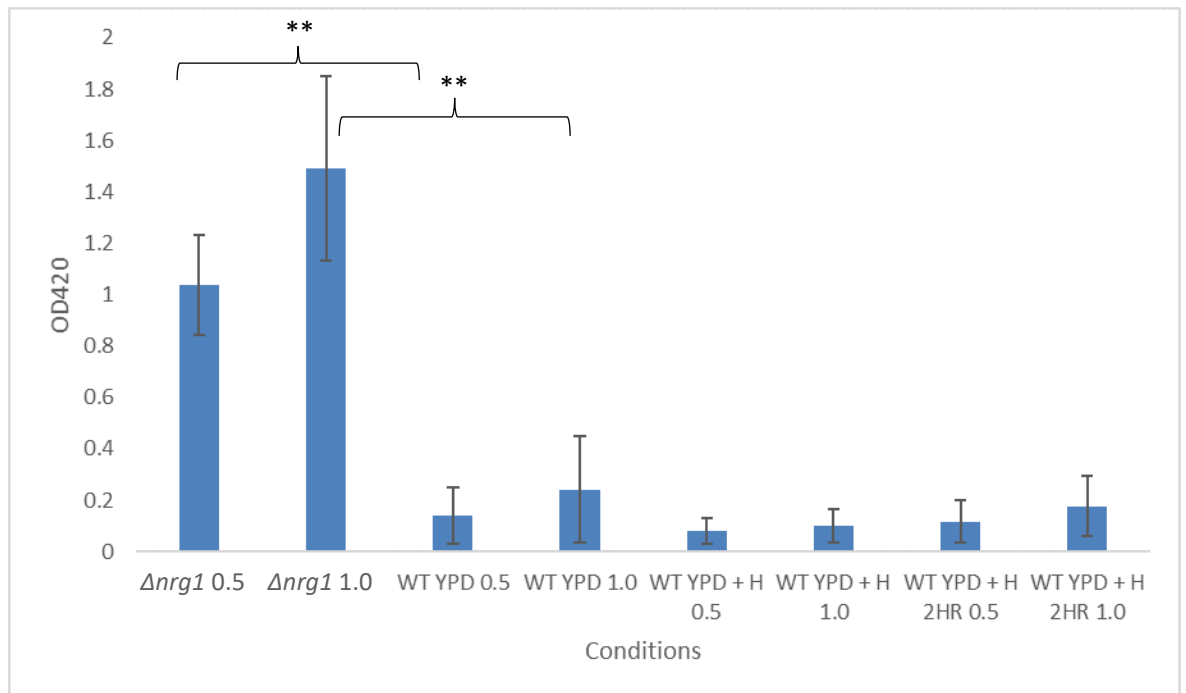
Figure 3.6 Beta-Galactosidase assay showing the effect of different starting cell densities and alkaline pH on Rsb1 expression. For the assay, the wild-type was either used at a starting OD_{600} of 0.5 or 1.0. In addition, HEPES buffer at pH 8 was also added at the beginning of the 4 hour incubation, after 2 hours, or not at all. A beta galactosidase assay was then done as described in the materials and methods section. ** corresponds to a P value = < 0.01.

To see if exposure time to the HEPES buffer affected was having an effect on the expression, the HEPES buffer at pH 8 was added either at the beginning of the 4hour inoculation period, or after 2 hours. The resulting data in **Figure 3.6** showed that increasing the cell density had significant effect on the amount of Rsb1 expressed in all cases, including the positive control (all P values = > 0.05). Adding the HEPES buffer at different time points had no significant effect on Rsb1 expression when compared to the untreated wild-type in YPD alone (all P values = > 0.05).
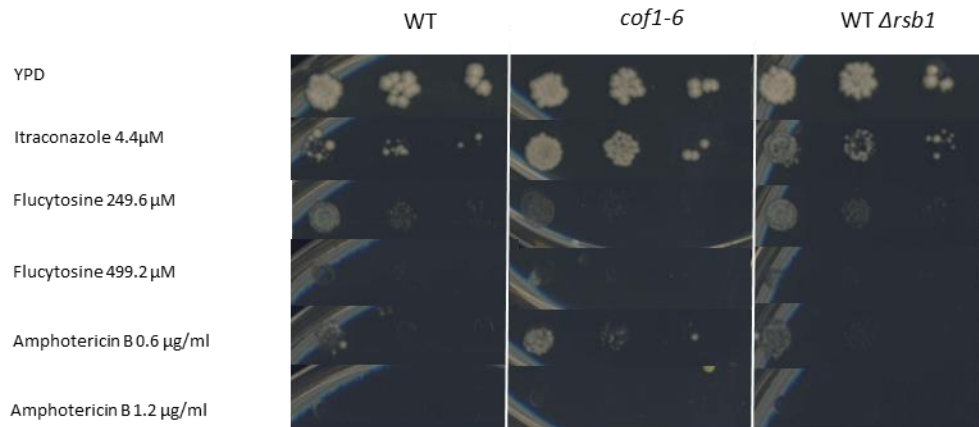
Figure 3.7 Spotting assay showing no change in resistance between resistant wild-type strain and its corresponding *RSB1* deletion. Overnight cultures of the wild-type, *cof1-6 and* wild-type *Δrsb1* strains were diluted to a starting OD_{600} of 0.1, and serially diluted 1 in 10. They were then grown for two days at 30°C on YPD plates in the presence of itraconazole, flucytosine and amphotericin at the different concentrations shown.

The *RSB1* knockout in the wild type background also showed no difference to the non-deleted wild-type strain, both showing inhibited growth in the presence of itraconazole at 4.4µM, flucytosine at 249.6µM and amphotericin B at 0.6µg/ml (**Figure 3.7**). Deleting *RSB1* showed had no effect on drug resistance in the wild-type background, however as we did not have the *cof1-6 Δrsb1* strain, it cannot be confirmed if Rsb1 is involved in drug resistance in the *cof1-6* mutant.

3.4 The effects of deleting *PDR5* on cofilin mediated drug resistance

We tested whether loss of the ABC transporter *PDR5* led to changes in multi-drug resistance using *Δpdr5* in both the wild-type and *cof1-6* background. Once the deletions had been acquired, they were used in a spotting assay to see how they would respond to different classes of drugs at different concentrations.
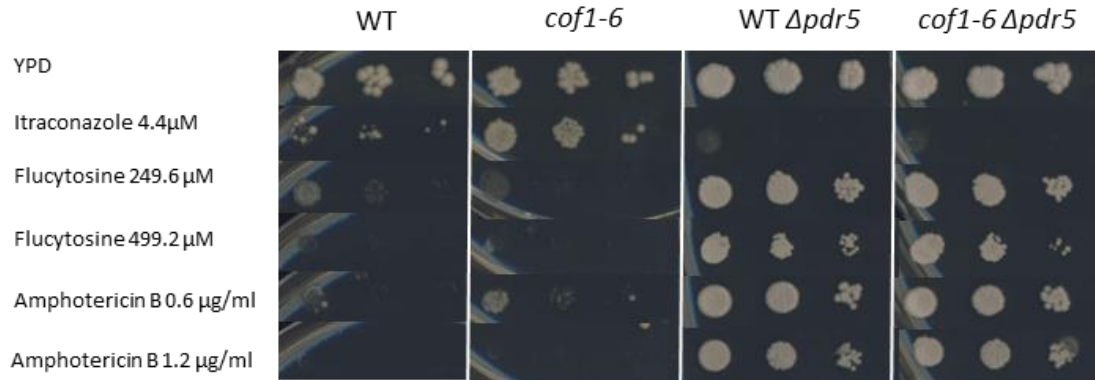
| | WT | cof1-6 | WT Δpdr5 | cof1-6 Δpdr5 |

Figure 3.8 Spotting assay showing the effect of deleting _PDR5_ on the wild-type and _cof1-6_ strains. Overnight cultures of the wild-type, cof1-6, wild-type Δpdr5 and _cof1-6 Δpdr5_ strains were diluted to a starting OD_{600} of 0.1, and serially diluted 1 in 10. They were then grown on YPD plates for two days at 30°C in the presence of itraconazole, flucytosine and amphotericin at the different concentrations shown. The _cof1-6_ strain loses resistance to Itraconazole but gains increased resistance to Amphotericin B at both 0.6μg/ml and 1.2μg/ml.

