Identifying novel interaction partners of the exocyst member Sec8 in the fission yeast *Schizosaccharomyces pombe*

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Abstract

The exocyst is a complex of proteins classically known for its role in tethering secretory vesicles during exocytosis, but it has since been shown to participate in a whole host of other cellular processes. Several human patients have been discovered in whom mutations in the exocyst appear to cause disease, but the underlying mechanisms are still poorly understood. This highlights an urgent need to better characterise these proteins. The exocyst complex is conserved in the fission yeast *Schizosaccharomyces pombe,* which is an ideal model eukaryote in which to examine conserved biological mechanisms. In this study, I used GFP-Trap combined with qualitative proteomics to reassess the role of exocyst component Sec8. Unexpectedly, this uncovered putative novel associations with both the nuclear envelope and the mitochondrial envelope. Using live-cell imaging, I further showed that cells expressing mutated Sec8 displayed altered mitochondrial morphology and a significant reduction in mitochondrial fusion. This indicates that Sec8 is somehow involved in mitochondrial distribution and dynamics, a role which has not been previously described. This may provide further insight into the role of the exocyst in both normal and pathological conditions.

Keywords: GFP-Trap, Exocyst, mitochondria

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Abbreviations used in this study

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CyPIN	Cytosolic PSD-95 Interacting Protein
DSBs	Double-stranded Breaks
Dvl	Dishevelled
EM	Electron Microscopy
ER	Endoplasmic Reticulum
FDR	False Discovery Rate
FOXO	Active form of Forkhead, O class
GluRs	Ionotropic glutamate receptors
JNK	c-Jun N-terminal Kinase
MAGUK	Membrane-associated Guanylate Kinase
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby Canine Kidney Epithelial (Cells)
MKS	Meckel-Gruber Syndrome
NHEJ	Non-homologous End Joining
NPC	Nuclear Pore Complex
NMDAR	N-methyl-D-aspartate receptor
NSF	N-ethylmaleimide sensitive fusion
PCP	Planar Cell Polarity
PKA	Protein Kinase A
Ral	Ras-like
SNARE	Soluble NSF Attachment Protein Receptor
TIRFM	Total Internal Reflection Fluorescence Microscopy
TJ	Tight Junction

A multitude of cellular roles for the exocyst complex

1.1 Exocytosis

1.1.1 Functions of Exocytosis

Exocytosis describes the process whereby secretory vesicles containing cargo are trafficked to the cell periphery, and fuse with the plasma membrane. Exocytosis is utilised for secretion and also participates in altering membrane composition.

Secretion describes the transport of certain cellular macromolecules to the cell exterior, in order to perform a function. Examples of secreted products include proteins required to form the extracellular matrix, and signalling molecules.

There are two main types of secretion: *constitutive* and *regulated* (Reviewed in Burgess *et al.*, 1987). Constitutive secretion occurs when cargo need to be released continuously. On production, secretory vesicles are immediately transported to the cell surface for release. An example is the release of matrix metalloproteinases to break down the extracellular matrix during growth, organogenesis and tissue turnover (Birkedal-Hansen *et al.*, 1993). Regulated secretion occurs when the release of cargo needs to be restricted to specific times and/or amounts. Secretory vesicles wait inside the cell until they receive a signal to fuse with the membrane and release their contents. Examples of this include neurosecretory cells which secrete endorphins and neurotransmitters (De Camilli *et al.*, 1990), and pancreatic cells which secrete insulin and glucagon (Burgess *et al.*, 1987). The exocytic signal depends on the cell type; for neurons it is a rise in intracellular Ca^{2+} (Lim *et al.*, 1990), for pancreatic β-cells it is increased blood glucose levels (Rutter, 2004).

On fusion, the vesicle membrane is incorporated into the plasma membrane. In this way, cells also use exocytosis to both expand the plasma membrane, and insert proteins into it. Vesicles are constitutively secreted during normal cell growth to provide the lipids required to expand the plasma membrane, and they are incorporated into the membrane by exocytosis (Zakharenko *et al.*, 1998). Any components embedded in the secretory vesicle membrane can therefore

be incorporated also. By this process, if cells need to alter the composition of the plasma membrane they can secrete vesicles containing particular lipids, membrane proteins and receptors, and insert them into the membrane by exocytosis. As a result, exocytosis is vital for processes such as cell polarity and cytokinesis (See Section 1.3.3).

1.1.2 Stages of Exocytosis

Secretory vesicles are transported to the cell periphery either passively by diffusion (Bendez *et al.*, 2012), or actively by motor proteins such as kinesins, dyneins and myosins, which transport the vesicle along cytoskeletal filaments to its destination. In yeast, vesicles are typically trafficked along actin cables (Pruyne *et al.*, 1998; Schott *et al.*, 1999; Evangelista *et al.*, 2002; Reviewed in Moseley *et al.*, 2006) or by passive diffusion (Bendez *et al.*, 2012), whilst in mammalian cells transport is primarily microtubule-based (Wacker *et al.*, 1997)

Once in the vicinity of the plasma membrane, the vesicle must then be 'tethered' and/or 'docked'. However, the distinction between these terms is not always clear. One hypothesis (eg. Toonen *et al.*, 2006) is that this step of exocytosis involves firstly an interaction between the vesicle and the accepting membrane (docking) which is promoted by mediating proteins. Other proteins then create a physical link (tether). Conversely, the more widely held view is that 'tethering' is in fact the initial step prior to docking, and that the difference between these steps is the distance between the vesicle and the plasma membrane.

The docking stage can be defined morphologically (Hammarlund *et al.*, 2007). Some authors refer to a vesicle as 'docked' when it is within approximately 30 nm of the membrane (Broadie *et al.*, 1995; O'Connor *et al.*, 1997). This accounts for the fact that fixation methods can cause changes in membrane structure, possibly dislodging docked vesicles. However, this can overlook variation between vesicles close to the membrane, so some authors alternatively define 'docked' vesicles as those which have a visible contact patch with the membrane (Harris *et al.*, 1995; Schikorski *et al.*, 2001; Xu-Friedman *et al.*, 2001). The presence of a strong association between vesicles and the plasma membrane has been demonstrated by two methods. Firstly, biochemical methods have shown that a large fraction of vesicles that appear docked remain associated with fragments of the plasma membrane even after homogenisation, washing and sedimentation

(Martin *et al.*, 1997). This demonstrates a strong physical connection. Secondly, analysis of the mobility of docked vesicles using Total Internal Reflection Fluorescence Microscopy (TIRFM) shows that docked vesicles display very restricted movements compared to undocked cytoplasmic vesicles (Oheim *et al.*, 1998; Nofal *et al.*, 2007), which also suggests a physical tether. As the boundary between tethering and docking is not well defined or understood on a molecular level, this often results in confusion and/or incorrect use of the terms interchangeably.

Another key step during vesicle docking/tethering is the interaction between Soluble N-ethylmaleimide sensitive fusion Attachment Protein Receptor (SNARE) proteins. These are present on both the surface of the arriving vesicle, and in clusters on the plasma membrane (Reviewed in Lang, 2007). They were therefore originally named vSNAREs (for vesicle) and tSNAREs (for target membrane) respectively. When in close enough proximity, the SNARE proteins on the vesicle and plasma membrane bind to each other, forming what are known as *trans*-SNARE complexes (Söllner *et al.*, 1993a,b). The formation of these complexes helps to bring the vesicle in close proximity to the plasma membrane for fusion to then occur. Membrane fusion is energetically costly due to the need to overcome the repulsive forces between the two charged lipid bilayers. Conformational changes in the SNARE complexes generate the force needed to overcome this repulsion, and bring the two membranes together (reviewed in Jahn *et al.*, 2006; Gao *et al.*, 2012).

During regulated secretion in mammalian cells, evidence suggests that initially only a small percentage of docked vesicles are actually capable of responding to the exocytic signal and fusing with the membrane (Rosenmund *et al.*, 1996; Xu *et al.*, 1998; Klenchin *et al.*, 2000). The majority must therefore undergo 'priming' to become fusion competent. Priming can involve several different ATP-dependent processes including lipid synthesis, protein phosphorylation and rearrangement of SNARE complexes (Reviewed in Klenchin *et al.*, 2000). However, the stage at which priming occurs is currently debated, and it is not clear if priming always occurs.

1.2 The Exocyst Complex

During the tethering/docking step of exocytosis, several mediating proteins are required to help target the secretory vesicle, secure its association with the membrane and ensure subsequent vesicle fusion. A heteromeric complex called the exocyst is one such example. It is formed of eight different protein subunits named Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 in yeast, and Exoc1 to Exoc8 respectively in mammalian cells.

1.2.1 Discovery

The constituents of the exocyst were originally discovered in the budding yeast *Saccharomyces cerevisiae.* Six of the components were first found during a genetics screen for secretory mutants, which identified 23 gene products required for exocytosis (Novick *et al.*, 1980). These proteins were named using the prefix 'sec-' for secretion, which gave rise to Sec1 - Sec23. Subsequent research showed that ten of these proteins are required specifically during protein transport from the Golgi-apparatus to the plasma membrane: Sec1/2/3/4/5/6/8/9/10/15 (Novick *et al.*, 1981).

Sec8 and Sec15 were the first to be identified as being part of a multiprotein complex, initially estimated to be between 1000-2000 kDa in size (Bowser *et al.*, 1991, 1992). It was then shown that the complex also contained Sec6 (along with five unidentified proteins), and was thus referred to as the 'Sec6/8/15 complex'. Its molecular weight was also more accurately estimated as 743 kDa (TerBush *et al.*, 1995). The same study also showed that mutation of Sec3, Sec5 or Sec10 disrupted the integrity of the complex, thus implicating their association. They were subsequently proved to be members of the complex using purification and peptide microsequencing, and this also unearthed the seventh member of the complex – Exo70 (TerBush *et al.*, 1996). It was in this study that the term 'exocyst' was first coined to describe the complex.

Efforts to discover the mammalian components of the exocyst continued in parallel to those in yeast. The last member of the exocyst - Exo84 - was discovered during purification of the mammalian exocyst complex (Kee *et al.*, 1997), and its yeast counterpart was later identified by co-immunoprecipitation (Guo *et al.*, 1999a).

1.2.2 Role in Exocytosis

The exocyst's classical role is thought to be in tethering secretory vesicles in close enough proximity to the plasma membrane to allow SNARE complexes to form, as outlined in Section 1.1.2, and/or in regulating SNARE complex assembly. However, this is a rather simplistic summary as the relationship between the exocyst complex and SNAREs is also not fully understood, and the tethering function is yet to be experimentally demonstrated. Studies in yeast have shown that Sec6 binds to SNARE complexes once they are assembled, which suggests that it promotes complex assembly, likely by a mechanism similar to other tethering complexes (Dubuke *et al.*, 2015). Further evidence that the exocyst interacts with SNARE regulating proteins and possibly helps to localise them adds a further level of complexity (Carr *et al.*, 1999; Morgera *et al.*, 2012). It is therefore clear that the exocyst is involved in not just simply the tethering of vesicles, but also in mediating SNARE complex formation.

The mechanism by which the exocyst complex assembles is also currently debated. Many studies in the past proposed that only Sec3 and Exo70 interact with the plasma membrane. The original model was therefore that these two components acted as landmarks for the rest of the complex, and thus defined the sites of exocytosis (Finger *et al.*, 1998; Boyd *et al.*, 2004; He *et al.*, 2007; Zhang *et al.*, 2008). However this model has been called into question, most notably by evidence that the high affinity association of the GFP construct used by Finger *et al.* with the plasma membrane was not replicated by studies of the native Sec3 protein (Roumanie *et al.*, 2005). This therefore suggests that the strong plasma membrane localisation original observed was likely an artefact of the GFP-tag. In addition, the observation of other exocyst components localising to the plasma membrane (Tsuboi *et al.*, 2005; Songer *et al.*, 2009) and of Sec3 and Exo70 localising to the cytoplasm (Cubelos *et al.*, 2005; Xu *et al.*, 2005) further refute this model. So just as the structure of the assembled complex remains elusive, the spatial and temporal dynamics of its assembly are yet to be confirmed.

1.2.3 Biochemical & Structural Studies

To date, the full-length crystal structures of Exo70 and Sec10, and partial structures of Sec3, Sec6, Sec5, Sec15, Exo84 have been resolved in various organisms (Fukai *et al.*, 2003; Dong *et al.*, 2005; Jin *et al.*, 2005; Wu *et al.*, 2005; Sivaram *et al.*, 2006; Moore *et al.*, 2007; Baek *et al.*, 2010; Yamashita *et al.*, 2010; Zhang *et al.*, 2015; Chen *et al.*, 2017). Unfortunately progress in this area remains slow, primarily due to the lack of soluble protein available for analysis (Croteau *et al.*, 2009). Studies have shown that all of the exocyst components have related structures (Croteau *et al.*, 2009) that share the same bundle topology, but each have unique helical packing angles, residue indels, and electrostatic and hydrophobic surface patterns (Sivaram *et al.*, 2006).

As the full-length structure of all eight components has not been solved in any one organism, the subunit organisation of the complete holo-complex remains unknown. Some studies have given an indication of how the fully-assembled complex may appear. One study used a quick-freeze electron microscopy (EM) technique to look at the mammalian exocyst complex from brain cells, and found that the assembled complex resembles a "T" or a "Y" (Hsu *et al.*, 1998, see Fig. 9). Other studies indicated that at least four of the exocyst components have extended rod-like structures, and that the assembled yeast exocyst complex has an elongated appearance, consistent with rods being packed together (Dong *et al.*, 2005; Munson *et al.*, 2006; Heider *et al.*, 2015). A recent study utilised live-cell imaging and fluorescence microscopy to reconstitute the 3D structure of the complex *in vivo*, and found that the exocyst has an "open hand conformation made of rod-shaped subunits that are interlaced in the core" (Picco *et al.*, 2017).

1.2.4 Evolutionary Conservation

As exocytosis is utilised by many organisms, ranging from yeast to higher plants (Elias *et al.*, 2003) to metazoans (Guo *et al.*, 1997), it is not surprising that parts of the exocytic machinery are also highly conserved; the exocyst complex is one such example (Koumandou *et al.*, 2007).

The individual subunits vary in their conservation across eukaryotes (See Fig. 4, Koumandou *et al.*, 2007). Many Opisthokonta, as well several Amoebozoa and

Archaeplastida possess eight subunits, whereas studied Excavata and Chromalveolata only possess between one and six subunits. It has even been found that trypanosomes (part of the Kinetoplastida) possess a ninth exocyst subunit in addition to the other eight (Boehm *et al.*, 2017). This variation in conservation, combined with the fact that the subunits share the same bundle topology (discussed in the previous section), may suggest that one exocyst component could perform different roles in different organisms, or even that there may be redundancy among components. This would also explain why not all of the subunits are essential in all organisms. It is thought that the different components evolved by multiple gene duplication and divergence events (Croteau *et al.*, 2009) to enable more functions in more complex multicellular organisms. This perhaps explains why some organisms can function with say only three of the components, as they can sufficiently perform the roles of the complex required in that organism. Alternatively, it is possible that the other subunits are in fact present, but just cannot be found computationally at this stage.

In yeast and metazoans, the exocyst components are typically each encoded by one gene. However, more complex multicellular organisms usually have a large number of isoforms. Taking Exo70 as the most extreme example, in *S. cerevisiae* it is encoded by one gene which produces one isoform (taken from NCBI Gene entry). In humans, Exo70 (Exoc7) is encoded by one gene, which is post-transcriptionally spliced to produce seven different mRNA isoforms (taken from NCBI Gene entry). In plants however, exocyst subunits are often encoded by duplicated genes or multiple different paralogs (Cvrčková *et al.*, 2012). In *Arabidopsis thaliana* for example, Exo70 is encoded by 23 different paralogs, and splicing variants have been proposed for most *Arabidopsis* subunits on top of this (Lamesch *et al.*, 2012). This further supports that different mechanisms of functional specialisation, and indeed levels of redundancy, have evolved among different species.

Since its discovery, research has shown that the exocyst is in fact fundamental to a whole host of cellular processes beyond just exocytosis. This is reflected by the range of phenotypes caused by its perturbation, which are apparent in both unicellular and multicellular organisms. In fungi for example, the majority of the genes encoding exocyst components are essential for viability. Exocyst mutants

are typically defective in polarity determination, branching, and in some cases plant pathogenicity or cytokinesis (reviewed in Martin-Urdiroz *et al.*, 2016). In mammals, the exocyst components are similarly essential, with existing knockouts in mice showing early embryonic lethality (Friedrich *et al.*, 1997; Mizuno *et al.*, 2015).

Conditional or heterogeneous knockouts display a range of developmental defects (reviewed in Martin-Urdiroz *et al.*, 2016), so it is not surprising that mutations in human exocyst components have also been linked to disease. Current implicated diseases/syndromes include ciliopathies (See Section 1.1.8), cancers (Camonis *et al.*, 2005; Armaghany *et al.*, 2012), intellectual development and electrophysical stability (Fruhmesser *et al.*, 2013), as well as facial defects and craniofacial abnormalities (Wen *et al.*, 2013).

1.3 Cellular Roles of Sec8

Despite being one of the earliest members of the exocyst to be identified, Sec8 is the only component currently with no crystal structure characterisation (see Section 1.2.3). Nonetheless, studies have demonstrated its involvement in a host of cellular processes, including several beyond the exocyst's canonical role in vesicle trafficking and tethering. These are summarised in Figure 1, and discussed below. The molecular mechanism of Sec8's involvement is not well understood in many of these cases. In addition, most of these studies only examine a limited number of exocyst components, meaning that whilst they demonstrate novel roles, these cannot be assigned as specific to Sec8 itself. It is highly likely that the rest of the complex participates in these functions, and that the whole complex is disrupted by perturbation of Sec8. However, for the purposes of this thesis, I will summarise the literature specifically pertaining to Sec8.

1.3.1 Discovery and Conservation

As discussed in Section 1.2.1, Sec8 was originally discovered in a screen for secretion mutants in *S. cerevisiae* (Novick *et al.*, 1980), and it was one of the earliest to be identified as a member of the exocyst complex (Bowser *et al.*, 1992). Mammalian Sec8 (Exoc4) was first identified in the rat brain (Ting *et al.*, 1995). It was part of a soluble 17S particle, thought to assist in the regulation of vesicle docking and fusion in the brain. This particle showed homology to the putative exocyst complex that had been described in yeast at the time, and was subsequently named the 'sec6/8 complex' (Hsu *et al.*, 1996). This complex localised to the plasma membrane in nerve terminals, and co-immunoprecipitated with the tSNARE syntaxin. These results gave the first implications that the mammalian exocyst complex had a role in vesicle docking and fusion. Since its initial discovery, studies have demonstrated its role in multiple cellular processes, even before the discovery of the other exocyst components.

Sec8 is highly conserved among eukaryotes; homologs or orthologs can be found from unicellular yeast to humans. The NCBI entry for human *sec8* lists 193 bilaterian organisms alone with an ortholog. Table 1 lists examples from multiple taxonomic groups. This conservation leads it to be often referred to as

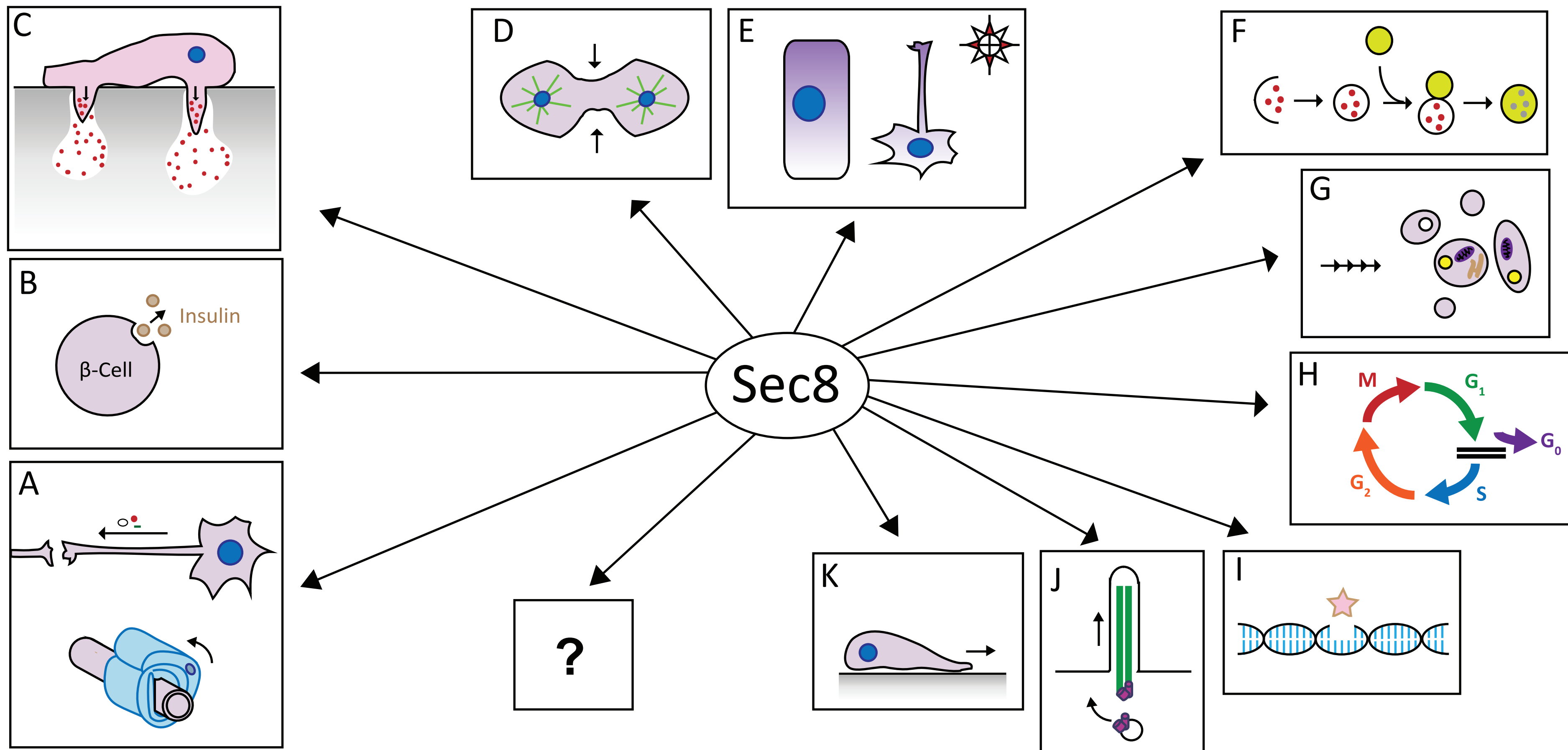


Figure 1: Sec8 has described roles in a wide variety of cellular processes, including many beyond exocytosis
The leftmost panels represent cellular processes in which Sec8 has shown to be involved in secretion; (A) Neuronal secretion and myelination (B) Insulin secretion (C) Matrix degradation. Either directly or indirectly through its role in exocytosis, Sec8 can also influence (D) Cytokinesis and (E) Polarity. The rightmost panels represent emerging non-canonical roles of Sec8, where it is thought to either regulate protein expression or protein localisation, or coordinate protein/enzymatic interactions: (F) Autophagy (G) Apoptotic Signalling (H) Cell cycle control (I) DNA Repair (J) Ciliogenesis and (K) Cell migration. (See text for details). Sec8 likely performs many more roles which are as yet undiscovered.

Table 1: Sec8 homologues and orthologues retrieved by the NCBI HomoloGene tool for three taxonomic groups.[1]

	Gene	Organism	Protein Accession	Protein Identity (%)
Bilateria	***exoc4***	***H. sapiens***	**NP_068579.3**	-
	exoc4	*P. troglodytes*	XP_001139207.1	99.9
	exoc4	*M. mulatta*	XP_001101640.2	98.8
	exoc4	*C. lupus*	XP_539369.	97.0
	exoc4	*B. taurus*	NP_001095677.1	96.4
	exoc4	*M. musculus*	NP_033174.2	95.9
	exoc4	*R. norvegicus*	NP_446327.1	95.4
	exoc4	*G. gallus*	NP_001186249.1	89.9
	exoc4	*X. tropicalis*	NP_001025305.1	87.3
	exoc4	*D. rerio*	NP_730996.2	80.9
	sec8	*D. melanogaster*	XP_321399.5	38.1
	sec-8	*C. elegans*	XP_002931915.2	35.3
Ascomycota	***sec8***	***S. cerevisiae***	**NP_015380.1**	-
	klla0e11419g	*K. lactis*	XP_454464.1	38.0
	agos_adl317c	*E. gossypii*	NP_983779.2	40.3
	sec8	*S. pombe*	NP_587846.2	22.5
	mgg_03985	*M. oryzae*	XP_003719868.1	25.1
	ncu04190	*N. crassa*	XP_961150.2	23.6
Mesangio-spermae	***sec8***	***A. thaliana***	**NP_566372.1**	-
	os08g0318500	*O. sativa*	NP_001061521.1	63.7

a ‘core’ exocyst component, as it is one of the six members with a wide taxon distribution (Koumandou *et al.*, 2007).

Since the exocyst’s canonical role in vesicle tethering during the late secretory pathway was described, studies have gone on to demonstrate that Sec8 is required to perform this function during both regulated and constitutive

[1] Protein identities were calculated by the HomoloGene system using blastp. Each protein is compared against the first gene/organism in its taxonomic group (highlighted in bold).

secretion, in a wide range of cellular contexts. Some examples of this will be discussed below.

1.3.2 Sec8 is Directly Involved in the Secretion of Multiple Proteins

Neuronal Secretion

Due to its discovery in the brain, much of the original research into Sec8's function in mammalian cells was conducted in neurons. It has been shown to be required for multiple types of secretion in neuronal cells.

The exocyst complex localises at regions of neuronal growth (Hazuka *et al.*, 1999) and is involved in both vesicle targeting (Vega *et al.*, 2001) and membrane trafficking (Teodoro *et al.*, 2013) at synapses. It has also been shown to assist RalA - a GTPase and upstream regulator of the exocyst (Moskalenko *et al.*, 2002) – in the modulation of synaptic strength by influencing the pool of primed synaptic vesicles (Polzin *et al.*, 2002). Sec8 was shown to directly interact with the effector domain of active RalA, and when this interaction is inhibited, neurosecretion is suppressed. However, the consensus from more recent studies seems to be that the exocyst is involved in the proper function of synapses, but not directly in neurotransmitter release (Murthy *et al.*, 2003) .

Trafficking by the exocyst has also been shown to help localise proteins at synapses that are required for 'synaptic plasticity' - this describes the ability of neurons to alter their response to a certain stimulus over time, which is required for processes such as learning to occur in higher organisms. PSD-95 is a synaptic scaffolding protein of the MAGUK family, which plays a key role in correctly localising proteins at excitatory synapses, and subsequently synaptic plasticity. PSD-95 has been shown to physically interact with Sec8 (Riefler *et al.*, 2003), and CyPIN - a PSD-95 interactor, thought to regulate dendrite morphology (Akum *et al.*, 2004) - can competitively disrupt this interaction with Sec8. As to the importance of this interaction, the authors speculate that as PSD-95 associates with vesicles and microtubules as it localises to the synapse (El-Husseini *et al.*, 2000), it likely associates with Sec8 during this trafficking, and CyPIN prevents targeting to the synapse by blocking this interaction. However, it is still not understood exactly how the exocyst helps to traffic/target PSD-95. Perhaps Sec8 is on the surface of vesicles travelling to the synapse, and binds to PSD-95 thus transporting it.

The exocyst is also involved in the trafficking of receptors. Ionotropic glutamate receptors (GluRs) are a class of receptors that localise to excitatory synapses and play a key role in regulating excitatory neurotransmission (Dingledine *et al.*, 1999). NMDAR is one such receptor. SAP102 is the major MAGUK protein expressed in neurons (Sans *et al.*, 2000; Standley *et al.*, 2000), and is speculated to interact with NMDARs during transport and function (Sans *et al.*, 2003). Sec8 was shown to physically interact with Sap102, and together they complex with NMDARs in the ER (Sans *et al.*, 2003). In addition, this Sec8-Sap102 complex is required for NMDAR to be delivered to the cell surface in neurons. Sec8 has also been shown to assist in both the delivery of AMPAR, another GluR, to the synapse (Gerges *et al.*, 2006). Together this demonstrates an essential role for the exocyst in delivering receptors to the excitatory synapse. Again, it is not fully elucidated how the exocyst performs this transport, but the physical interaction indicates it plays a direct role.

Sec8 has also been implicated in myelination, a process whereby oligodendrocytes secrete layers of myelin that surround neuronal axons, thus creating an insulating layer (Pfeiffer *et al.*, 1993; Barres *et al.*, 1999). Myelination serves to protect the axon, facilitate signalling between oligodendrocytes and neurons, and increase the efficiency of signal transduction. Sec8 physically interacts with OSP and CASK, two proteins that are shown to be essential for myelin synthesis (Anitei *et al.*, 2006). Sec8 clearly plays a central role, as its inhibition reduces myelin production and its overexpression promotes myelin-like membrane formation. It is thought that Sec8 forms a complex with OSP and CASK that regulates the transport of myelin proteins to sites of membrane growth by influencing vesicle recruitment, but the exact mechanism of Sec8's involvement remains to be confirmed. Sec8 has also been implicated in the regulation of membrane homeostasis in myelination through an interaction with Dlg1 – a scaffold protein involved in polarised vesicle trafficking and membrane addition (Lee *et al.*, 2003; Gorczyca *et al.*, 2007). It is thought that Dlg1 regulates the balance between Sec8-mediated membrane addition and Mtmr2-mediated membrane remodelling (Bolis *et al.*, 2009). This is an example of Sec8's role in exocytosis being fine-tuned to in order to execute a specific cellular process.