The cells were diluted to an optical density (OD600) of 0.1 and then diluted 1 in 10 in three subsequent serial dilutions. They were assayed in the presence of the azole Itraconazole, the polyene amphotericin B, and the nucleoside analogue flucytosine. As expected, removing the _PDR5_ gene in the _cof1-6_ strain reversed the drug resistant phenotype normally seen in the _cof1-6_ strain (**Figure 3.8**). This was expected as azoles are known to be substrates for ABC transporters. We also noticed that deleting _PDR5_ in both the wild-type and _cof1-6_ strains caused the strains to become resistant to both flucytosine and amphotericin B (**Figure 3.8**). This was unexpected as we hypothesised that knocking out the most prevalent drug efflux pump in the cell would lead to sensitivity to amphotericin B but this was not the case. An explanation for this could be that when _PDR5_ is deleted, it allows other drug efflux pumps within the cell such as Pdr10 and Pdr15 to be upregulated

and these have the ability to remove flucytosine and amphotericin B from the cell more effectively, increasing resistance. Further analysis is required to test this hypothesis.
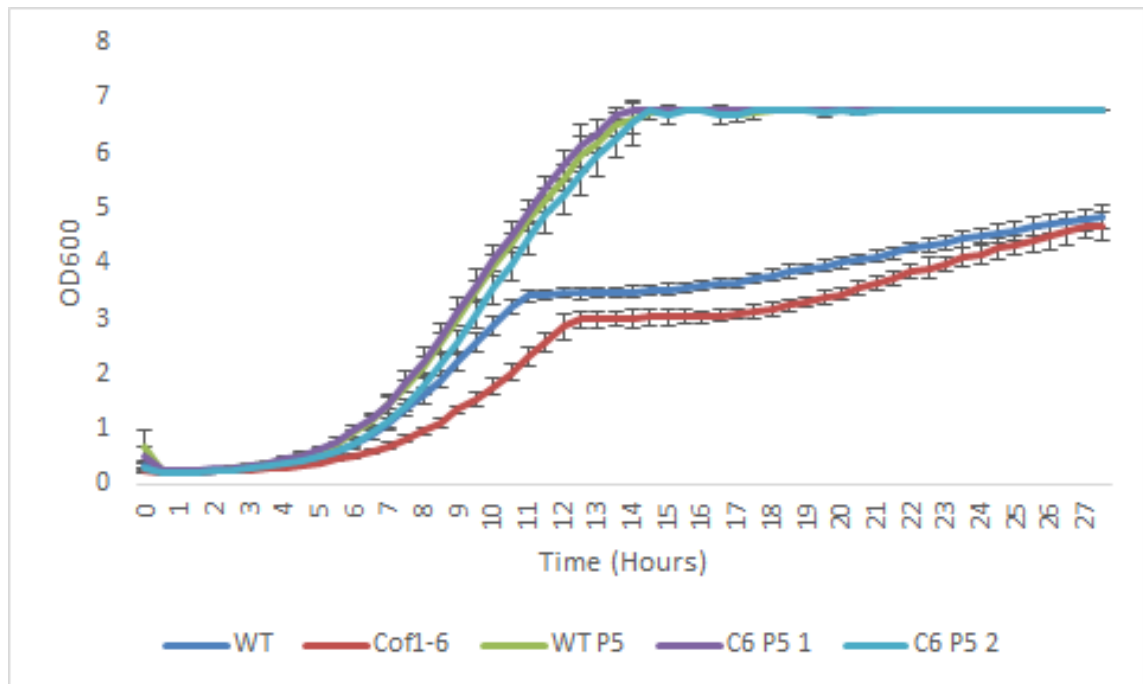
Figure 3.9 Growth curve showing the growth rate of the *PDR5* deletions compared to the wild-type and *cof1-6* strains. The strains were grown overnight and inoculated to a starting OD_{600} of 0.1 in YPD. They were then measured at 30 minute intervals for 27 hours. The *PDR5* deletions showed increased growth rate and a higher final density than the wild-type and *cof1-6* strains.

Our data suggest that deletion of *PDR5* leads to sensitivity to itraconazole but resistance to flucytosine. Itraconazole and Flucytosine resistance is often seen in tandem so further study was required in the *PDR5* knockout to see why this was not the case. It has been reported that resistance to drugs that reduce growth by targeting anabolic processes such as RNA or DNA biosynthesis which is inhibited by flucytosine, can occur in strains simply because they grow more slowly. To test whether this may be the case for cells lacking *PDR5* we tested the effects of removal of this ABC transporter on growth rate. Surprisingly in all

cases the deletion of *PDR5* led to a dramatic increase in growth rate and led to a much higher final density. This suggests that Flucytosine resistance observed in *PDR5* knockout strains does not occur as a result of reduced growth. The increased rate of growth may indicate that Pdr5 production requires a lot of energy or resource expenditure from the cell and when deleted it allows the cell to channel this energy into growth. Alternatively, Pdr5 may be involved in signalling processes that medicate growth rate and biomass accumulation. The *PDR5* knockouts all reached a plateau at an OD_{600} of 6.7, which is most likely when they exhausted the supplies of nutrients required or growth from the growth media. Alternatively, it could indicate that the *Δpdr5* strains are unable to use ethanol as a carbon source once the supply of glucose is used up.
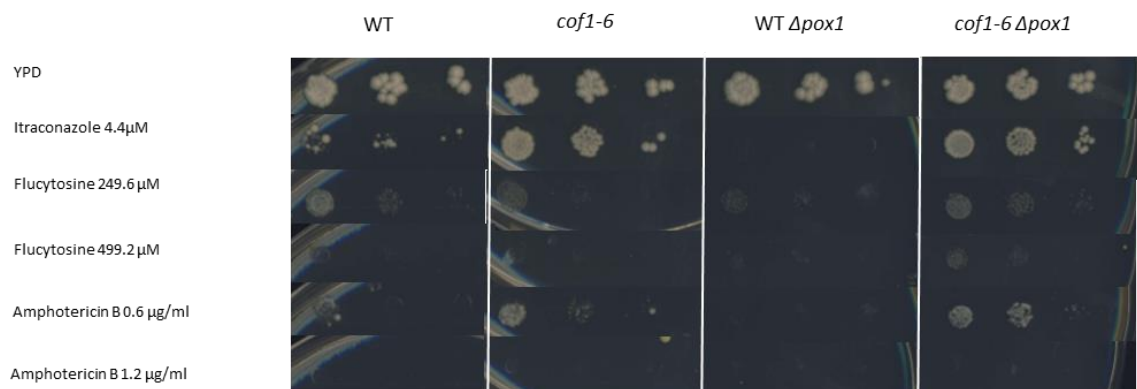
Figure 3.10 Spotting assay showing no change in resistance between resistant *cof1-6* strain and its corresponding *POX1* deletion. Overnight cultures of the wild-type and *cof1-6,* wild-type *Δpox1* and *cof1-6 Δpox1* strains were diluted to a starting OD_{600} of 0.1, and serially diluted 1 in 10. They were then grown on YPD plates for two days at 30°C in the presence of itraconazole, flucytosine and amphotericin at the different concentrations shown. The wild type and *Δpox1* strains also showed no effect. This shows that *POX1* is not essential for the drug resistant phenotype.

The *POX1* deletion in the *cof1-6* background did not lead to measurable differences in resistance to the three drugs tested. The *cof1-6 Δpox1* strain seemed to be resistant to Itraconazole at 4.4μM as well as amphotericin B at 0.6μg/ml. The concentration of amphotericin B at 1.2μg/ml was too high for the *cof1-6* strain and so no growth was seen despite *cof1-6* being resistant. The *cof1-6 Δpox1* strain showed no change in growth from the *cof1-6* indicating that the *POX1* gene had no effect on the resistance of the *cof1-6* strain. However, deleting the *POX1* gene in the wild-type background seemed to result in a slight increase in sensitivity to flucytosine at both concentrations.

3.5 Control of Pdr5 expression is not regulated by PKC or activated by lipid stress

Our unpublished experiments (Dr. Patrick Rockenfeller, Gourlay lab) suggest that cofilin interacts with the PKC/MAPK cell wall integrity pathway. One possibility is that the *cof1-6* mutation leads to activation of PKC and that this signal mediates the expression of Pdr5 and/or the multi-drug resistance response via MAPK signalling.
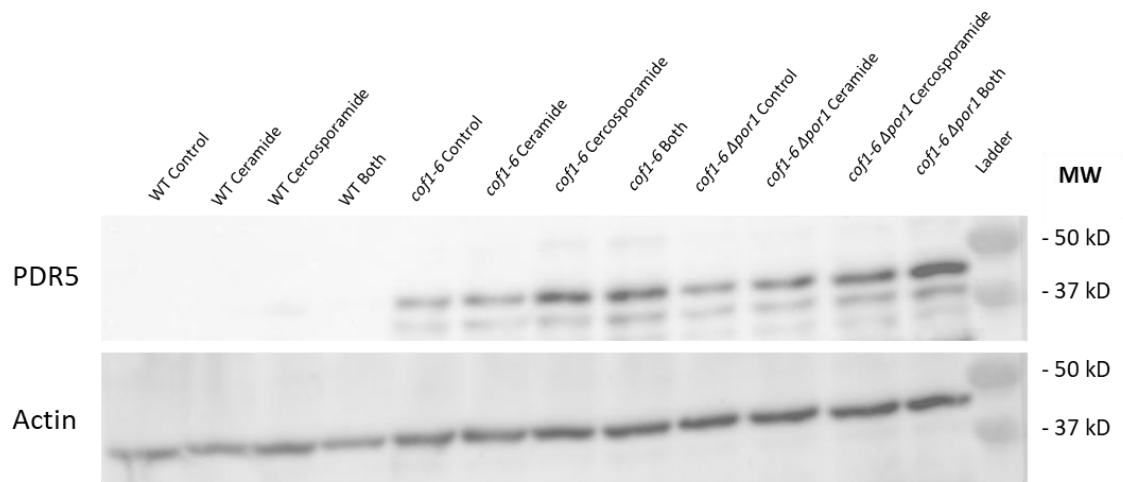
Figure 3.11 Western blot showing changes in Pdr5 expression after Ceramide and Cercosporamide treatment. Strains were grown up overnight in YPD before being inoculated for 4 hours in YPD with either ceramide, cercosporamide or both. Whole protein extracts were then taken as described in the materials and methods. Pdr5 expression did not change in the presence of any of the treatments. Pdr5 expression was elevated in both the *cof1-6* and *cof1-6 Δpor1* compared to the wild-type but this was not affected by the treatments. Also shown is the actin loading control.

To determine if the Pkc1-mediated cell wall integrity pathway was involved in cofilin mediated drug resistance, we tested the wild-type, *cof1-6* and associated *POR1* deletion strains by inoculating them for four hours in YPD with either Cercosporamide, Ceramide, or both. Ceramide is a sphingolipid precursor that causes mitochondrial cell death in high doses. In this case we used ceramide to test whether lipid stress was sufficient to activate upregulation of a drug resistance response and whether this was mediated via PKC signalling. This could be achieved by using Cercosporamide, which specifically inhibits protein kinase C (Pkc1). The treated strains were also used to make protein extracts and run on a western blot to check for Pdr5 levels and activation of the MAPK signalling pathway.

Initially we conducted we did a spotting assay to see the effect of the treatments, ceramide and Cercosporamide, on drug resistance phenotype (**Figure 3.13**). The wild-type showed small amounts of growth on the Itraconazole in all treatments, while being susceptible to both concentrations of Amphotericin B. Neither ceramide nor cercosporamide treatment resulted in a change in drug resistance in the *cof1-6* compared to the control, with the strain showing resistance to Itraconazole in all treatments. Removing porin also showed no effect compared to the control, with the *cof1-6 Δpor1* showing sensitivity to all the antifungals used regardless of treatment with cercosporamide, ceramide or both. This indicated that the resistance seen in the *cof1-6* is not regulated by the Pkc1-mediated cell wall integrity pathway, and it also confirms that it is not a response to lipid stress upon ceramide treatment.

To confirm the effects of ceramide and cercosporamide we also carried out a western blot for both Pdr5 (**Figure 3.11**) and for the activated form of the terminal MAPK Slt2 which is a protein kinase that is activated by Pkc1, and which is responsible for the activation of a whole plethora of genes involved in cell wall integrity.

The Slt2 probe showed no specific binding in any of the strains or treatments, resulting in the blot seen in **Figure 3.12**. Indicating that Pck1 activity was not upregulated in response to the cell was stress caused by ceramide, or decreased in the presence of the Pkc1 inhibitor, Cercosporamide. However a positive control was omitted from these experiments and so it remains possible that a lack detection of activated Slt2 may have arisen for technical reasons. Repetition of this experiment with the inclusion of a positive control, such as the addition of cell wall stress, would be required to confirm this.

The Pdr5 probe showed very little signal in the wild-type when compared to the *cof1-6* and *cof1-6 Δpor1* strains in all treatments, indicating that Pdr5 expression is increased in the *cof1-6* background. The upregulation of Pdr5 in *cof1-6* cells was not inhibited upon deletion of porin and in fact levels were significantly elevated. This result was unexpected as deletion of porin renders cells sensitive to itraconazole treatment. (**Figure 3.9**).
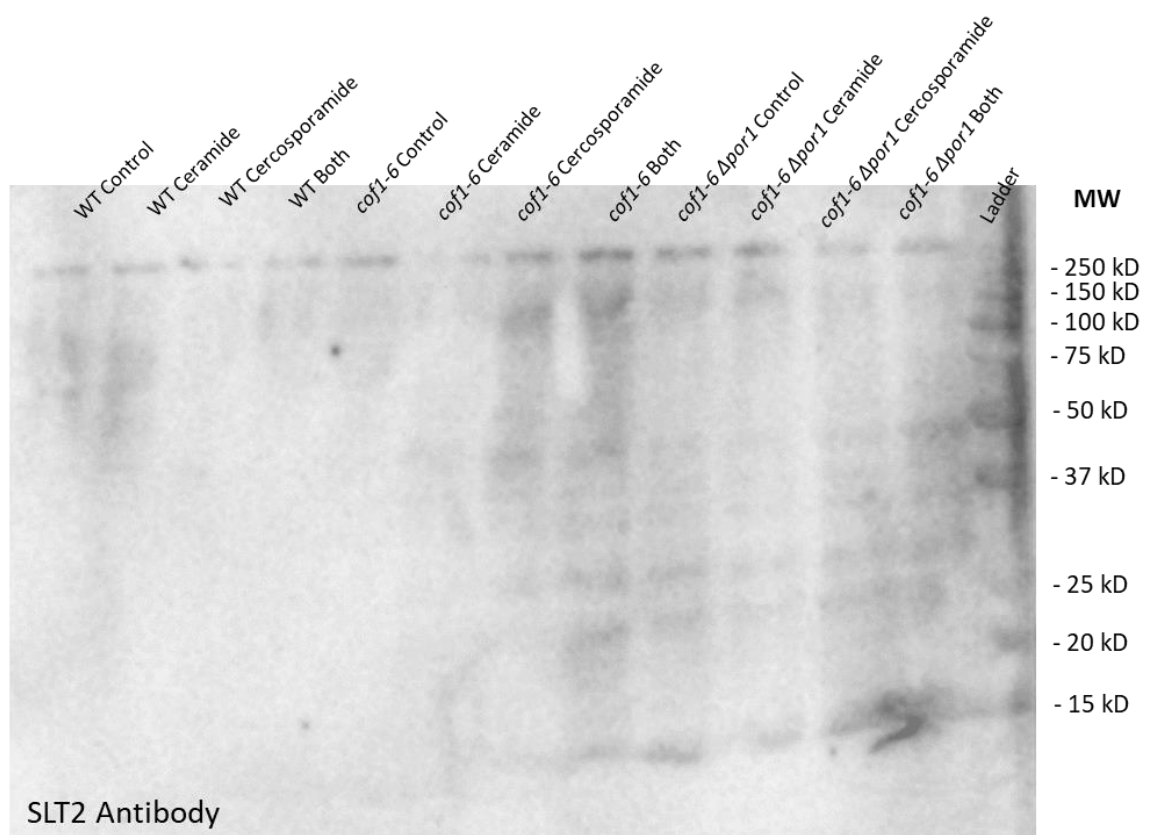
Figure 3.12 Western Blot to see Slt2 expression in strains treated with Cercosporamide, Ceramide or both. Strains were grown up overnight in YPD before being inoculated for 4 hours in YPD with either ceramide, cercosporamide or both. Whole protein extracts were then taken as described in the materials and methods. Nonspecific binding of Slt2 was observed in all strains, indicating that the *cof1-6* does not induce Slt2 activity through the CWI pathway in response to cell wall stress.
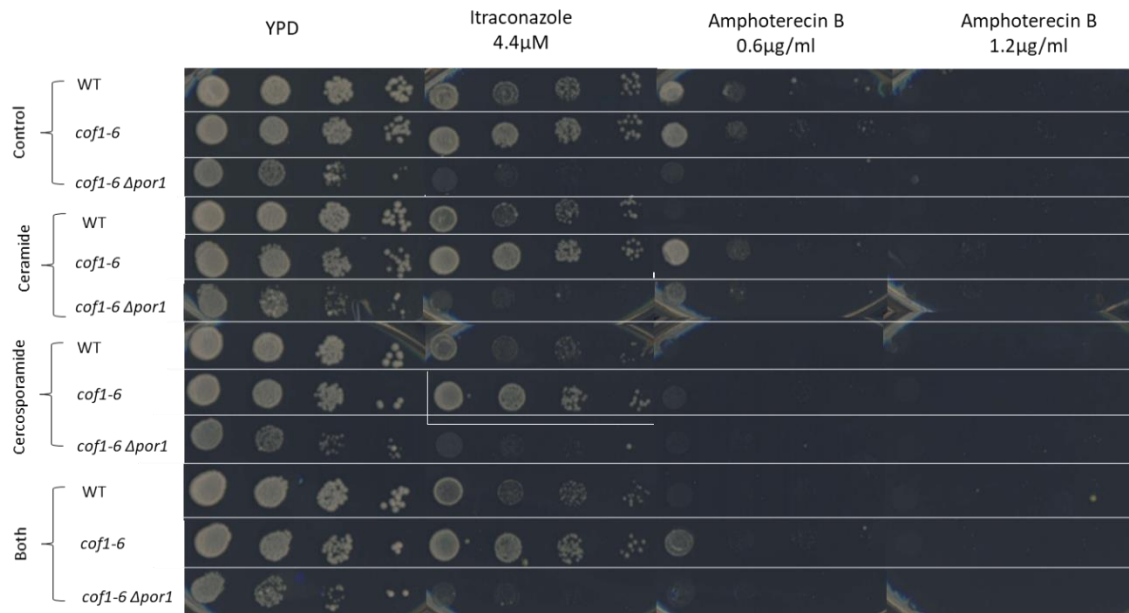
Figure 3.13 Spotting assay showing the effect of Cercosporamide and Ceramide treatment on drug resistance. Strains were grown up overnight in YPD before being inoculated for 4 hours in YPD with either ceramide, cercosporamide or both. After inoculation, the strains were diluted to a starting OD_{600} of 0.1, and serially diluted 1 in 10. They were then grown in the presence of itraconazole at 4.4μM and amphotericin B at 0.6μg/ml and 1.2μg/ml. The plates were left to grow for 2 days at 30°C. The wild-type and *cof1-6 Δpor1* were sensitive to both Itraconazole and Amphotericin B. The *cof1-6* mutant showed resistance to Itraconazole and Amphotericin B even when treated.