Insulin Secretion

As mentioned in Section 1.1.1, secretion of insulin by pancreatic β-cells is a prime example of regulated secretion. Large insulin-containing vesicles remain in the cytoplasm until a rise in external blood glucose concentration causes changes in intracellular ATP and Ca^{2+} concentrations, which in turn stimulates exocytosis (for details see Rutter, 2004). There are thought to be 'reserve', 'intermediate' and 'primed/docked' pools of vesicles, which are both morphologically and biochemically distinct (Rorsman, 1997; also discussed in Section 1.1.7; Bratanova-Tochkova *et al.*, 2002). This results in a bi-phasic release of insulin – the primed pre-docked vesicles are exocytosed immediately upon stimulation, thus forming the first phase, and the reserve/intermediate pools are recruited and then exocytosed, this delay forming the second phase (Barg *et al.*, 2002). The exocyst complex is required to dock insulin vesicles at the plasma membrane prior to exocytosis. Perturbation of Sec8 inhibits this process (Tsuboi *et al.*, 2005; Xie *et al.*, 2013), and thus it has been speculated that exocyst defects could potentially have links to diabetes. Sec8 has also been shown to directly bind to MyRIP, a synaptotagmin-like protein that functions as a PKA-anchoring protein. Sec8 and MyRIP co-localise in the perinuclear region in INS-1 cells (Goehring *et al.*, 2007). It is therefore suggested that together, a complex of MyRIP, the exocyst and the PKA holoenzyme regulate the mobilisation and membrane recruitment of insulin vesicles.

In addition, Sec8 has been shown to localise at the plasma membrane of MIN6 β-cells, whereas Sec6 and Sec10 were shown to localise on the vesicles and in the cytoplasm respectively (Tsuboi *et al.*, 2005). This is contrary to the proposed model discussed in Section 1.2.2 where Sec3 and Exo70 are the exocyst components that associate with the plasma membrane. It is therefore suggested that Sec8 may be recruited to the membrane (along with other exocyst members), and Sec6 is delivered on the arriving insulin vesicles.

Extracellular Matrix Degradation

During the maturation of cancer tumours, malignant cells can often spread to other parts of the body in a process known as metastasis. These invasive cells can escape the tissue surrounding the primary tumour, penetrate the walls of the blood vessels and travel to a new site, where they invade secondary tissues (Reviewed in Ha *et al.*, 2013; Alizadeh *et al.*, 2014). Invasive cells must be able

to degrade the extracellular matrix of tissues and epithelial layers during this process in order to migrate through the three-dimensional environment (Mignatti *et al.*, 1993; Stetler-Stevenson *et al.*, 1993; Ray *et al.*, 1994; Reviewed in Brown *et al.*, 2015). They therefore form specialised actin-rich membrane protrusions called invadopodia, which help to degrade the extracellular matrix, amongst other functions (Weaver, 2006; Murphy *et al.*, 2011).

Sec8 has been shown to be required for the secretion of matrix-degrading enzymes from invadopodia. Upon reduction or mutation of Sec8, matrix degradation is drastically inhibited (Sakurai-Yageta *et al.*, 2008), and the secretion of multiple different matrix-degrading enzymes is reduced (Yamamoto *et al.*, 2013). This indicates that the exocyst likely plays an essential role in the targeting and trafficking of these enzymes during invasion. In addition, Sec8 interacts with IQGAP1 (Sakurai-Yageta *et al.*, 2008), a master polarity protein whose expression promotes matrix degradation (Nabeshima *et al.*, 2002; Mataraza *et al.*, 2003; Sakurai-Yageta *et al.*, 2008). When IQGAP1 cannot bind to the exocyst, this enhancement of matrix degradation is lost, suggesting that Sec8 may also be involved in more complex regulation of invadopodial processes.

1.3.3 Through Its Role in Exocytosis, Sec8 Controls Cell Division and Polarity

The exocyst's role in targeting membrane and vesicle trafficking has also been shown to function in other processes where cellular traffic needs to be directed to specific sites. Examples include both cell division, and the induction and maintenance of polarity. Establishing polarity is essential for the functions of cells in a variety of contexts, on both the cellular and tissue level. Examples range from governing the location of cells in the developing embryo, to the formation of neural networks and the organisation of epithelial cell layers. Sec8 has been shown to participate in polarity induction and maintenance in multiple cell types.

Cytokinesis

There are several lines of evidence which demonstrate that the exocyst is involved in cytokinesis – the culminating step of the cell cycle which sees the cytoplasm physically divided, and the two daughter cells fully separated. Exocyst components interact with several trafficking proteins that are required for

cytokinesis, including Rab11 (Zhang *et al.*, 2004; Neto *et al.*, 2013), Arf6 (Prigent *et al.*, 2003) and the Ral GTPases (Chen *et al.*, 2006. Other examples discussed throughout Section 1.3).

Sec8 shows a high degree of colocalisation with Rab11 (Rivera-Molina *et al.*, 2013), and depletion of Rab11 results in a decrease in Sec8 levels. During mitosis, mammalian Sec8 associates with the mitotic spindle and spindle poles, and during cytokinesis it associates with the central spindle and the midbody (Chen *et al.*, 2006). When at the midbody, Sec8 is anchored within a ring-like structure by centriolin (Gromley *et al.*, 2005). Mutation of Sec8 results in delayed abscission, and an increased proportion of binucleate cells (Gromley *et al.*, 2005; Neto *et al.*, 2013), indicating it is required for the final stages of cytokinesis. Prior to abscission, secretory vesicles are delivered asymmetrically to the midbody (Gromley *et al.*, 2005). It is likely that Sec8 is required for the targeting and/or docking of these vesicles.

Examination of Sec8 in fission yeast supports this deduction. Cytokinesis in such yeast has an additional step in that the division septum (formed of cell wall components) must be broken down. Sec8 localises to the growing tips, and in rings on either side of the contractile ring at the division site (Wang *et al.*, 2002). Mutation of Sec8 causes severe extracellular septation defects, and 'presumptive secretory vesicles' accumulate at either side of the formed septum, which indicates a tethering/docking defect. This is thought to block the secretion of enzymes that degrade the primary septum during cytokinesis, but has not yet been demonstrated. So Sec8 is required for the late stages of cytokinesis, and its role is likely in the tethering and/or docking of secretory vesicles.

Neuronal Polarity

Neurons are a highly specialised cell type. In order to properly transmit signals, maturing neurons must break symmetry and establish polarisation. This enables the formation two cellular compartments – axons and dendrites – which are functionally and structurally distinct (Arimura *et al.*, 2007; Barnes *et al.*, 2008).

The relocation of the PAR/aPKC complex to the developing axon is a key step during neuron polarisation (Y. M. Chen *et al.*, 2006; Arimura *et al.*, 2007). Sec8 physically interacts with both Par-3 (a member of the PAR complex) and aPKC in polarising neurons (Lalli, 2009). The exocyst is a known effector of the Ral

GTPases RalA and RalB (Moskalenko *et al.*, 2002). In the absence of either RalA or Sec8, neurons fail to polarise and Par-3 does not relocate to the developing tip (Lalli, 2009). Together, this demonstrates a RalA-regulated association between the exocyst and the PAR/aPKC complex, which is essential for the latter's relocation during neuron polarisation. The mechanism by which the Sec8 participates in trafficking the complex remains to be confirmed, but this is clearly required to establish neuronal polarity.

Epithelial Polarity

Sec8 also has a described role in polarity induction and maintenance in epithelial cells. When epithelial cells (such as MDCK cells, from kidney tissue) form a monolayer, they adhere to their substrate, adhere to their neighbours, and form cell-cell junctions. This in turn induces apical-basal polarity. In mammalian epithelia, the apical and basolateral regions are separated by tight junctions (TJs), protein complexes which join adjacent cell membranes to form an impermeable barrier and seal the monolayer. An important requirement when establishing polarity is the asymmetric trafficking of vesicles. Basolateral traffic is delivered to the plasma membrane region adjacent to TJs. The exocyst is known to be involved in this process. Par-3, the polarity protein discussed previously in the context of neuronal polarity, is the receptor which targets the exocyst to these sites (Ahmed *et al.*, 2017).

Sec8 is required for trafficking vesicles specifically to the basolateral membrane of MDCK cells (Grindstaff *et al.*, 1998; Lipschutz *et al.*, 2000). In its absence, proteins which are destined for the basolateral membrane are not delivered. Sec8 has also been shown to localise closely with the TJ protein occludin (Charron *et al.*, 2000), indicating an interaction at TJs also.

1.3.4 Emerging Non-conventional Roles of Sec8

Beyond roles in vesicle transport and tethering, the exocyst has been shown to be involved in several key signalling pathways. The only known structural motifs identified within Sec8 are a 'sec8 specific domain' of unknown function, and a PDZ-binding domain identified in the rat sequence (Sans *et al.*, 2003). PDZ domains are commonly found in cellular signalling proteins (Reviewed in Jeleń *et al.*, 2003; Lee *et al.*, 2010), and recognise specific sequences in order to help assemble protein complexes. As Sec8 has a binding region for this domain, this

supports that Sec8 likely interacts with proteins in signalling pathways. An increasing amount of literature seems to suggest that Sec8 can regulate protein expression, and that it may function as a physical platform to coordinate protein and enzymatic interactions. The evidence for such roles, described in different cellular contexts, will be discussed in this section.

Macroautophagy

Macroautophagy is the process by which cytoplasmic components are targeted for degradation. They are packaged in a double-membrane vesicle called an autophagosome, which then fuses with the lysosome, allowing breakdown of the contents by the lysosomal acid hydrolases (For Reviews, see Wong *et al.*, 2011; Feng *et al.*, 2014). The exocyst has been shown to act as a platform for the assembly and activation of different components of the autophagy machinery in mammalian cells; RalB, a regulator of the exocyst, acts a regulatory switch for autophagosome biogenesis. When activated, RalB binds to Exo84 and induces complexes of catalytically active autophagy proteins to assemble on the exocyst. When RalB is inactive, a suppressed complex of these proteins instead associates with Sec5 (Bodemann *et al.*, 2011; Farré *et al.*, 2011).

Whilst depletion of Sec8 suppresses autophagy (Farré *et al.*, 2011) indicating it too is essential, it would seem that its role is separate from that described above for Exo84/Sec5. Sec8 associates with a complex of Atg12 and Atg5, which is part of the autophagy machinery that specifically is involved in elongation of the isolation membrane. The exact nature of Sec8's contribution is yet to be determined. It is possible that Sec8 may be part of the Sec5 or Exo84 subcomplexes, but was not identified in the study. Perhaps its loss affects the stability and/or localisation of these complexes, or alternatively it may affect the localisation and/or function of Atg12/Atg5 by a mechanism which is not currently characterised.

Apoptosis

Apoptosis, or programmed cell death, is the process by which redundant or damaged cells are destroyed. Its induction and maintenance is tightly controlled by multiple signalling pathways (Reviewed in Ashkenazi *et al.*, 2014). JNK is a component of the mammalian MAPK signalling pathway (Johnson *et al.*, 2002), which when phosphorylated can trigger expression of proapoptotic proteins (Dent

et al., 2003). JIP4 is a JNK-interacting scaffolding protein and has been implicated in the p38 MAPK pathway (Kelkar *et al.*, 2005), which can also induce apoptosis under certain conditions (Zarubin *et al.*, 2005; Hasegawa *et al.*, 2012). JIP4 has been shown to physically interact with Sec8, but not Sec6 (Tanaka *et al.*, 2014). Furthermore, depletion of Sec8 resulted in suppression of apoptosis and increased viability of cells, by supressing the phosphorylation of JNK and p38. This suppression is caused by increased binding to and subsequently reduced activation of MKK4, a protein kinase kinase which phosphorylates JNK and p38 when activated (Kelkar *et al.*, 2005; Wang *et al.*, 2007).

Together this demonstrates an important role for Sec8 in both modifying protein expression, and in regulating the MAPK signalling pathway and subsequently apoptosis. However, the molecular mechanism by which it causes increased binding of JIP4 to MKK4 is not known. The authors speculate that Sec8 may function as a local adaptor protein, and may sequester JIP4 and its associated kinases to specific areas upon pathway activation, thus promoting their activation. This provides initial evidence that Sec8 can act to somehow regulate protein interactions.

Cell Cycle Control

Progression through the cell-cycle is controlled by cyclin-dependent kinases (CDKs), whose activity is in turn regulated positively by cyclin binding and negatively by CDK inhibitors (CKIs). When a cell reaches the G_1/S phase boundary, a checkpoint ensures that the DNA replication does not proceed if the DNA is damaged (Figure 2). Briefly, when a cell normally reaches this checkpoint at the end of G_1 phase, $p21^{Cip1}$ inhibits the activity of cyclins, and the Cdk2/cyclin E complex ensures complete phosphorylation of retinoblastoma protein (Rb). This then then releases E2F factor, which enables expression of S-phase genes. However if DNA damage has occurred, $p21^{Cip1}$ instead inhibits the activity of Cdk2, which results in hypophosphorylated Rb, which then binds the E2F factor, preventing expression of S-phase genes and causing the cell cycle to arrest (Cazzalini *et al.*, 2010; Hydbring *et al.*, 2010). This cell cycle arrest at the G_1/S boundary can also be promoted by the expression of FOXO transcription factors (Medema *et al.*, 2000).

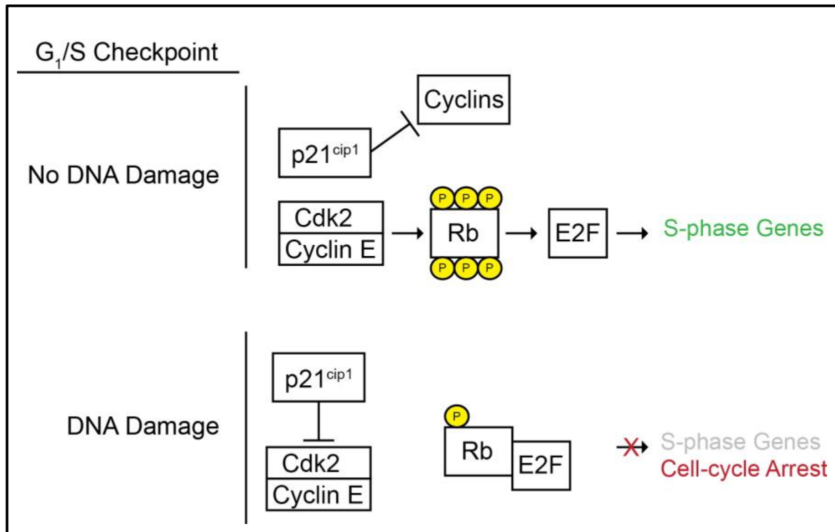


Figure 2: Regulation of p21 activity at the G_1/S Checkpoint. Based on Cazzalini *et al.*, 2010 and Hydbring *et al.*, 2010. See text for details.

It has been shown that Sec8 can regulate FOXO transcription factors, and in turn affect p21 and cell-cycle progression (Tanaka *et al.*, 2014a). This study showed that knockdown of Sec8 in human cell lines resulted in reduced mRNA levels of Mdm2, an E3 ligase which targets FOXO factors for degradation by promoting their ubiquitination (Brenkman *et al.*, 2008; Fu *et al.*, 2009). This influenced the polyubiquitination of FOXO factors, and resulted in the increase of either FOXO1 or FOXO4 mRNA levels (depending on the cell type). This in turn resulted in increased levels of p21 mRNA, which causes reduced cell proliferation, reduced phosphorylated Rb and ultimately cell-cycle arrest. This demonstrates an important role for Sec8 in regulating cell-cycle arrest, but the mechanism by which it controls Mdm2 expression is not known. The authors speculate that Sec8 may be involved in nuclear export of mRNA or may influence other related signalling pathways, but this would be quite a contrast to the exocyst's currently described cellular functions.

DNA Repair

Several different types of DNA damage can stimulate the p53 tumour suppressor network (Lakin *et al.*, 1999), which as its name suggests acts to suppress tumorigenesis and promote genomic stability, amongst other roles. p53 is a transcription factor, which is phosphorylated in response to stress such as DNA damage. This activation results in an increase in both the levels and the DNA-binding affinity of p53 (Reviewed in Lakin *et al.*, 1999). Depending on the severity of the damage, this results in the expression of genes promoting either apoptosis, cell-cycle arrest, DNA repair or differentiation (Hupp *et al.*, 1992; el-Deiry, 1998).

Sec8 has been shown to play a role in modulating the DNA damage response. Depletion of Sec8 results in increased genomic instability, and also promotes the expression of p53 network downstream target genes (Torres *et al.*, 2015). Furthermore, it was shown that Sec8 assists in the repair of double-stranded breaks (DSBs), as its depletion not only sensitised cells to the formation/detection of DSBs, but also reduced the persistence of the ensuing repair response. Sec8 may play a role in repair pathway choice (Torres *et al.*, 2015). It directly interacts with 53BP1, an adaptor protein involved in repair pathway choice, and this interaction is disrupted upon induction of DNA damage. Depletion of Sec8 increased the resolution of DSBs by homologous recombination (HR) as opposed to non-homologous end joining (NHEJ), and altered the levels of histone-modifying proteins AFT2 and RNF20 which promote either the HR or NHEJ repair pathways. The authors therefore suggest that the exocyst complex acts a "spatially constrained platform from which to effectively coordinate localized enzyme/substrate interactions", and that in this context it limits the activity of chromatin remodelling proteins until DNA damage actually occurs. In the absence of Sec8, the DNA repair that occurs has a high level of recombination but low fidelity, which ultimately results in chromosomal aberrations and genomic instability.

Given what is already demonstrated about the cellular functions of the exocyst complex, an intranuclear involvement in DNA repair seems very unlikely, and it is hard to fathom a mechanism for such a role. Yet, it would seem that the evidence for Sec8's roles in both DNA repair and cell-cycle regulation (Section 1.3.4) are closely linked. Sec8 appears to alter the expression of Mdm2, as previously discussed in relation to cell-cycle progression. However, Mdm2 can also inhibit p53, both by competitively binding to its N-terminus thus blocking its interaction with the transcriptional machinery, and by modulating the stability of the p53 protein (both reviewed in Lakin *et al.*, 1999; Alarcon-Vargas, 2002). Also, p53 has been shown to stimulate increased transcription of p21 (Reviewed in Ko *et al.*, 1996; Levine, 1997), which inhibits cell-cycle progression as previously discussed. It is therefore possible that these two roles are connected. Further evidence is required however to elucidate a mechanism by which Sec8 is involved.

Cell Migration

As discussed in Section 1.1.1, Sec8 is involved in the secretion of matrix-degrading enzymes from invadopodia during cancer cell invasion. However, knockdown of Sec8 was also shown to suppress cell migration in a metastatic cell line (Tanaka *et al.*, 2015). Cytokeratin8 is an epithelial-specific intermediate filament, which has been implicated in cell migration and tumour metastasis (Bordeleau *et al.*, 2010; Pan *et al.*, 2013). Two MAPK signalling proteins – ERK and p38 – which are known to be essential for cell migration (Reviwed in Huang *et al.*, 2004), can induce the phosphorylation and reorganisation of cytokeratin8, thus promoting cell migration (Ku *et al.*, 2002). Knockdown of Sec8 affects the expression of two upstream regulators - Pirh2 and Siah1 - which ultimately culminates in reduced phosphorylation of ERK and p38, subsequently reduced phosphorylation of cytokeratin8, and suppression of cell migration. Thus Sec8 can regulate the expression levels of proteins which are required to promote cell migration. However the physical mechanism by which this regulation is achieved this remains unclear.

1.3.5 The Exocyst can Perform Multiple Different Roles in Order to Orchestrate a Cellular Function

A further example of the exocyst's broad and far reaching roles can be found in ciliogenesis, where it performs multiple different roles in order contribute to an overall function: the formation and maintenance of a vital organelle.

Cilia are eukaryotic organelles, consisting of a microtubule-based axoneme that projects from the cell surface and is encased by the plasma membrane. Found in virtually every cell type throughout the mammalian body, cilia can have both motile functions (Reviewed in: Ibañez-Tallon *et al.*, 2003) and immotile signalling roles (Reviewed in: Berbari *et al.*, 2009; Goetz *et al.*, 2010).The base of the cilium becomes a focal point for vesicular and membrane trafficking, and several groups of proteins are required to achieve this. Three Rab GTPases – Rab8, Rab10 and Rab11 - have well-described roles in this process, particularly with regard to membrane and vesicle trafficking (Babbey *et al.*, 2010; Reviewed in Das *et al.*, 2011). They execute these roles by influencing their downstream effectors, one of which is the exocyst. Several exocyst members have been shown to localise at the base of cilia in MDCK cells (Rogers *et al.*, 2004; Park *et al.*, 2008), and

interact with the Rab proteins (Guo *et al.*, 1999b; Zhang *et al.*, 2004; Wu *et al.*, 2005).

Sec8 has been shown to co-localise with Rab10 and Sec6 in apical rings prior to ciliogenesis, and at the ciliary base once formed (Babbey *et al.*, 2010). Sec8 also physically interacts and complexes with Rab10. The study suggests that the exocyst and Rab10 may be involved in mediating membrane transport to the primary cilium. Examination of the exocyst in photoreceptor rod outer segments (a specialised ciliary-derived structure) showed that, at the base of the photoreceptor-connecting cilium, Sec8 co-localised with Rab8 at sites of vesicle fusion (Mazelova *et al.*, 2009). This suggests that in addition to membrane transport, the exocyst may perform a tethering role at the base of cilia similar to its one in polarised exocytosis.

One study has also implicated the exocyst in the formation of cilia, specifically in the migration and docking of the centrosome during ciliogenesis (Park *et al.*, 2008). During ciliogenesis, the centrosome/basal body migrates to the cell periphery. It is used to nucleate a ciliary axoneme either directly at the plasma membrane, or within an intracellular ciliary vesicle which subsequently fuses with the plasma membrane (Sorokin, 1968). Dishevelled – a protein member of the planar cell polarity (PCP) signalling pathway - was shown to be essential for both the apical positioning of the basal bodies and their association with ciliary vesicles (Park *et al.*, 2008). Interestingly, whilst in wildtype multi-ciliated cells Sec8 localises in puncta that associate with basal bodies, knockdown of Dishevelled causes this association to be lost. A disorganised cytoplasmic localisation was instead observed, with Sec8-positive structures often localising at the apical membrane in the absence of any docked basal bodies. This suggests that Sec8 is in fact a component of the ciliary vesicle, which requires Dishevelled in order to associate with a basal body, but can still dock at the apical membrane in the absence of one. This poses a role for Sec8 in not only trafficking to the base of formed cilia, but also in the regulation of the apical membrane docking of basal bodies during ciliogenesis.

Dysgenesis or dysfunction of cilia causes a severe class of syndromes collectively known as ciliopathies (Reviewed in: Badano *et al.*, 2006; Waters *et al.*, 2011). To the author’s knowledge, there is only currently one described case

of a human patient with a mutation in a coding region of Sec8, and they displayed symptoms aligning with the severe lethal ciliopathy MKS (Shaheen *et al.*, 2013). The fact that mutation of exocyst components can cause ciliary-related disease highlights that their role in ciliogenesis and maintenance must be an essential one, but it is yet to be fully uncovered.

1.4 Perspectives and Outstanding Questions

1.4.1 The Possibility of Sub-complexes

Currently, a controversial issue regarding the exocyst complex is the possible existence of sub-complexes. It is widely debated as to whether the complex assembles and functions as a whole, or if subcomplexes of exocyst components actually perform unique and separate functions within the cell. Recent evidence (Heider *et al.*, 2015) indicates that the yeast exocyst is present as a complete complex. However, subcomplexes appear to be present in mammalian cells (Moskalenko *et al.*, 2003; Bodemann *et al.*, 2011). I will discuss the evidence for the existence of subcomplexes below.

Several studies have observed, by immunofluorescence, that different exocyst components also have different subcellular locations. However, a recent paper raised important questions about the effect of both antibody epitopes and exocyst conformation on subcellular localisation. Inamdar*et al.* (2016) used antibodies that recognised 20 different epitopes in six regions of mSec6, with the theory that conformational changes in the exocyst may cover/uncover different binding sites. They found that the different antibodies showed different subcellular localisations. Firstly, this demonstrates that exocyst exists in different conformations in different areas of the cell, likely to perform its different cellular roles. It also suggests that the results and subsequent deductions made from immunofluorescence and immunoprecipitation studies could have been unknowingly influenced by the choice of antibody. These are important considerations going forward, as perhaps different subcellular localisations of exocyst components would indicate not subcomplexes, but the detection of different conformations of the fully assembled complex. It also raises the possibility that localisations, interactions and roles of Sec8 have not yet been discovered, as available antibodies may not detect it in certain conformations of the exocyst complex.

Studies using methods other than immunofluorescence have also shown that subsets of exocyst components exist separately prior to assembly (Shin *et al.*, 2000; Moskalenko *et al.*, 2003; Boyd *et al.*, 2004). However, this only demonstrates that such sub-complexes exist in different subcellular locations. As to whether these sub-complexes have independent and/or unique functions prior to the assembly of the whole complex, the field is yet to be convinced. Some

studies have demonstrated distinct roles for individual exocyst components, such as Gerges *et al.* (2006) who when examining the delivery of AMPAR to excitatory synapses found that whilst Sec8 was required for the directional transport of AMPAR towards the synapse, Exo70 was required for insertion of the receptors into the membrane. This was supported by multiple observations. This poses the question as to whether these proteins are in fact functioning independently, or whether they are complexed with other members of the exocyst when performing these functions. When elucidating the molecular mechanisms of Sec8's involvement in cellular processes, it will be important to determine whether it is part of the complete complex when it is performing these roles, as this would perhaps influence protein folding and binding ability.

There is also a possible argument for the existence of subcomplexes from an evolutionary perspective. As discussed in Section 1.2.4, the exocyst components share structural similarities that indicate they likely evolved from multiple gene duplication and divergence events, and can therefore possibly perform each others roles in some contexts. Many eukaryotes only possess between one and six of the exocyst components, which either suggests that the other components cannot be detected computationally, or that these 'sub-complexes' can actually function sufficiently. This could give evidence to the idea that sub-complexes of the components in mammalian cells could in fact perform some specific or less specialised functions. Additionally, in the eukaryotic protist *Trypanosoma brucei,* there are in fact nine exocyst components (Boehm *et al.*, 2017) - could this mean that even the mammalian exocyst is actually a 'subset' of components?

Knowing how the complete complex fits together would be a helpful contribution to the discussion on this issue, but this cannot be known for sure until the full structure of each of the subunits is resolved (See 1.2.4). Only then can we begin exploring the possibility of different interactions between subunits, and how these may contribute to different binding combinations.

1.4.2 Elucidating the Roles of Exocyst Components

One question raised by the research into the exocyst's cellular roles is the issue of assigning function to specific exocyst components, versus the exocyst complex as a whole, especially given the speculation surrounding subcomplexes. As the importance of the exocyst complex was recognised relatively early on, research into its cellular functions in different organisms exploded, often before homologues of all the components had actually been identified. As a result, many papers only examined exocyst components which had been identified at the time. More recent studies have the same issue, but that is now likely due to the availability of antibodies and the fact that it is not time or cost-efficient to replicate experiments with the eight different components.

Many studies therefore often use between one and three individual exocyst components as a 'markers' for the whole complex. They then test their hypotheses using the marker(s), and suggest that any results extend to the rest of the complex. Just to select one example, Hazuka*et al.* (1999) use Sec6 and Sec8 as markers for immunofluorescent localisation of the whole exocyst complex because they are "stable components". However, it could be possible that a subcomplex including Sec6 and Sec8 displays this localisation, and that other components of the exocyst could be localised elsewhere.

On the other hand, some studies that only test (or report that they only test) a marker of the complex can therefore rightly only attribute the results to that one component. For example, Park *et al.*(2009) state that because they speculated a role for the exocyst in the interaction between the protein 'Dvl' and basal bodies during ciliogenesis, they immunostained for Sec8 to test their hypothesis. The study goes on to demonstrate a role for Sec8 in basal body docking. However, it is entirely possible that the rest of the exocyst is involved in this interaction, and that this not an exclusive role of Sec8 – we just do not know. The other components of the exocyst have demonstrated roles in an even wider range of cellular process, including endocytosis, cell adhesion, nanotube formation, calcium signalling, tubulin polymerisation, pre-mRNA splicing and lipid interaction to name but a few (Reviewed in Tanaka *et al.*, 2017). It is possible that Sec8 functions in these processes too, but its role has not yet been described.

1.4.3 Undiscovered Functions

With the exocyst being implicated in so many different and far-ranging cellular processes, it is almost easier to ask what it is *not* involved in. However this ubiquity can make it difficult to tease apart specific interaction pathways and determine the direct mechanism by which the exocyst is contributing to these processes. One of the main issues limiting more detailed functional analysis of Sec8's roles is the fact that deletion of Sec8 is embryonically lethal in higher organisms. Together, this results in many publications about Sec8 reporting phenotypes but no molecular mechanism.