3.6 Porin is required for Pdr5 activation
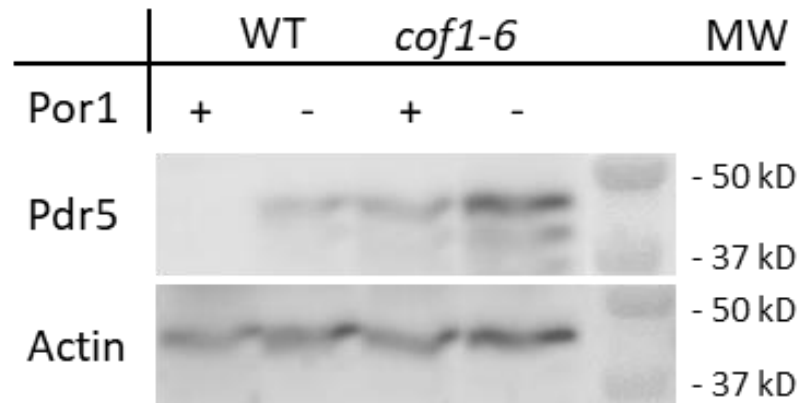
Figure 3.14 Western blot displaying increased expression of Pdr5 in the *cof1-6* when compared to the wild-type. Strains were grown up overnight in YPD before a whole cell protein extract was taken as described in the materials and methods. Lane 1 contains the wild-type, lane 2 contains *Δpor1*, lane 3 contains the *cof1-6* mutant and lane 4 contains the *cof1-6 Δpor1* mutant.

Current theories link the presence of drug transporters to the presence of the drug resistant phenotype. In order to determine if there was a difference in expression levels of Pdr5 between the wild-type and *cof1-6* mutant, a western blot was undertaken. As predicted, the *cof1-6* mutant had significantly higher elevated Pdr5 expression compared to the wild-type. What was more surprising was the *Δpor1* also showed elevated expression of Pdr5 in relation to the wild-type, although much less than seen in the *cof1-6* mutant. The most surprising of all, however, was the elevated Pdr5 levels seen in the *cof1-6 Δpor1*, which showed the highest expression levels of any of the strains. This may suggest that Por1 has a down-regulatory role in Pdr5 expression, and when deleted, expression levels are upregulated. It also suggests that the expression of Pdr5 regulated by por1 and the

expression of Pdr5 in the *cof1-6*, are controlled through two separate pathways. We predicted that the presence of Pdr5 would correlate to drug resistance but this clearly is not the case in the *cof1-6* mutants. It is more likely that por1 has a role in the activation of the Pdr5 transporter and that is why, when porin is deleted, the drug resistant phenotype is no longer seen.

3.7 Porin levels play a role in drug resistance
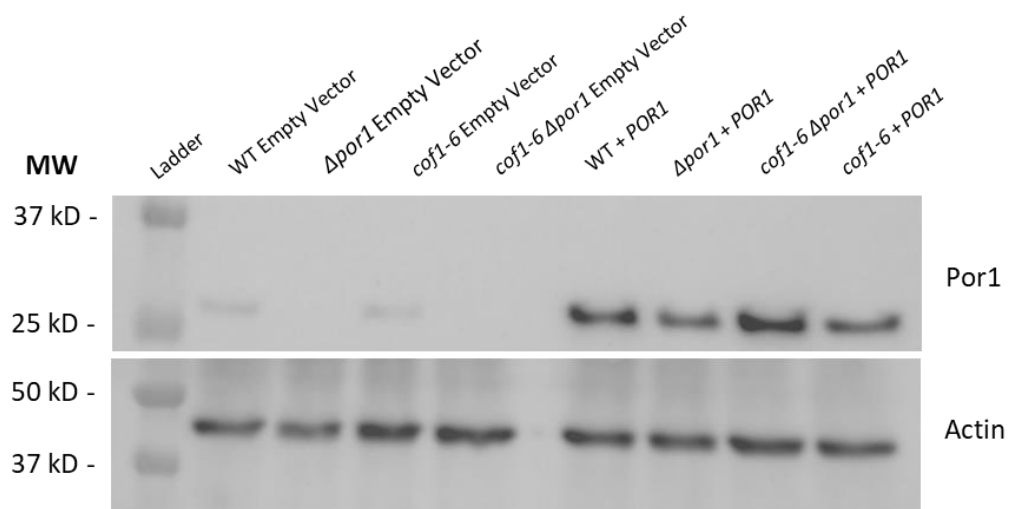
Figure 3.15 Western blot to confirm the overexpression of porin. The first probe is with a porin specific mouse antibody, whereas the second probe is the actin loading control. Lanes 1-4 contain the empty vector control strains, and lanes 5-8 contain the porin overexpression strains. Porin overexpression is clearly seen in lanes 4-8 in relation to lanes 5-8.

Our experiments suggest that Porin plays a role in the control of Pdr5 level and activity. To investigate this further we introduced a Porin1 expression plasmid into wild-type, *cof1-6* and associated porin deletions. We also transformed an empty vector into the strains as a control. To confirm the porin overexpression, a western blot was done using a porin specific mouse antibody (**Figure 3.15**). The resulting western blot showed elevated levels in all of

the plasmid inserted clones. It is important to note that Por1 levels were significantly higher than observed in the empty vector control strains (**Figure 3.15**). Having confirmed Porin overexpression we investigated its effect upon resistance we performed a spotting assay in the presence of itraconazole and amphotericin B (**Figure 3.16**) .
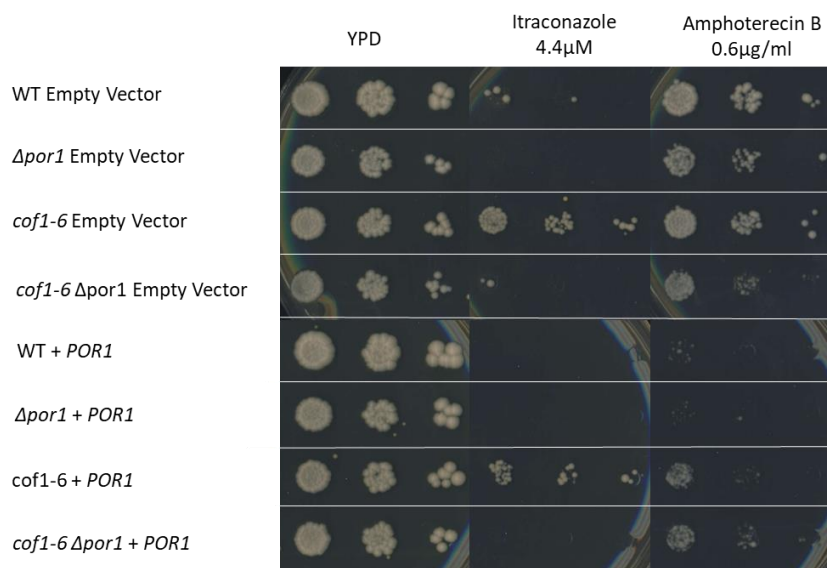
Figure 3.16 Spotting assay showing the effect of porin overexpression on drug resistance. Overnight cultures of strains were diluted to a starting OD_{600} of 0.1, and serially diluted 1 in 10. They were then grown in the presence of itraconazole at 4.4µM and amphotericin B at 0.6µg/ml. The plates were left to grow for 2 days at 30°C. Porin overexpression does not affect resistance to Itraconazole but increases sensitivity to Amphotericin B.

Deletion of porin leads to sensitivity to all drugs tested in a wild type background while Expression of *cof1-6* control demonstrated resistance to Itraconazole as before. Deletion of porin prevented resistance to itraconazole while porin overexpression reduced resistance in all cases. The overexpression of Porin also led to a reduction in resistance to amphotericin B in all strains. These results suggest that porin level and/or its function is

important in drug resistance. One possibility is that porin plays a role in the control of lipid maturation within the mitochondria and that this leads to changes in transporter activity or localisation or to the lipid composition of the cell.