It also begs the question – what other roles have we not discovered yet? The exocyst is hypothesised to likely be involved in many diseases, including ciliopathies, retinal degeneration, diabetes, cancers, obesity, kidney disease, neural degeneration and many more. But as it is still not fully understood how the exocyst functions in these cellular processes at the molecular level, it is only speculation at this point. The emergence of several human patients with exocyst mutations underlying serious disease however (see Section 1.2.4), demonstrates the urgent need to rectify this.

1.5 Aims of This Study

This project aimed to a) better characterise the known cellular roles of Sec8 and b) potentially discover new cellular roles, through two key research avenues: firstly, through identification of its interaction partners by combining a GFP-Trap with mass spectrometry and qualitative proteomics, and secondly, through characterisation of cellular phenotypes upon perturbation of Sec8. The model organism *Schizosaccharomyces pombe* was chosen for its fast generation time and genetic tractability, and because whilst a large amount of research surrounding the exocyst has been performed in other yeast species, it is comparatively less characterised in *S. pombe*.

Methods

2.1. Yeast Culture and Genetics

2.1.1. Media and Culture

The list of *Schizosaccharomyces pombe* strains used in this study is shown in Appendix Table 1. Yeast cells were maintained and cultured as described in (Moreno *et al.*, 1991). The composition of the rich medium (YE5S), minimum medium (EMM), freezing medium (YFM), mating medium (ME4S), supplements and stock solutions is described in Appendix Tables 2-5. Plates contained 20 % agar (VWR Chemicals). Most experiments were carried out in YE5S, but EMM was used for auxotrophic selection and for microscopy (see below).

Cells were woken up (from storage at -80 ^{o}C) on YE5S plates, and incubated at 27 ^{o}C for 2- 8 days. Cells were inoculated into sterile glass flasks containing 50-200 mL of either YE5S, or EMM supplemented with amino acids. Flasks were shaken at 180-200 rpm. All experiments were carried out at 27°C. To ensure cells were exponentially growing prior to starting experiments (mid-log phase in YE5S = 1.10^{6}- 1.10^{8} cells/ mL; in EMM= 1-6.10^{4} cells/ mL), cell density was measured with a spectrophotometer, and the OD_{600nm} value was converted into a cell concentration from an existing lab standard curve.

The temperature-sensitive *sec8-1* mutant was obtained from the Balasubramanian lab, and is the only published *sec8* mutant in *S. pombe* (Wang *et al.*, 2002). Sequencing has not been performed, so the nature of the mutation(s) in the gene is unknown.

2.1.2. Crossing Strains

To cross strains of opposite mating types, small pellets of both strains were taken from a YE5S plate, mixed together with 10 µl of sterile water, and spread on an ME4S plate. Cells were left to conjugate and sporulate at 27°C for 2 days. A small pellet of these cells was mixed with 200 µl of sterile water, and 10 µl of this mixture was transferred to a new YE5S plate. Using a tetrad dissector (MSM 400, Singer Instruments) up to 18 asci were separated into a grid. Asci were once again left at 27°C to allow endolysis of the ascus wall. The four spores of each ascus were then further dissected, and the plate left to grow at 27°C for 4-5 days. Cells were

replicate-plated onto selective conditions. Geneticin was used in YE5S at a final concentration of 100 µg/ml. The *sec8-1* mutant is temperature-sensitive, and clones were therefore identified by an absence of growth at 36ºC. The *coxIV-dsrfp* strain (used for visualising mitochondria) was originally constructed by insertion of this cassette at the *leu1* locus (Chiron *et al.*, 2008), and CoxIV-dsRFP-positive clones were selected on EMM containing no leucine. Only tetrads that yielded four colonies and that displayed normal allelic segregations were selected. Two successful clones were re-streaked on selection, assigned a strain collection number and frozen in YFM at -80°C until required.

2.2. GFP-Trap

2.2.1. Protein Extraction and Immuno-precipitation

Two independent replicate GFP-Trap experiments were carried out (R1 and R2). The methodology is summarised in Figure 3.The experiments were performed on two different strains: a strain in which a *gfp* cassette was inserted in the 3′ region of the endogenous *sec8* gene, thus expressing Sec8-GFP at its endogenous level (Wang *et al.*, 2002). These cells show no apparent growth or morphological defect that may suggest that the GFP fusion protein affects the function of Sec8. A wild-type strain was also used, as a control.

The protein extraction prior to the GFP-TRAP was performed as in Jourdain *et al.*, 2012 .In brief, approximately 0.5-1x10^9 cells were pelleted by centrifugation (4000 rpm, 5 min, room temperature) and re-suspended in 50 µl of Extraction Buffer (EB - see Appendix Table 6). Cells were lysed using the mechanical action of acid-washed glass beads in a ribolyser (FastPrep FP120 apparatus, Savant. Co.). They were ribolysed (4ºC) for two 25 second periods at 5.5 power, with a one-minute interval. The base of the 1.5 ml microcentrifuge tubes containing the ribolysed cells were pierced using a hypodermic needle (21G x 1 1/1”), and the solution was centrifuged (4ºC) at 10,000rpm for 60 seconds into a new 1.5 ml microcentrifuge tube (Figure 4). This yielded whole cell extracts (WCE). Protein concentrations were determined by Bradford assay (BioRad, California), and 40 µg of WCE was retained for western blotting.

For each reaction, 50 µl of GFP-Trap®_A beads (Chromotek GmbH, Germany) were centrifuged to remove the 20% ethanol suspension. Beads were then

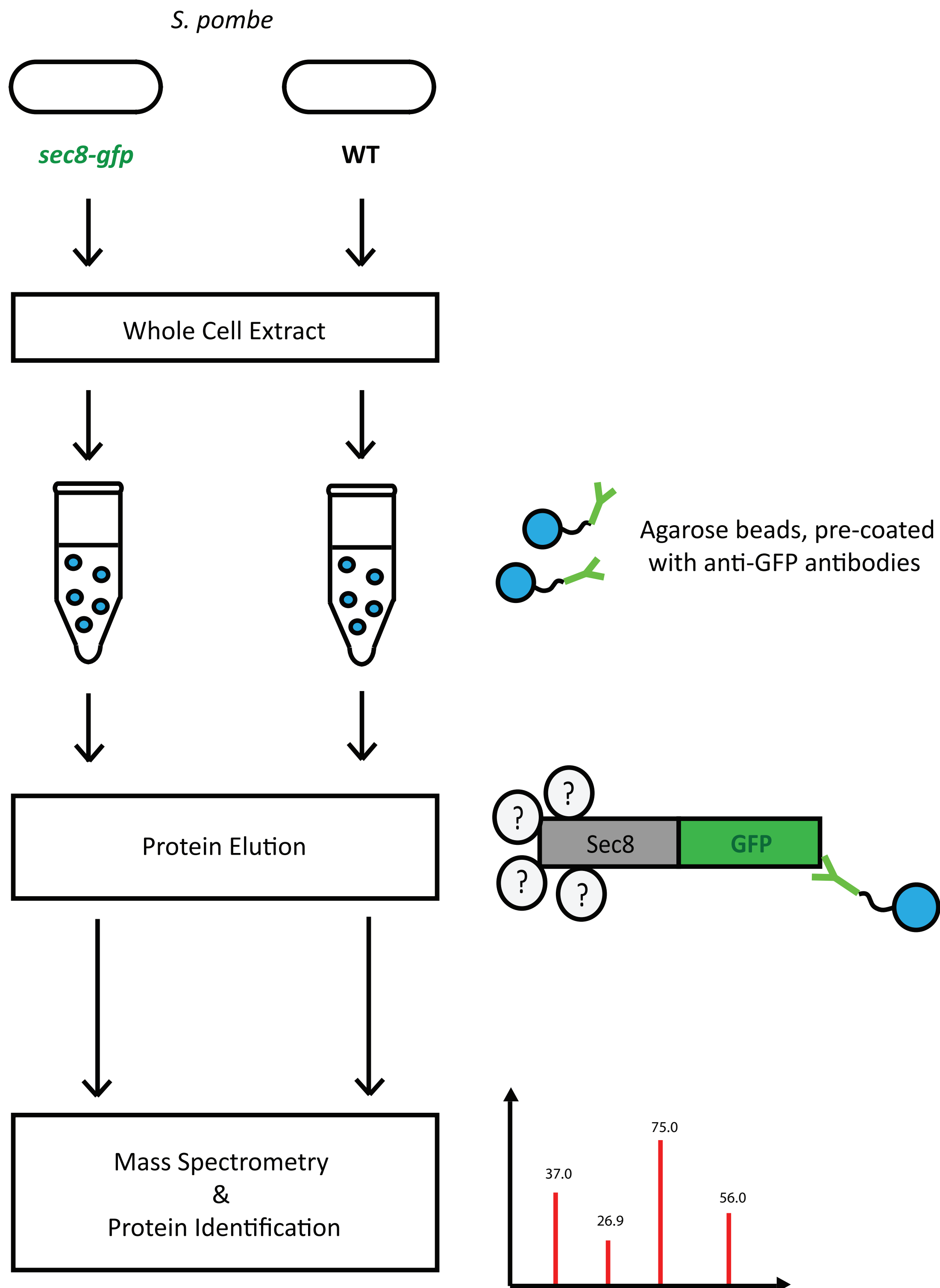


Figure 3: An illustration of the GFP-Trap methodology.
Whole cell extracts (WCE) were collected. The WCE was then incubated with pre-coated Chromotek GFP-Trap beads at 4°C for 18 hours. Proteins were washed and eluted, before being analysed by mass spectrometry and subsequent comparison to the *S. pombe* proteome. Duplicate experiments were performed for both of the strains shown; *sec8-gfp* and wildtype as a control.

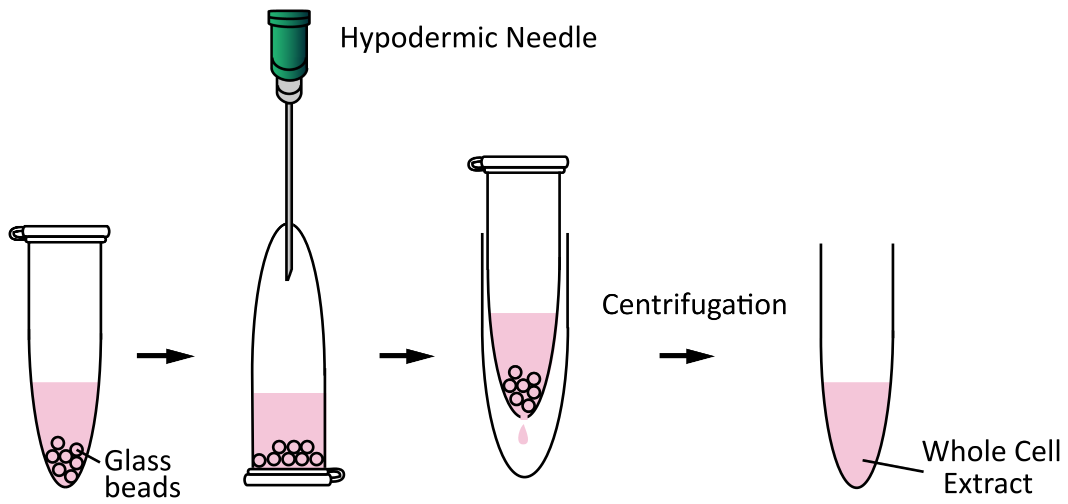


Figure 4: The whole cell extract (WCE) filtering process
Diagram illustrating the filtering process used to extract whole cell extract (WCE). After cells were ribolysed using glass beads, the microcentrifuge tube was pierced, and the supernatant centrifuged into a new tube.

washed once in 500 µl of EB, and re-suspended in 50 µl of EB. The washed beads were then mixed with 10 mg of WCE. The solution was left to rotate at 4°C overnight (approximately 18 hours). The immunoprecipitated proteins were washed twice in 400 µl of EB, and 10% of the final volume was retained for western blotting (IP). The remaining protein/bead complexes were stored at -80°C. These were then boiled in laemmli buffer (to elute the proteins), and analysed by LC-MS/MS (liquid chromatography – tandem mass spectrometry) at the University of Bristol Proteomics Facility.

2.2.2. ***Western Blotting***

Whole cell lysates and the IP solutions were separated on a 10% polyacrylamide gel using SDS-PAGE (see Appendix Table 7). Gels were transferred to methanol-activated PVDF membranes overnight at 30 V. Membranes were blocked in PBS 5% nonfat milk, then incubated with 1:1000 anti-GFP antibody (Sigma-Aldrich, Cat #11814460001) for 60 minutes at room temperature, and washed three times in PBS 0.1% Tween. The membranes were then incubated with 1:40,000 anti-mouse IGG secondary antibody (Sigma-Aldrich, Cat #A9044) for 60 minutes at room temperature. All antibodies were diluted in PBS 2.5% nonfat milk. Membranes were developed using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Cat #RPN2106). The membrane from replicate one was developed for 5 minutes, and the membrane from replicate two was developed for 60 minutes.