3.8 Investigating lipid organisation *cof1-6* and porin mutant cells

Our data suggests that cofilin and porin may function to influence the lipid environment of the cell and that this in turn may influence drug resistance. Mutations in the *cof1-6* strain have been seen to result in the upregulation of fatty acid beta-oxidation in cells. In addition, mitochondria are involved in the maturation of phosphatidylserine to phosphatidylethanolamine. To investigate lipid organisation, we made use of GFP labelled probes that recognise specific phospholipids using fluorescence microscopy. Using these probes, we investigated phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 3-phosphate (PI3P) localisation in the wild-type, *cof1-6* and corresponding porin deletions. PI3P is a phospholipid that is located in the plasma membrane and is responsible for recruiting protein trafficking proteins to the membrane (Gillooly *et al*, 2000). PI4P is a phospholipid located in the Golgi apparatus which has a role in the recruitment of proteins that are destined for the cell membrane (Stefan *et al*, 2011). Overnight cultures of the strains were inoculated for four hours and visualised in early log phase, or inoculated for 24 hours and visualised at stationary phase.

Figure 3.17 GFP localisation phenotypes observed in the GFP-tagged PI3P strains. The cells were inoculated

from an overnight culture and grown to stationary phase for 24 hours. The images were analysed in ImageJ. The

cells either showed a number of different phenotypes like localisation to the cell membrane (A), localisation to

the membrane but also with a single foci (B), localisation with multiple foci (C), a single foci with no membrane

localisation (D), or a diffuse signal (E). Bar chart shows the average number of each phenotype in each strain. At

least 180 cells were counted in total for each strain, with at least 4 technical repeats. * corresponds to a P value =

<0.05, ** corresponds to a P value = <0.01, *** corresponds to a P value <0.001.

PI3P could be observed at a number of different locations in the wild-type and mutant strains. The wild-type had predominantly GFP localisation at the plasma membrane with a multiple foci also at the plasma membrane. They also displayed a large percentage of cells with membrane localisation but no foci (**Figure 3.17, C**). The *Δpor1* showed a similar phenotype to the wild-type showing no significant difference in single foci (P value = 0.0797) or multiple foci (P value = 0.3553). The *cof1-6* had a similar percentage of cells with membrane only localisation to the *Δpor1* strain but less multiple foci with membrane localisation than the wild-type (P value = 0.039). The *cof1-6 Δpor1* had the most distinct phenotype of all, with almost all GFP-expressing cells showing a diffuse signal, a phenotype that was also seen in the *Δpor1* but only in 1% (**Figure 3.17**). Some *cof1-6 Δpor1* displayed single foci but no membrane localisation (**Figure 3.17, D**). This suggest that porin is important for PI3P membrane localisation in the *cof1-6* background.
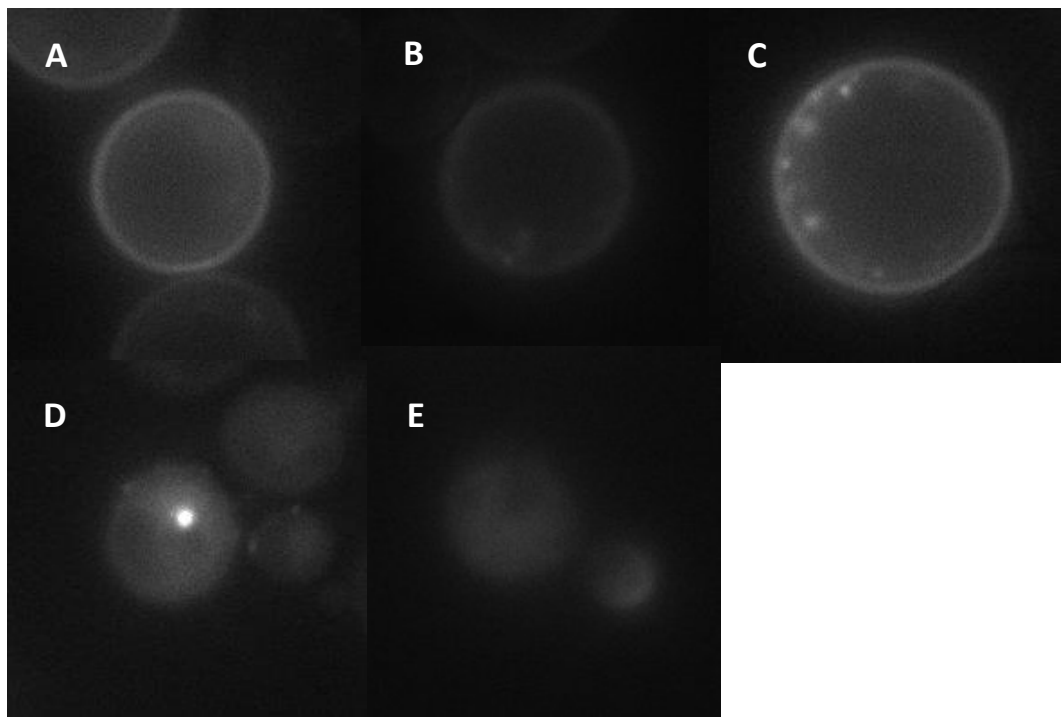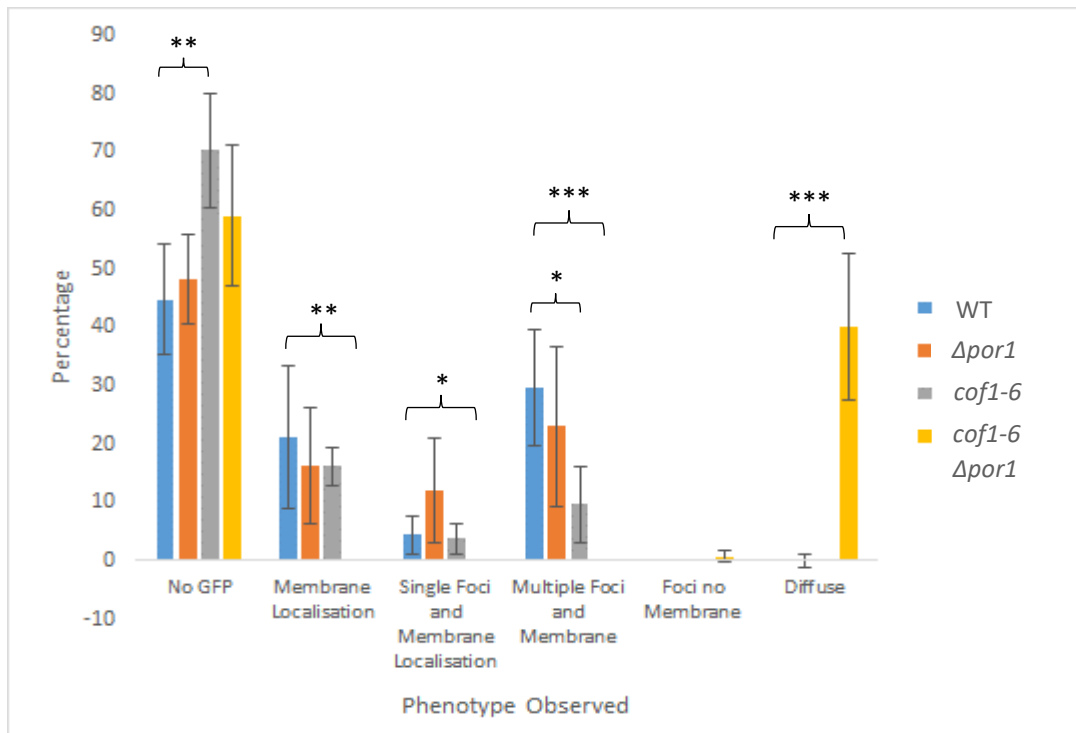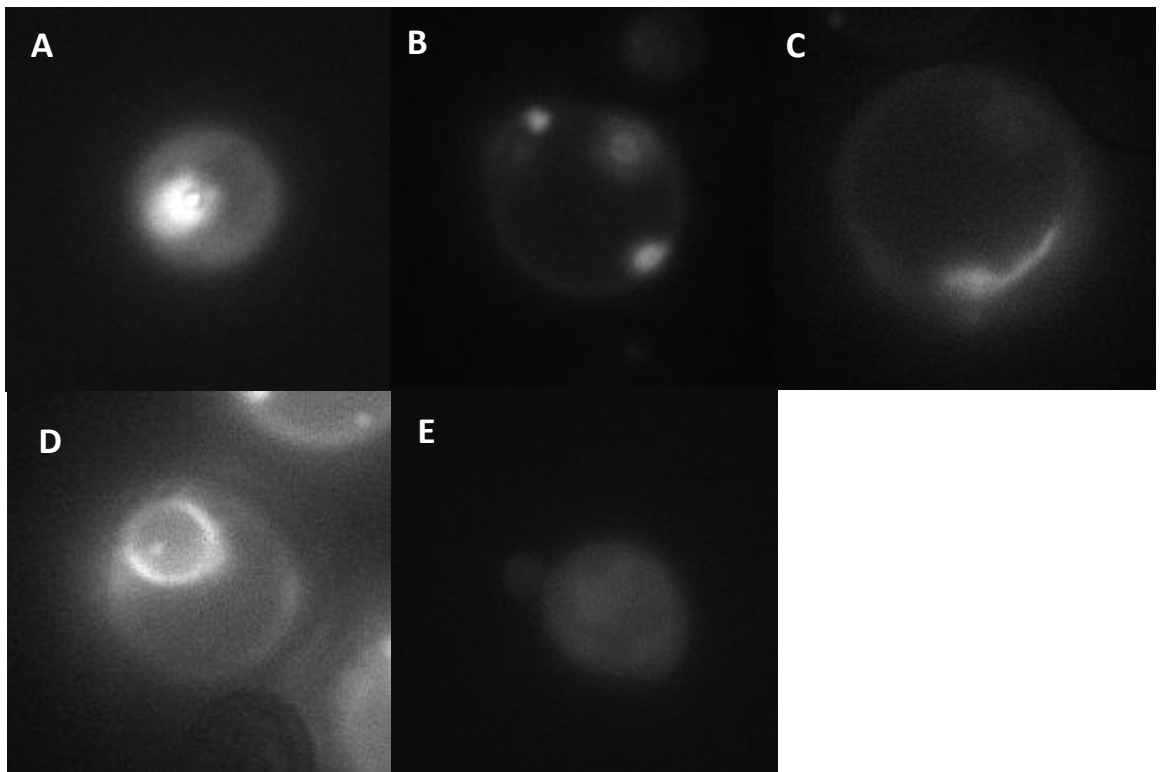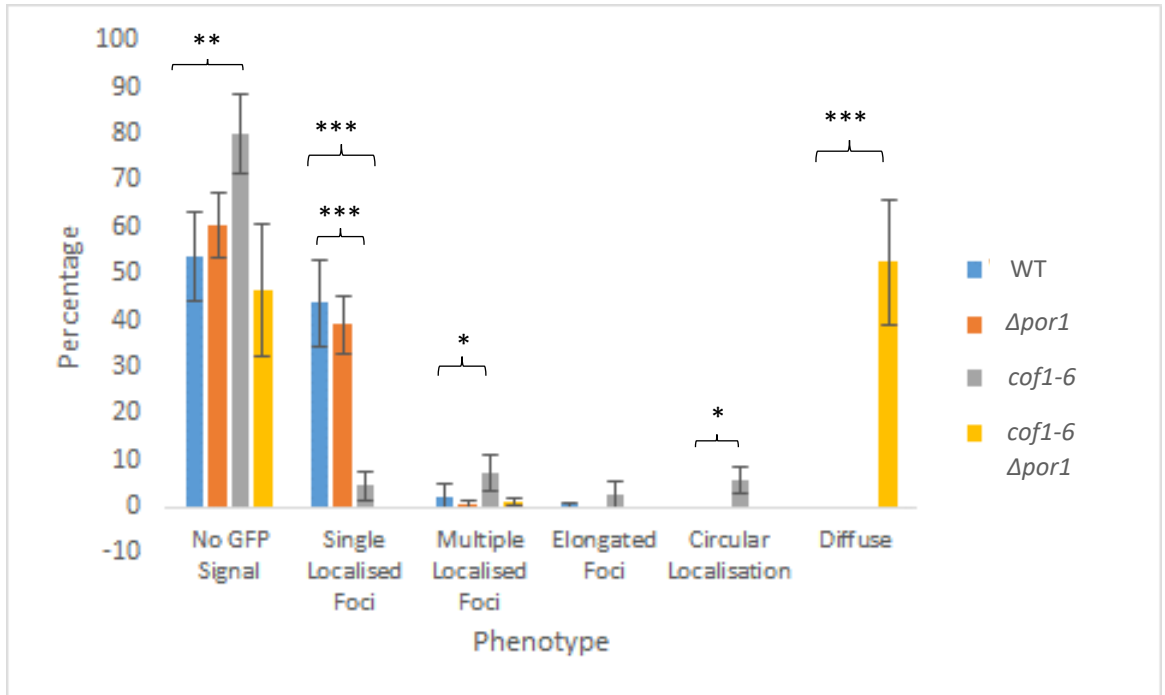
Figure 3.18 GFP localisation phenotypes observed in the GFP-tagged PI4P strains. The cells were inoculated from an overnight culture and grown to stationary phase for 24 hours. The images were analysed in ImageJ. The cells either showed a number of different phenotypes like a single localised foci (A), multiple localised foci (B), localisation to a single foci elongated along a membrane (C), localisation to an internal circular membrane (D), or a diffuse signal (E). Bar chart shows the average number of each phenotype in each strain. At least 550 cells over 3 technical repeats were counted from each strain. * corresponds to a P value = <0.05, ** corresponds to a P value = <0.01, *** corresponds to a P value <0.001.