2.2.3. ***Proteomics Analysis***

Proteins were identified by the Proteomics Facility using a Sequest search against the *S. pombe* Uniprot database. Low confidence peptides with less than 5% false-discovery rate (FDR) were removed. These identified proteins were received as a spreadsheet which detailed the statistics for each protein hit, such as the number of proteins detected, number of unique peptides detected, area and percentage coverage. A 'score' for each protein hit was also given - this considers several variables and gives an overall indication of the quality of a hit (the higher, the more significant). In the wildtype strain, replicate 1 had 2182 protein hits and replicate 2 had 1886 protein hits. In the *sec8-gfp* strain, replicate 1 had 2210 protein hits and replicate 2 had 2170 proteins hits. These subsequently needed to be filtered to eliminate false positives. Figure 5 shows

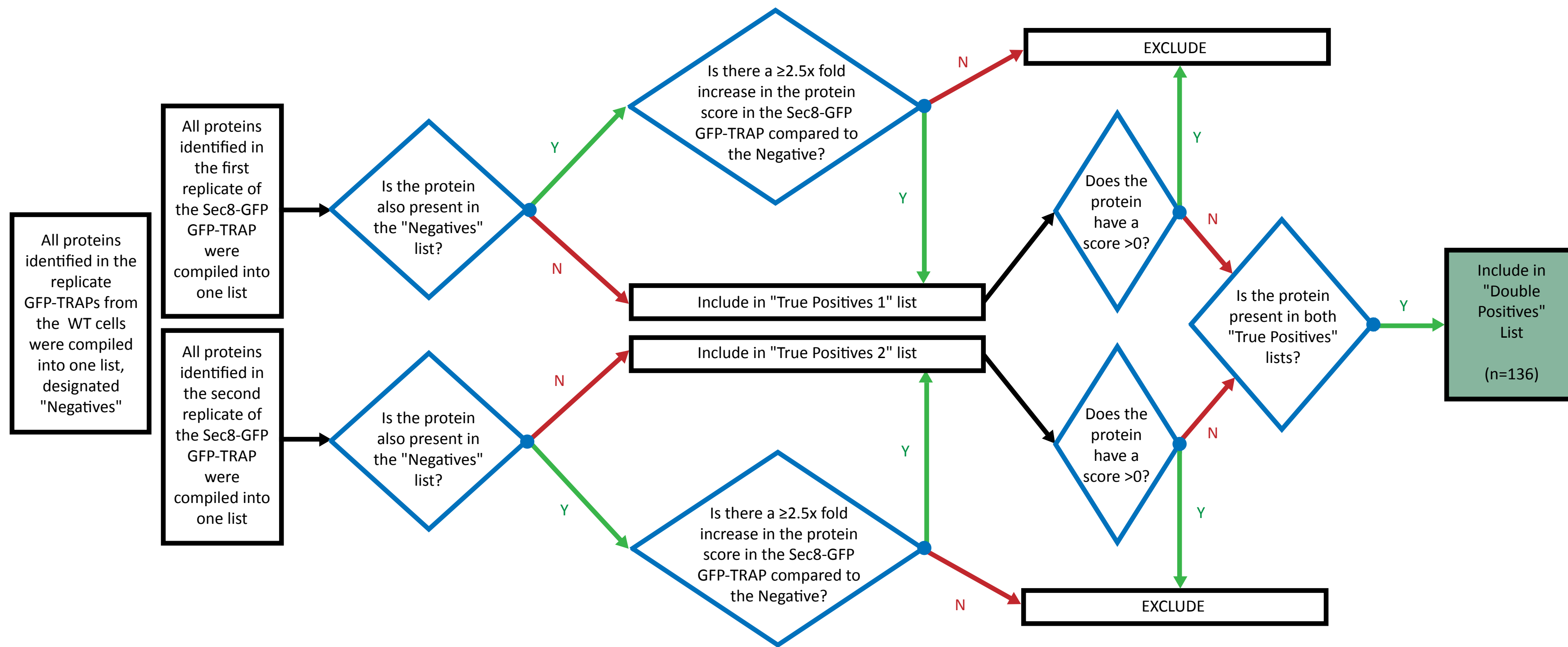


Figure 5: Thresholding of the GFP-Trap data identified 136 double-positive Sec8-interacting proteins
All protein hits were collated in a Microsoft Excel spreadsheet. The filtering schematic shown above was used to eliminate false-positive and low-confidence hits from the data, in order to produce a list of high-confidence interacting proteins (see in text for further details). This analysis identified 136 double-positive Sec8-interacting proteins.

how this data was filtered to produce a list of high-confidence hits. Firstly, the proteins identified in the two replicate wildtype GFP-Traps were combined, and this list was designated ‘Negatives’ – proteins that were pulled down by the anti-GFP antibody in the absence of GFP (n=4068). The data from each *sec8-gfp* replicate was initially analysed separately. Both were compared against the Negatives list to remove any non-specific hits; any proteins detected exclusively in the Sec8-GFP Traps were considered true positives, and any proteins which were in the Negatives list, but showed at least a 2.5-fold increase in score in the Sec8-GFP Trap were also considered true positives. This produced a list of ‘True Positives’ for each replicate (n=649 and n=874 for R1 and R2 respectively). Any protein with a score of 0 was then removed from the true positives lists, reducing them to R1 n=415 and R2 n=613. These two lists were compared, and any protein which appeared in both lists was deemed a ‘Double-Positive’ (n=136). These double-positives were considered high-confidence Sec8-interacting proteins, and were used for subsequent analyses.

2.3. Cell Biology

2.3.1. Microscopy and Image Acquisition

For microscopy, cells were grown and observed in EMM medium supplemented with amino acids. This was for two main reasons: firstly, EMM creates less fluorescent background than YE5S. Moreover, CoxIV-dsRFP is under the control of a repressible *nmt* promotor which is shut off in the presence of thiamine (Chiron *et al.*, 2008). Thiamine is naturally present in YE5S, but not in EMM. Therefore, to be able to visualise the CoxIV-dsRFP signal, cells were cultured in EMM for at least 18 hours.

An agarose pad was prepared by pipetting 800 µl of EMM 2% agarose in an imaging chamber gasket (20-mm diameter, 0.5-mm depth, CoverWell™, Molecular Probes). One millilitre of cells was concentrated by centrifugation (13000 rpm, 30 sec) and 5 µl was spread on the polymerised agarose pad. The chamber was then sealed with a 22 × 22 mm glass coverslip.

When visualisation of the cell’s border was required, 0.7 µl of Calcofluor White (fluorescent brightener 28, Sigma-Aldrich) prepared at 5mg /mL in water was pipetted on the top of the cells before the coverslip was sealed.

Mitotracker Red CMXRos (Molecular Probes) was dissolved in DMSO at a concentration of 1 mM. This stock solution was diluted in EMM (supplemented with amino acids) to 1 μM. Nine hundred microlitres of cells was incubated with 100 μl of this 1 μM Mitotracker dilution for 15-30 minutes. Cells were washed three times in EMM supplemented with amino acids, placed on an agarose pad and observed immediately.

Samples were examined using a Zeiss Z1 AxioObserver inverted fluorescence microscope, equipped with a motorised stage, in a temperature-controlled incubation chamber. Images were captured at 27°C using a 100x, 1.4 NA oil-immersion lens and a Cool-Snap HQ2 CCD camera (Photometrics) controlled by Axiovision software (ZEISS).

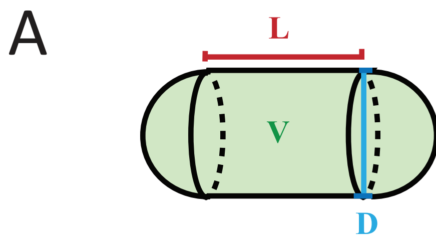
To assess nuclear morphology, z-stacks taken through the full depth of the cell were acquired, with slices 0.5 μm apart. To assess mitochondrial morphology, z-stacks through the full depth of the cell were acquired with slices 0.4 μm (replicate 1) or 0.5 μm (replicate 2) apart. To monitor mitochondrial dynamics, time-lapse imaging was performed. Each field of view was imaged every 30 seconds for 10 minutes. At each timepoint, a Z-stack through the full depth of the cell was acquired with slices 0.5 μm apart.

2.3.2. Image Analysis

All image analysis was performed using ImageJ (Schindelin *et al.*, 2012), and counts and measurements were exported to Microsoft Excel.

For the analysis of nuclear and cellular volume, the two z-stack slices at which the nucleus had the widest diameter (presumed to be the centre) were merged to create a maximum intensity projection using ImageJ. Measurements were then taken using this image. The cell was treated as a capsule, and the nucleus as a prolate ellipsoid (Neumann *et al.*, 2007). Measurements were taken as shown in Figure 6A and 6B, and volumes were calculated using simple geometries. For the analysis of nuclear positioning, measurements were taken as shown in Figure 6C. They were used to calculate the true centre of the cell, and the centre of the nucleus. The difference between these two values was calculated in micrometres, and then converted to a percentage of the total cell length.

A

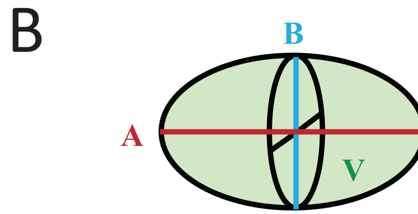


Capsule

D ÷ 2 = r

$$\text{Volume} = \pi r^2 \left(\frac{4}{3} r + L\right)$$

B

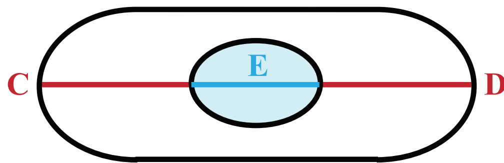


Prolate Ellipsoid

A ÷ 2 = a
B ÷ 2 = b

$$\text{Volume} = \frac{4}{3} \times \pi b^2 a$$

C



$$\text{Centre of cell} = \frac{C + D + E}{2}$$

$$\text{Centre of Nucleus} = C^{*} + \frac{E}{2}$$

Figure 6: Geometric measurements used to determine cellular and nuclear properties
Diagram illustrating the measurements taken and formulas subsequently used to determine (A) cell volume (B) nuclear volume and (C) nuclear position.
(A) For cell volume, *S. pombe* cells were treated as capsules. "L" was measured as the length along one side between the cell tips. "D" was measured as with the width just before the cell tip. To calculate volume, these measurements were fed into the equation shown above.
(B) For nuclear volume, the nucleus was treated as a prolate ellipsoid. "A" was measured as the longest nuclear width (in any direction). "B" was measured as the perpendicular width to A. To calculate volume, these measurements were fed into the equation shown above.
(C) "C" and "D" were measured as the length from the centre of the cell tip to the nuclear envelope, from either end respectively. "E" was measured as the width of the nucleus parallel to the length of the cell, between "C" and "D". The centre of both the cell and the nucleus were calculated using the equations shown. *For this equation, the longest value out of "C" or "D" was used here.

For mitochondrial analysis, images were exported to ImageJ and the out of focus slices removed. A maximum intensity projection was created for each timepoint. Mitochondrial morphology and fission/fusion events were scored manually from each maximum intensity projection.

2.3.3. ***Statistical Analyses***

Frequency counts (for mitochondrial morphology) were assessed using Chi-Squared analysis. All quantitative data (mitochondrial fission/fusion evens) were tested for normality using the Shapiro-Wilk test, and parametric (t-test) or non-parametric (Mann-Whitney/U test) tests applied, as appropriate, using the GraphPad software.

Identifying Novel Interaction Partners of the Exocyst Member Sec8

The fission yeast *Schizosaccharomyces pombe* has a short generation time, a relatively small genome, and is genetically tractable. Indeed with a completely sequenced and well-annotated genome, *S. pombe* is easy to manipulate (Wood *et al.*, 2002). Its small size (approximately 2-3 µm by 7-10 µm) and uniform rod shape make morphological defects easy to observe. *S. pombe* is also bigger than the budding yeast *S. cerevisiae*, making its cytology easier to observe by light microscopy. Its proteome is also much simpler than that of higher eukaryotes. Importantly the exocyst complex is conserved in *S. pombe*, with four of its members sharing ~20% sequence identities and aligning along their full length with their orthologues in other organisms (Wang *et al.*, 2002), indicating that the exocyst plays important conserved roles across eukaryotic evolution. Taken together, these qualities make *S. pombe* an ideal model organism to investigate and characterise cellular roles of the exocyst complex.

3.1. GFP-Trap Identifies 136 Interaction Partners of Sec8

As a first step to test the hypothesis that there are uncharacterised cellular roles for Sec8, I sought to identify novel interaction partners of Sec8 in *S. pombe* that may either shed some light on known mechanisms, or indicate new cellular functions of Sec8.

GFP-Trap is one of several of techniques that allow the identification of previously unknown interaction partners. Others include yeast two-hybrid, pulldown assays using recombinant proteins, and tandem affinity purification (TAP-tagging). GFP-Trap is a versatile tool to investigate GFP-fusions and their interacting partners (Agarwal *et al.*, 2007; Schermelleh *et al.*, 2007; Trinkle-Mulcahy *et al.*, 2008; Frauer *et al.*, 2009). Its high affinity and the lack of heavy and light antibody chains is a major advantage over conventional immunoprecipitation. Moreover, it is much less time-consuming than yeast-two hybrid.

The technique requires a cell line that is expressing the protein of interest (POI) tagged with GFP. Whole cell extract is incubated with commercially-available agarose beads that have been pre-coated with GFP antibody. The antibody-GFP-

POI complexes (along with proteins that are interacting with the POI) are eluted by boiling. These proteins are then identified by mass spectrometry and subsequent comparison to the organism's proteome.

GFP-Trap was performed to identify Sec8 interacting partners in *S. pombe*. Duplicate samples of wild type *S. pombe* and cells expressing Sec8-GFP under the control of the native promoter (Wang *et al.*, 2002) were subjected to GFP-Trap. To confirm that Sec8-GFP could be extracted successfully, immunoblotting of whole cell extract and GFP-Trap samples (IP) was performed with anti-GFP antibody. Western blotting revealed a band of the appropriate size (~150 kDa, Figure 7), in both the whole cell extract (WCE) and the precipitate (IP) from post-incubation with the GFP-TRAP beads, indicating successful pull-down of the fusion protein. No bands were detected in a WT strain lacking the fusion protein, indicating specificity of the antibody. The identity of the smaller bands in the IP sample is unknown, but is hypothesised to be proteolysis products. However, these were not detected in the WT control and therefore are likely to be specific to Sec8-GFP.

Samples from two independent replicates were therefore taken forward, and analysed by mass spectrometry to identify the proteins that had been pulled down alongside the GFP fusion protein. This identified 2000+ protein hits in each sample. These were filtered further to produce a list of high-confidence hits, as illustrated In Figure 5. Proteins that were pulled down by the anti-GFP antibody in the absence of GFP (n=4069) were designated "negatives". The hits from each Sec8-GFP replicate were filtered against the Negatives list to remove non-specific hits. Proteins detected exclusively in the Sec8-GFP Traps were considered true positives, as were proteins in the Negatives list that showed at least a 2.5-fold increase in score in the Sec8-GFP Trap. Data from the two Sec8-GFP replicates were combined, and the 136 proteins that appeared in both lists - termed 'Double-Positive' - were considered to be high-confidence Sec8-interacting proteins (listed in Table 2, scores in Appendix Table 8). Many of these 'double-positives' are likely indirect interactors due to the nature of the technique, and will need to be confirmed by alternative techniques (see discussion).

Anti-GFP:

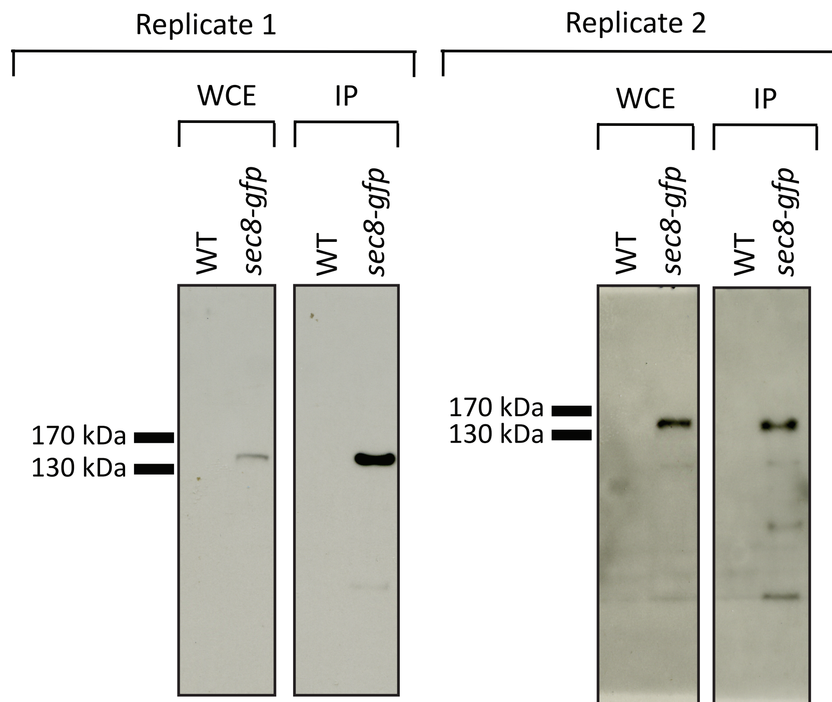


Figure 7: The presence of Sec8-GFP was successfully immunodetected
Either wildtype or *sec8-gfp* cells were lysed to produce whole cell extract (WCE). This WCE was then incubated with GFP-Trap beads, producing a bead precipiate. WCE and 10% of the bead precipitate (IP) were subjected to western blotting and probed with anti-GFP antibody. Blots are shown for each of the two replicate GFP-Traps, which were performed and developed separately.