The most common localisation phenotype observed with the GFP PI4P probe was a single localised foci, most likely at the Golgi apparatus as documented, although some cells also displayed multiple localised foci (**Figure 3.18**). The *Δpor1* showed a similar phenotype compared to the wild-type with most of cells showing a single localised foci (P value = 0.3304) or multiple foci (P value = 0.1741). The *cof1-6* strain showed a lot less single foci (P value = < 0.0001) and more multiple foci compared to the wild type (P value = 0.0231) (**Figure 3.18**). The *cof1-6* cells mostly showed either no signal, elongated foci or localisation to an internal circular membrane. The *cof1-6* was the only strain that showed localisation to an internal circular membrane. The *cof1-6 Δpor1* cells mostly had a diffuse signal (**Figure 3.18, E**), a phenotype not seen in the other strains. These results may indicate that that there is mis-localisation of PI4P in the *cof1-6* strain, but without porin there localisation is dispersed, or that PI4P levels become reduced.
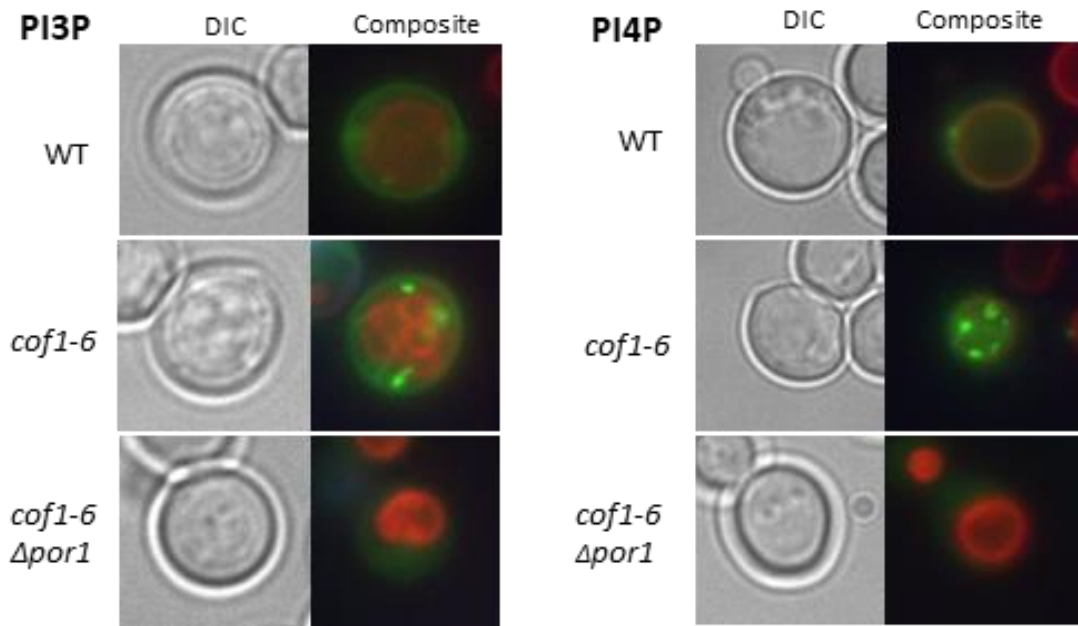
Figure 3.19 Staining of the vacuole and PI3P and PI4P sensors showed fragmented vacuoles and mis-localisation of GFP signal in the cof1-6 strain. The strains were transformed with GFP-tagged PI3P and PI4P sensors, as well as being stained with FM4-64, a vacuole stain. The cof1-6 strain shows both fragmented vacuoles and mis-localisation of PI3P and PI4P that was prevented by deletion of porin. This suggests a role for both cofilin and porin in lipid organisation.