Table 2: The GFP-Trap identified 136 double-positive Sec8 interacting proteins [2]

Uniprot Accession	Gene Name	Uniprot Accession (cont.)	Gene Name (cont.)
O74562	*sec8*	Q9USJ4	*nte1*
O74846	*sec6*	Q9USZ2	*mtr10*
O75006	*sec15*	O42963	*nup44*
O13705	*sec10*	P33277	*gap1*
O94598	*sec5*	O42854	*bbc1*
O14226	*exo84*	Q9P792	*nbr1*
Q10339	*exo70*	Q9P7H8	*ecm29*
Q10411	*spo15*	Q10222	*pta1*
O14065	*tcb1*	O42998	*sip1*
Q10324	*sec3*	Q1MTQ5	*mug89*
Q9UUE2	*csx2; cnt5*	O94548	Unassigned
Q1MTR3	*vid27*	Q1MTN8	Unassigned
O14072	*cta4*	Q9UTF8	*ugo1*
Q09891	Unassigned	O74738	*set10*
Q09857	*uso1*	O74547	*bit61*
O74319	*taf73*	O74541	Unassigned
O13791	*slt1*	O14130	Unassigned
Q9UT35	*gdp1*	Q9P382	*nup82*
O74369	*css1*	Q9UT67	*imt1*
Q10064	*tra2*	Q9UQX0	*sod2*
O14283	*prr1*	P87114	*fft1*
P78847	*nup186*	O42930	*vps10*
O14290	Unassigned	O74481	*pdi5*
O74349	*rkr1*	O94516	*pex16*
Q10435	Unassigned	O94520	*emc4*
Q7Z9H9	*fig4*	P78871	*rst2*
O42901	*orm1*	O14068	Unassigned
Q10243	*vps26*	Q9P7J5	*yta4*
O13282	*taf5*	Q9UTL2	*klp8*
Q10496	*gyp51*	Q10361	*etp1*
O14197	Unassigned	O59747	*pdf1*
Q9P782	*aha1*	Q8WZK2	*its8*
O74534	*sly1*	Q9UTK6	Unassigned
O60081	*trm72*	O94689	*yme2*
P50524	*rpn12*	Q9Y7N9	Unassigned
Q9P7W8	*rsc9*	Q9UUI8	*pet1*
Q9Y7K2	*tor2*	P27584	*gpa1*
O14301	*aip1*	Q09922	Unassigned
O74925	*pep3*	Q09787	Unassigned
Q9UTH0	*nup132*	O42929	*tma22*
O42954	*mbx1*	O94691	*jmj3*
Q9Y7J8	*psy2*	Q10347	*any2*
Q9P7X5	*ppk32*	Q09830	Unassigned
O14340	Unassigned	O42973	Unassigned
O94374	*imp1*	Q1MTQ1	*tea2*
Q9UTK7	*dsc2*	O74308	*gsf1*
Q9UUH0	Unassigned	Q9US60	*klp3*
Q10331	*nup107*	O14002	*mak2*
O94712	*gid1; vid30*	Q9UTK4	*nup189*
O13897	*fsh2*	Q10245	*ifa38*
Q09849	*arp42*	Q92359	*puf4*
Q9P7R8	*gea1*	Q9UT38	*vps16*
O74773	*msh2*	Q9UTN3	*cid14*
Q10429	*cnd3*	O74823	*red5*
Q09779	*tho2*	P78875	*tpp1*
Q9C100	*ogm2*	O43092	*oxa102*
Q10108	*edc1*	Q10093	Unassigned
P78953	*mid1*	Q10366	*pik1*
O74504	Unassigned	P87234	*gyp3*
O13636	*tim50*	O60108	*cbh2*
Q09743	*ste20*	O94733	Unassigned
Q9P7W0	Unassigned	O14076	*mpp6*
Q10165	*cnt6*	Q9HGN7	*sec63*
Q10268	*mac1*	O59741	*prt1*
P00046	*cyc1*	Q76PC3	*mme1*
O14188	*rng2*	Q10110	*rrn3*
O59712	Unassigned	Q9Y802	*lsd1*
Q09682	*pre9*	O13960	*ecm33*

[2] The Uniprot accession ID and gene name is listed for each double-positive Sec8-interacting protein. Interactors are listed from highest score (averaged across both Sec8-GFP replicates) to lowest. “Unassigned” is used when there is no gene name assigned in the PomBase NAR Database entry for that protein (Wood *et al.* 2012).

Of the double-positive proteins identified, the highest scoring hit was Sec8 itself, with both a score and number of unique peptides 2.5 times higher than any other hit. As Sec8 was the bait protein, this is expected. The other members of the exocyst complex were the next highest scoring hits, providing evidence for the success of this approach.

To examine the pathways and processes that the putative interaction partners were involved in, each of the 136 double-positives was analysed by functional annotation clustering according to gene ontology using DAVID (Huang *et al.*, 2009a, 2009b). Grouping of the Sec8 interaction partners by cellular compartment (GOTERM_CC_5) showed that the most significant clusters include 'exocyst complex', 'Vesicle', 'Microtubule', 'Cortical Actin' and 'Golgi' (Figure 8A). Grouping by biological process (GOTERM_BP_5) showed that the most significant processes were exocytosis and vesicle tethering, and that within the top 20 terms there was also endosomal transport, vesicle fusion and protein targeting to membrane (Figure 8B).

These top clusters are not surprising, as they all align with Sec8's well-documented role in the late secretory pathway. Combined, they are a good indication that, to an extent, the GFP-Trap has been successful. However, in addition to these known interaction partners, the GFP-Trap data revealed an unexpected novel association of Sec8 with proteins found in the nucleus (Figure 8, blue bars) and in mitochondria (Figure 8, red bars).

3.2. <u>Clustering of the Sec8 Interaction Partners Reveals Nucleus-associated Proteins</u>

Further inspection of the grouped interactors revealed several nucleus-associated clusters which were intriguing because they did not necessarily align with the exocyst's documented roles. When grouped by cellular compartment (CC), 'Nuclear envelope' and 'ER' were the clusters with the highest significance after 'exocyst', and indeed there were several other nuclear-related clusters (highlighted blue in Figure 8A) including 'transcription factor complex',

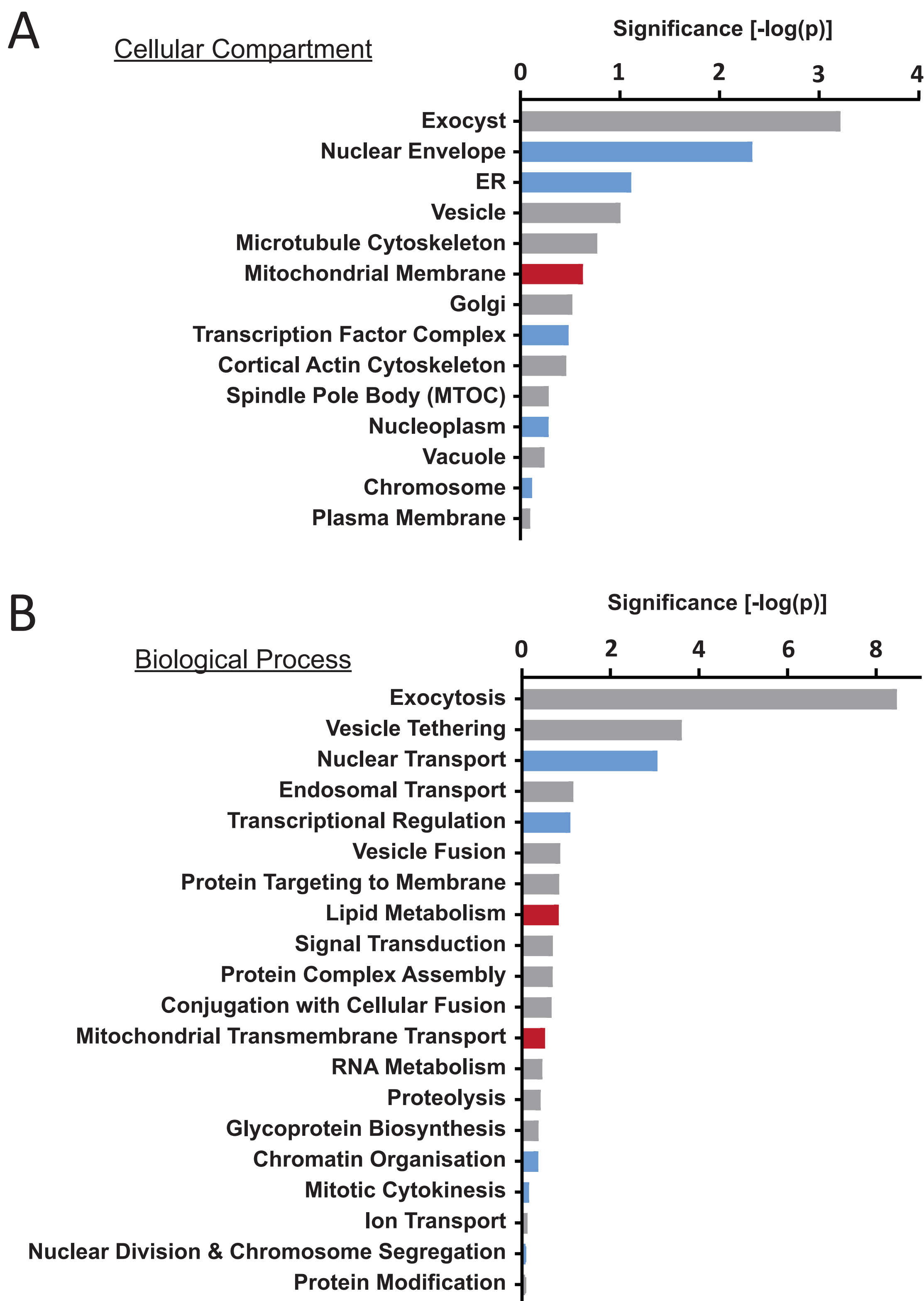


Figure 8: Gene ontology grouping of the Sec8-interacting proteins reveals both expected and novel clusters.

Double-positive interacting proteins were clustered according to either (A) cellular compartment, using GO TERM CC_5 or (B) biological process, using GOTERM BP_5. Typically, the name of each cluster was taken from the GO_TERM in that cluster with the highest number of proteins, but in some cases a summary name was manually chosen. Significance was calculated as the –log10 of the p value calculated for that cluster. Nuclear clusters of interest are shown in blue, and mitochondrial clusters of interest are shown in red.

‘nucleoplasm’ and ‘chromosome’. Grouping by biological process (BP) also revealed significant nuclear-related clusters (highlighted blue in Figure 8B), with nuclear transport’ the third most significant cluster, and ‘transcriptional regulation’ the fifth. Both had a higher significance than ‘vesicle fusion’, which is one of the exocyst’s most characterised roles. ‘Chromatin organisation’, ‘mitotic cytokinesis’ and ‘nuclear division & chromosome segregation’ were also in the top 20.

To examine the localisation of these nucleus-associated Sec8-interacting proteins more closely, I retrieved those with the following CC GO annotations: ‘Nuclear envelope’, ‘nuclear pore’ and ‘nucleoplasm’. These are listed with their respective scores in Figure 9A. Of the Sec8-interacting proteins in the ‘nuclear envelope’ cluster, eight also fall within the ‘nuclear pore’ cluster (Figure 9A, highlighted red). All proteins have also been illustrated in Figure 10 to give some indication of their localisation *in situ.*

For each CC GO-term, I calculated what percentage of the total number of proteins in that term were detected as double-positive interactors in this experiment (Figure 9B). 8.5% of Nuclear Envelope proteins, 2.7% of Nucleoplasm proteins and 16% of Nuclear Pore proteins were detected. This showed firstly that the nuclear envelope was better represented than nucleoplasm, which could perhaps suggest that a greater interaction is occurring outside the nucleus and at the periphery. It also showed that ‘nuclear pore’ was the most represented cluster; 16% of components were detected as double-positive interacting partners, although below the threshold a further 35% were detected as showing a positive score increase in at least one replicate. I therefore looked again at more specific CC GO-terms to see which structural components of the nuclear pore complex (NPC) were detected: outer ring, cytoplasmic filaments and basket (Figure 9C). The GFP-TRAP detected 30% of the Outer Ring proteins, none of the nuclear basket proteins and 50% of the Cytoplasmic Filaments. The cytoplasmic filaments are therefore the best represented structure within the nuclear pore complex.

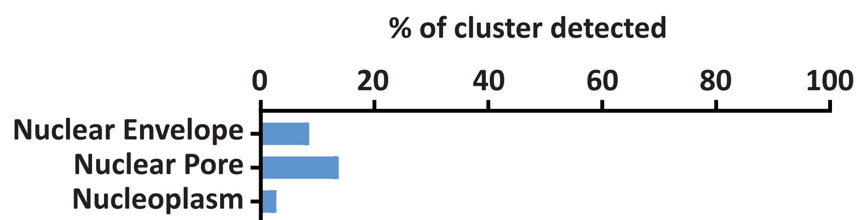
Some of the Sec8-interacting proteins detected by the GFP-TRAP therefore indicate a putative novel interaction between Sec8 and the nuclear envelope, specifically with the nuclear pore complex and nuclear import/export.