In order to determine whether the foci seen in the *cof1-6* strains in the PI3P and PI4P were associated with the vacuole we made use of the stain FM6-64. The resulting images are seen in **Figure 3.19**. In the PI3P stained wild-type strain, the signal was localised to the cell embrane as expected, with the foci localised at points along that membrane. In the wild-type the vacuole was intact and usually observed as a single entitiy. In contrast the vacuole in *cof1-6* cells appeared to be fragmented, with the GFP signal at the membrane with the foci dotted along it. The *cof1-6 Δpor1* strain showed a difuse signal throughout the entire cell with a vacuole similar to the wild-type cells. In the PI4P microscopy the signal localised

to the vacuole with single foci in most cells that presented the GFP tagged PI4P. The *cof1-6* showed foci within the often fragemented vacuole with some localised at the inner vacuole membrane. In the *cof1-6 Δpor1*, as seen in the PI3P localisation, had a diffuse signal throught the cell with a normal looking vacuole the same as the wild-type. These data implicate porin in vacuole integrity and lipid organisation within the cell.

Discussion

4. Discussion

The actin cytoskeleton is a dynamic structure which is capable of rapid assembly and disassembly, and has roles in many cell processes. Previous research has linked the actin regulatory protein Cofilin to drug resistance in yeast. There has also been links between cofilin and drug resistance in cancer, although the exact mechanisms of resistance are yet to be determined. Phosphorylation of cofilin has been linked to HDAC resistance in hepatocellular carcinoma, and cofilin has also been associated with apelin-13 regulated cell migration in adenocarcinoma(Liao *et al*, 2016; Lv *et al*, 2016). Cofilin, encoded by the *COF1* gene, acts to depolymerises F-actin. We studied a mutation in cofilin, *cof1-6*, that elevates mitochondrial function and activates a robust drug resistance phenotype.

Here we show that *cof1-6* mutant cells express high levels of the ABC transporter Pdr5 in a manner that is dependent upon the mitochondrial membrane protein Porin. We hypothesise that cofilin mutation leads to overexpression of Pdr5 and that porin is essential for its activation. Our data suggests that mitochondria participate in drug resistance via their role in lipid organisation.

4.1 Evidence for the presence of an adaptive drug resistance response to cell wall or plasma membrane damage by antifungal agents

The mechanisms by which yeast cells elicit a multi-drug resistance response are unclear. One possibility is that chemical damage at the level of the cell wall or plasma membrane is linked to a multi-drug resistance response. We entertained the possibility that cofilin may play a role in sensing cell wall or cell membrane stress, given its known affinity for

phospholipids, and that changes in its binding to lipids in the plasma membrane may mediate an adaptive drug resistance response. As an extension of this hypothesis we postulated that the change from positively charged lysine to alanine in *cof1-6* leads to changes in plasma membrane association that in turn activate a drug resistance response.

Our original hypothesis was that drug resistance was response to cell wall stress in the *cof1-6* mutant, and this was induced through the PKC/MAPK CWI pathway. However, treatment of *cof1-6* cells with Cercosporamide, an inhibitor of PKC, did not affect in drug resistance associated with *cof1-6*. This might indicate that Pkc1 activation is not involved in cofilin mediated drug resistance. and that the CWI pathway is not the trigger for drug resistance within the *cof1-6* mutant. In agreement with this we saw no activation of Slt2, the terminal MAPK activated by Pkc1 signalling within the CWI pathway.

4.2 Rsb1 expression dependant on expression of Porin

Interestingly in the *cof1-6* mutant the expression of the lipid transporter Rsb1 are increased. As Rsb1 is involved in the regulation of lipid within the plasma membrane bilayer we hypothesised that the *cof1-6* mutation mimicked a membrane stress response that also led to the upregulation of a number of plasma membrane transporters that could influence drug resistance, such as Pdr5. In fact, Pdr5 is known to act both as a drug pump and a lipid transporter (Mahé *et al*, 1996). We investigated if lipid stress may influence Rsb1 levels and/or multi-drug resistance. However, treatment with the sphingolipid precursor ceramide, which is lethal to *S. cerevisiae* in high doses, also had no effect upon drug resistance in strains investigated. Rsb1 expression is upregulated by the RIM101 pathway which in turn is activated in response to changes in cell surface charge(Ikeda *et al*, 2008).

Ikeda et al hypothesised that changes in the lipid asymmetry could be detected by the RIM21 sensors, part of the RIM101 pathway thus activating *RSB1*. These changes in lipid asymmetry could result in negatively charged phospholipids such as PS and PI being exposed on the cell surface, and this negative charge being subsequently detected by RIM21. However, we did not observe changes in Rsb1 promoter activity in response to alkaline conditions.

As the *cof1-6* has shown dysregulation of the cell membrane, the elevated Rsb1 expression could be explained by these changes in membrane asymmetry, with PS and PI being exposed and detected by the RIM101 pathway. Interestingly deletion of the mitochondrial outer membrane protein porin prevented the increase in Rsb1 promoter activity observed in *cof1-6* cells. One possibility is that porin is involved in the formation of ERMES junctions. ERMES junctions are mitochondria/ER interaction channels which have many important physiological functions within the cell. The primary function of these channels is to allow the transfer of phospholipids from the ER to the mitochondria. The majority of phospholipids are made in the Endoplasmic Reticulum but as mitochondria are not part of the endomembrane system, they cannot receive phospholipids required for their inner and outer membranes through vesicular trafficking. One purpose of these ERMES junctions is to allow an alternative route by which mitochondria can receive these phospholipids it requires (Michel & Kornmann, 2012).

The mitochondria is also a crucial element in the post translational modification of important lipids required at the cell wall. The ERMES junction allows a contact point where lipids can enter the mitochondria to undergo decarboxylation. The mitochondria is the site where phosphatidylserine (PS) is decarboxylated to phosphatidylethanolamine (PE) by the

enzyme phosphatidylserine decarboxylase 1 (Psd1). Psd1 is an enzyme that is located in the mitochondria so the precursor, PS, must cross from the ER to the mitochondria, be converted to PE before crossing back to the ER again (Michel & Kornmann, 2012). In the ER PE is converted to phosphatidylcholine which is the most prevalent phospholipid found in the membranes of yeast cells (Choi *et al*, 2004).

We hypothesise that removing porin, and so damaging ERMEs connections to the ER, could result in less PS being transported to the plasma membrane which could in turn lead to the activation of the RIM101 pathway and Rsb1. Alternatively, it could be that direct and as yet undescribed signalling mechanisms are controlled by Porin leading to changes in Rsb1 expression.

4.3 Deletion of *PDR5* increases resistance to Amphotericin B and Flucytosine

Interestingly we saw a gain in resistance to Amphotericin B and Flucytosine in the *PDR5* deletion strains which is similar to results seen in a paper by Guan et al. in which they saw also saw a gain of resistance to Amphotericin after deleting *PDR5* in *S. cerevisiae*. Guan et al. hypothesised that Pdr5 was involved in the synthesis of ergosterol, the target of Amphotericin B, and when *PDR5* was deleted, ergosterol levels were reduced resulting in the resistance seen.

Research by Kotiadis et al. showed that although levels of ergosterol were reduced in the *cof1-6* mutant in log phase, the levels did not vary between the mutant and wild-type in stationary, concluding that there was no difference in ergosterol synthesis(Kotiadis *et al*, 2012). However, no such analysis of ergosterol synthesis in the *cof1-6 Δpdr5* mutant has

been done. Although no other papers linking Pdr5 to ergosterol synthesis have emerged, this hypothesis would also explain the gain in resistance to amphotericin seen in the wild-type *Δpdr5* mutant.

Other explanations for a gain in amphotericin could include mutations in *ERG6*, a gene involved in ergosterol biosynthesis which have been shown to bestow resistance to polyenes to mutants (Vandeputte *et al*, 2008). This could make sense for the *cof1-6 Δpdr5* mutant but couldn't explain the increase in resistance or gain of resistance in the wild type *Δpdr5* unless there *PDR5* and *ERG6* were linked, however Emter et al. showed that drug resistance via *ERG6* and *PDR5* occur through different distinct pathways(Emter *et al*, 2002).

Another possibility could be that the deletion of *PDR5* results in the upregulation of other drug pumps which are able to remove Amphotericin and Flucytosine. Other transporters seen to be upregulated in the *cof1-6* mutant were Pdr15 and Pdr10. Deleting *PDR10* has been seen to have no effect on amphotericin resistance, however Pdr15 has been seen to show similar properties to Pdr5(Rockwell *et al*, 2009)(Schüller *et al*, 2007). It could be possible that deleting *PDR5* induces increased Pdr15 expression to compensate for the loss of the most prevalent transporter.

4.4 Deletion of *PDR5* results increased rate of growth

Deleting *PDR5* in both the *cof1-6* mutant and the wild-type background resulted in an increased growth rate and final density when compared to the wild-type and *cof1-6* strains. This suggests that the production of Pdr5 within the cell requires large amounts of energy

expenditure that could be focused into growth, and when *PDR5* is deleted, it allows the redirection of this energy. Alternatively, Pdr5 could be involved in the control of cell cycle or response to quorum sensing mechanisms that limit cell number. This would require further research as Pdr5 has not previously been linked to growth regulation.

4.5 Porin and cofilin work in separate pathways to control Pdr5 levels

As well as in the *cof1-6*, Pdr5 upregulation was seen in both the *Δpor1* and *cof1-6 Δpor1* strains. Deleting porin in the wild-type induced Pdr5 expression to similar levels as seen in the *cof1-6* mutant. Furthermore, the *cof1-6 Δpor1* showed more expression than the *cof1-6*, indicating that this Pdr5 expression seen by deleting porin is cumulative. This result suggests that the regulation of Pdr5 levels is controlled independently by cofilin and porin via two distinct mechanisms. At this point both pathways are uncharacterised. Further evidence for a role for Porin in the control of drug resistance was observed upon its over-expression. The over-expression of porin in the *cof1-6* mutant, made it more sensitive to all of the drugs at the concentrations tested.

4.6 Porin possibly required for ERMES Junction formation

We hypothesis that porin plays a role in lipid regulation and that this is linked to a role in drug resistance. One possibility is that loss, or overexpression of Porin changes mitochondrial/ER contacts at ERMES junctions that in turn affects the localisation of plasma membrane transporters such as Pdr5. Interestingly our data suggest changes in PI3P and PI4P localisation, especially in the absence of porin, in the *cof1-6* strain, with a diffuse signal

throughout the cell. This could indicate the role of porin in the regulation of lipid organisation within the cell. One possibility is that this could occur via lipid maturation at ERMES junctions. Interestingly Psd1 has been seen to have a role in mediating Pdr5 expression (Gulshan *et al*, 2008). Gulshan et al. showed that the overexpression of Psd1 resulted in the expression of Pdr5 dependant of Pdr3. They also showed that deleting *PSD1* prevented Pdr5 expression. Finally, they showed that even when Psd1 was inactive and unable to form PE, Pdr5 levels were increased, showing that PE levels were not the mediating factor in drug resistance.

It has been shown that in mammalian cells, the oxysterol-binding protein (OSBP)-related protein 5 (ORP5) and ORP8 are involved in delivering PS from the ER to the plasma membrane where it is then exchanged for PI4P. This PI4P is delivered to the ER and exchanged for PS. The PI4P is then localised to the PI4P phosphatase Sac1 where it is degraded(Chung *et al*, 2015)(Galmes *et al*, 2016). In yeast cells this process has been seen to be conserved, with the ORP homologues Osh proteins, Osh6 and Osh7 respectively(Stefan *et al*, 2011)(Chung *et al*, 2015). The mis-localisation of PI4P in the *cof1-6 Δpor1* cells could show a problem with PI4P/PS transport linked to PS synthesis.

It is possible that porin has a role in the formation of these ERMES junctions, so when it is deleted in the *cof1-6* background, these junctions cannot be formed, resulting in the cell unable to make PC. This would explain the mis-localisation of the PI4P signal in the *cof1-6 Δpor1* mutant. Porin may also interact with Psd1 resulting in overexpression. Further study is required to determine how these different elements all interact.

4.7 Cofilin and Porin act to control Pdr5 levels and its localisation to the plasma membrane

In light of our findings we propose a model by which cofilin and porin act together to regulate drug resistance. Cofilin has been previously seen to upregulate ABC transporters seen in drug resistance, including Pdr5, through interaction with the mitochondria. We hypothesise that changes in the membrane charge such results changes cofilin/membrane interaction that signals the nucleus to activate the transcription factor Pdr1 which controls the expression of a battery of plasma membrane transporters. We speculate that porin has a role in the organisation of the lipid membrane, potentially through the formation of ERMES junctions, and as such, is involved in the localisation of Pdr5. In the absence of porin, this localisation of Pdr5 cannot occur and remains in some sort of lipid compartment. As a result, whilst there is high levels in the *cof1-6* mutant *Δpor1* deletion, it does not express the drug resistant phenotype.
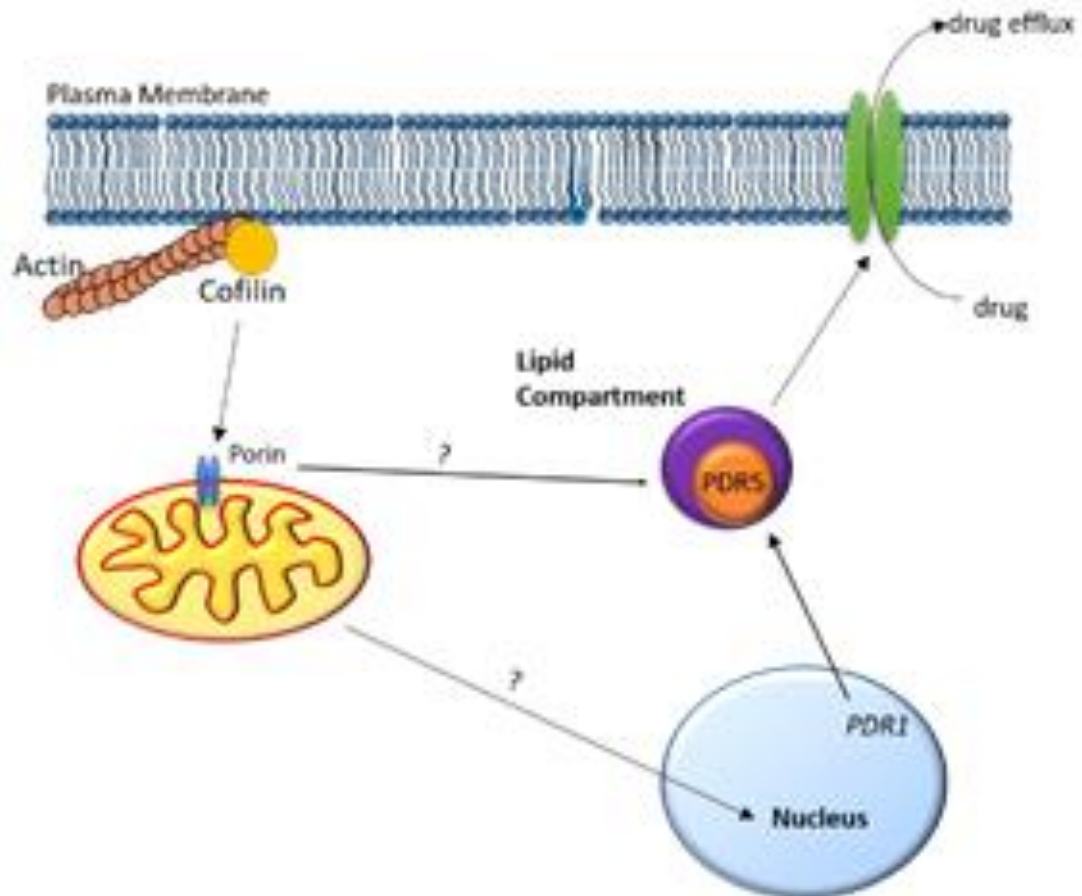
Figure 4.1 Model showing the proposed involvement of Porin and Cofilin in Pdr5 expression. We propose that
Pdr5 localisation to the lipid membrane is dependent on Porin. In the absence of Porin, Pdr5 remains contained in
an unknown lipid compartment and therefore in an inactivated state.

4.8 Future Work

- Due to time restraints, we were not able to determine the localisation of the Pdr5
  within the mutant strains. A GFP-tagged Pdr5 construct has been transformed into
  the wild-type, *cof1-6* and the porin deletions for use in microscopy which will be
  done in the future. Our hypothesis is that in *cof1-6 Δpor1* strain, the Pdr5 will not
  be localised to the cell wall whereas in the wild-type and *cof1-6* strains, the Pdr5
  should be targeted at this membrane. If this hypothesis proves correct, further work
  will be required to determine the compartment that Pdr5 is contained in.

- To determine the involvement of ERMES junctions, knockout the genes *MDM10*, *MDM34*, *MMM1*, *GEM1* in the *cof1-6* background to see if the same phenotype is observed as seen in the *cof1-6 Δpor1* strain. These genes are subunits of the ERMES complex so if porin is responsible for the formation, deleting them should remove the resistant phenotype seen, in the same way it is removed in the *cof1-6 Δpor1* strain.

- Levels of Psd1 within the mutant *cof1-6* needs to be determined as does its localisation within the mutant cell.

- Deletion of other pleiotropic drug resistance genes upregulated in the *cof1-6* mutant as seen in the paper by Kotiadis et al.

- Characterisation of all lipid concentrations at the plasma membrane needs to be done to see if the levels of PS, PE and PC vary in the *cof1-6* mutant in comparison to the wild-type.

- The porin overexpression strains should be tested by western blot to see how elevated porin effects the expression Pdr5. We hypothesise that the levels of Pdr5 will be reduced in the overexpression strain in comparison to the standard strains.

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