A

	Protein Name	Pombase Identifier	Control Score (R1)	Sec8 Score (R1)	Control Score (R2)	Sec8 Score (R2)
Nuclear Envelope	Vid27 Family Protein	SPBC1685.14c	11.8	35.5	6.8	22.0
	Nup186	SPCC290.03c	6.8	18.8	6.0	20.4
	Nup132	SPAC1805.04	1.9	21.2	0	2.4
	Mid1	SPCC4B3.15	0	12.9	0	4.1
	Imp1	SPBC1604.08c	5.1	16.6	0	5.1
	Nup107	SPBC428.01c	5.4	19.1	0	1.7
	Mtr10	SPBC11G11.07	0	10.6	0	2.8
	Nup44	SPBC19G7.15	2.2	10.6	0	2.6
	Nup82	SPBC13A2.02	0	8.1	0	1.7
	Nup189	SPAC1486.05	0	2.8	0	1.9
	Sec63	SPBC36B7.03	0	1.6	0	2.0
Nucleoplasm	Tra2	SPAC1F5.11c	3.4	35.3	0	6.7
	Taf73	SPBC15D4.14	8.4	23.7	5.5	23.0
	Taf5/Taf72	SPCC5E4.03c	7.6	22.3	0	7.9
	Imp1					
	Arp42	SPAC23D3.09	0	8.6	2.9	10.6
	Pta1	SPAC1071.01c	1.7	8.0	0	4.3
	Red5	SPBC337.12	0	2.3	0	2.3

*Also present in CC_Nuclear Pore

B



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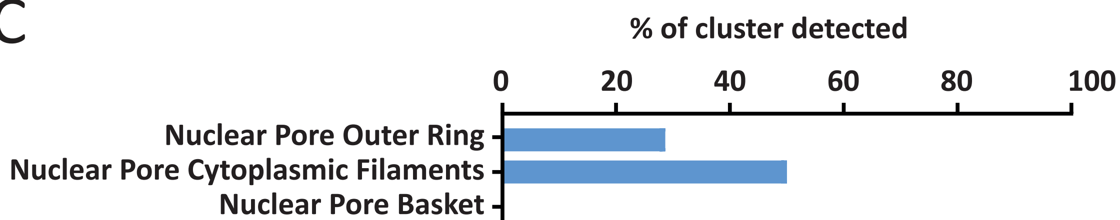


Figure 9: Analysis of the nuclear-related Sec8-interacting proteins reveals components of the nuclear pore complex

(A) Table detailing the Sec8-interacting proteins with the nuclear CC_GO annotations shown. Proteins that also had the annotation “CC_Nuclear Pore” are highlighted in red. The protein scores for each replicate are shown, rounded to 2 d.p. Theses scores are an overall measure of the quality of a protein hit (see text for details). Where a protein appears in both groups, its details have not been listed a second time.

(B) and (C) are graphs demonstrating the representation of different CC_GO terms within the Sec8-interacting proteins. The number of proteins detected is shown as a percentage of the total number of proteins in the genome with that annotation. (B) is examining differenct nuclear structures, and (C) is examining specific components of the nuclear pore complex.

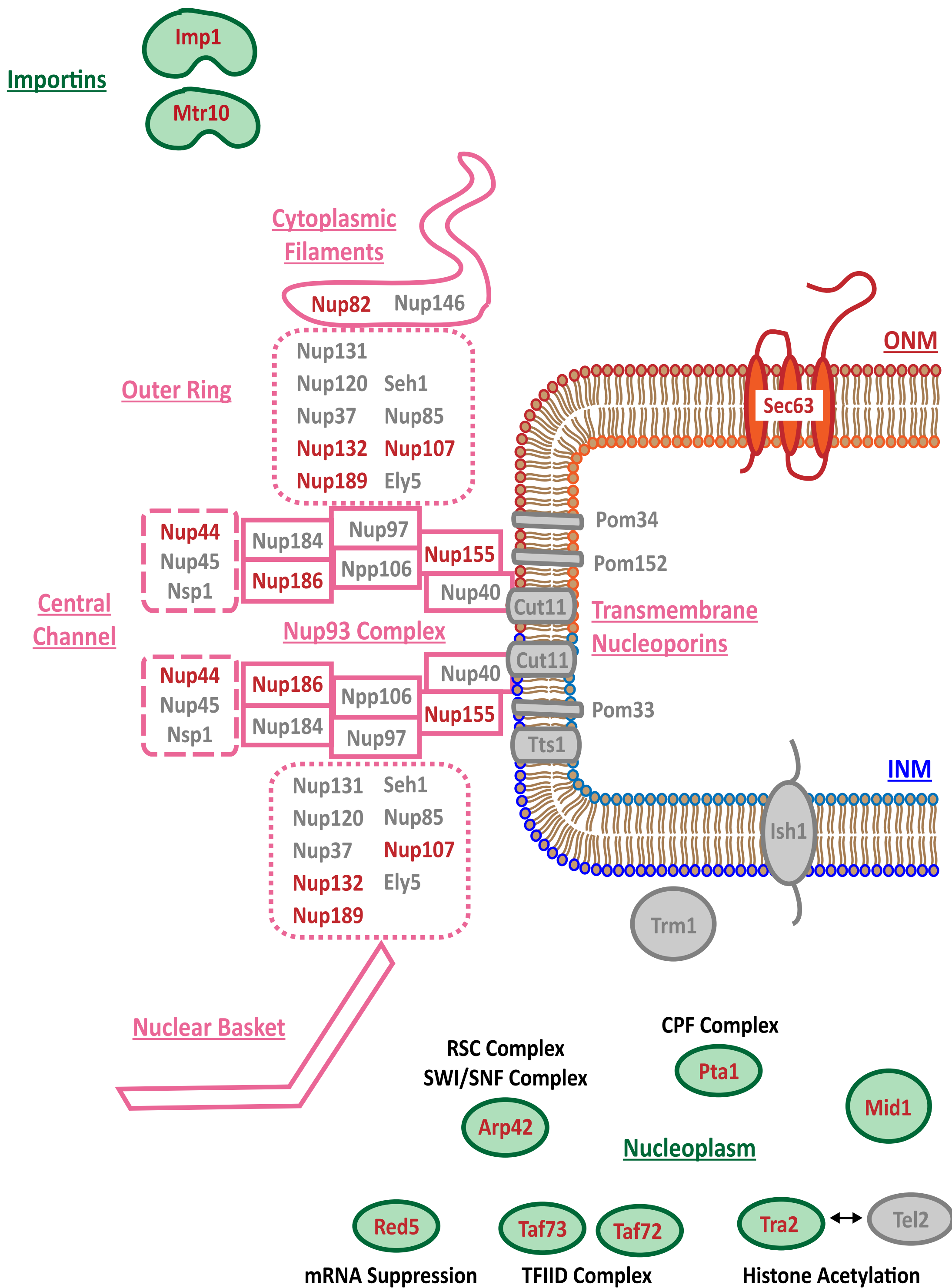


Figure 10: Illustration of the nuclear envelope, nuclear pore and nucleoplasm components captured by the Sec8 GFP-Trap.
Proteins with the GO annotation "CC_Nuclear Envelope" or "CC_Nucleoplasm" are diagrammed above, and labelled with red text. Proteins labelled in grey were not detected in the GFP-Trap, but have been shown to give context. Components of the outer nuclear membrane (ONM) are shown in red, the inner nuclear membrane (INM) in blue, the nuclear pore complex in pink and the nucleoplasm in green. Annotations have been added to give some indication of protein function, where known. The organisation of the nuclear pore complex is based on Asakawa *et al.,* 2014.

3.3. Perturbation of Sec8 Affects Nuclear Volume, but Not Positioning

As the proteomics data suggests an interaction with the nuclear envelope, I sought to examine the nature of this interaction by examining nuclear phenotypes in the *sec8-1* mutant (Wang *et al.*, 2002). This temperature-sensitive mutant exhibits a severe septation defect at all temperatures, but more so at higher temperatures (36°C). The population displays a high septation index even at the supposedly permissive temperature (27°C, which will henceforth be referred to as the semi-permissive temperature). However, as it is currently the only existing *sec8* mutant *S. pombe* strain, it was therefore used in this study.

I created a *sec8-1* strain expressing nuclear envelope marker Pom152-GFP. As it was not detected as a Sec8-interacting protein, it is expected that its localisation will not be affected by mutation of Sec8, making it a suitable choice for a marker. Cells were stained with calcofluor (to mark the cell wall) and live-imaged at the semi-permissive temperature, alongside wildtype cells expressing Pom152-GFP. From these images, measurements were taken and used to calculate the nuclear to cellular volume (N/C) ratio, and the position of the nucleus in relation to the cell centre.

Visual comparison of *sec8-1 pom152-gfp* to wildtype *pom152-gfp* showed no immediately apparent phenotypes (Figure 11A). In both strains, Pom152-GFP appeared to be homogenously distributed along the nuclear periphery. Compared to WT cells, *sec8-1* nuclei showed no obvious defects in size or morphology. However, measurement of the Nuclear/Cellular (N/C) ratio revealed that there is in fact a subtle increase in the mean N/C ratio in the *sec8-1* cells (Figure 11B) (mean N/C ratio WT= 0.10 +/-0.003, n=70 cells; *sec8-1* = 0.11 +/-0.003, n=62 cells).

In fission yeast, the position of the septum is defined by the position of the nucleus in interphase (Daga *et al.*, 2005). I speculated as to whether the severe septation phenotype observed in the *sec8-1* mutant could be linked to a defect in nuclear positioning. To test this possibility, I measured the position of the nucleus relative to both cell ends in WT and *sec8-1* cells (see Methods). The offset of the nucleus was measured as a percentage of the total cell length. These measurements

Figure 11: Mutation of Sec8 mildly affects nuclear volume, but does not affect nuclear positioning

(A) Images of both wildtype and *sec8-1* cells expressing the nuclear envelope marker Pom152-GFP. Cell peripheries have been stained using calcofluor. Scale bar represents 5 µm. (B) Nuclear to cellular volume ratios for both wildtype (n=70) and *sec8-1* (n=62) cells (see text for details on calculation). (C) Frequency distribution showing the displacement of the nucleus from the cell centre, measured as % of cell length, for both wildtype and *sec8-1* strains (see text for details on calculation). n > 50 cells.

were binned into one of 11 categories and displayed in a frequency distribution (Figure 11C). The distributions of the wildtype and *sec8-1* populations appear similar, so mutation of *sec8* does not appear to affect nuclear positioning.

These preliminary data indicate that mutation of *sec8* mildly affects nuclear volume, but does not affect nuclear positioning. However as only one replicate was performed for these datasets, it was not possible to perform statistical analyses to confirm this. Due to time constraints of this study, and the fact that the nuclear phenotype observed was relatively mild, I decided to focus on pursuing the mitochondrial avenue of investigation from this point onwards.

3.4. Clustering of the Sec8 Interaction Partners Reveals Mitochondria-associated Proteins

As well as revealing nuclear-related clusters, inspection of the grouped Sec8-interacting proteins also revealed several mitochondrial-associated clusters. As no interaction between the exocyst and the mitochondria has been previously described, I decided to investigate this putative novel association further.

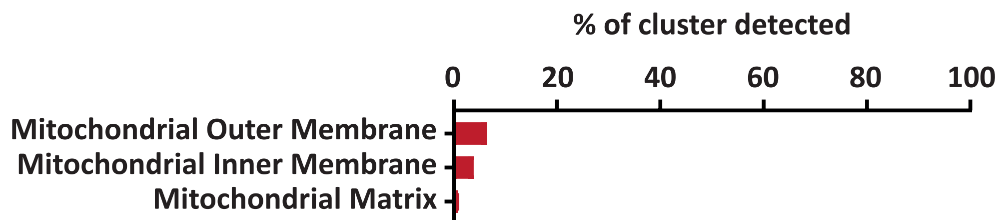
When the Sec8-interacting proteins were grouped by cellular compartment (CC), ‘Mitochondrial membrane’ was the cluster with the sixth highest significance (highlighted red, Figure 8A). It placed higher than clusters such as ‘Golgi’, ‘Cortical Actin Cytoskeleton’ and ‘Plasma membrane’ which link to the exocyst’s know roles in the late secretory pathway. Grouping by biological process (BP) showed that ‘Lipid Metabolism’ and ‘Mitochondrial Transmembrane Transport’ both place in the top 20 significant clusters (highlighted red, Figure 8B).

To further examine the localisation of the mitochondrial Sec8-interacting proteins, I retrieved all with the following CC GO annotations: ‘Mitochondrial outer membrane’, ‘mitochondrial inner membrane’ and ‘mitochondrial matrix’. These are listed with their respective scores in Figure 12A, and have been illustrated in Figure 13 to give some indication of their localisation *in situ.* For each CC GO-term, I calculated what percentage of the total number of proteins in that term were detected as double-positive Sec8 interactors (Figure 12B). 6.4% of outer membrane proteins, 3.8% of inner membrane proteins and 0.7% of matrix

A

	Protein Name	Pombase Identifier	Control Score (R1)	Sec8 Score (R1)	Control Score (R2)	Sec8 Score (R2)
Mitochondrial Outer Membrane	Css1	SPBC32F12.01c	7.1	24.9	1.9	17.5
	Ugo1	SPAC1B2.02c	0	8.1	0	2.6
	Msp1/Yta4	SPCC24B10.10c	0	4.8	0	2.7
Mitochondrial Inner Membrane	Tim50	SPBC17A3.01c	0	8.9	0	7.3
	Cytochrome C	SPCC191.07	0	2.1	2.1	13.5
	Etp1	SPAC22E12.10c	0	5.4	0	2.1
	Mitochondrial RNA-binding Protein	SPBC83.05	0	4.1	0	2.2
	Oxa102	SPAC23D3.09	0	8.6	2.9	10.6
	Mme1	SPAC1071.01c	1.7	8.0	0	4.3
Mitochondrial Matrix	Sod2	SPAC1486.01	0	2.5	2.4	6.6

B



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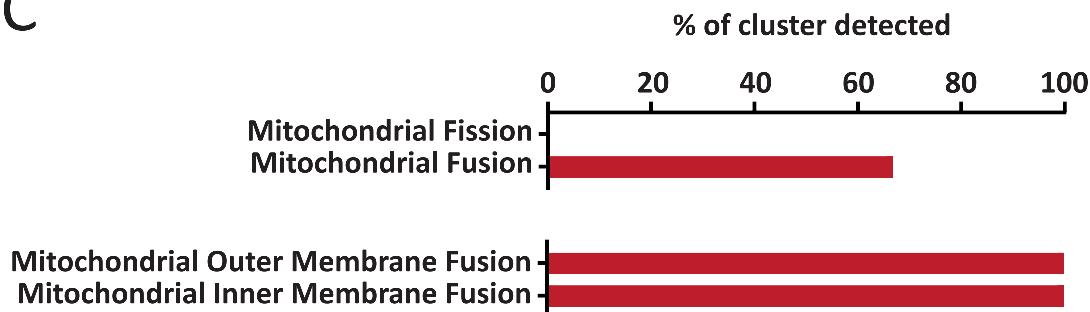


Figure 12: Analysis of the mitochondria-related Sec8-interacting proteins reveals components of the mitochondrial fusion mahcinery.
(A) Table detailing the Sec8-interacting proteins with the mitochondrial CC_GO annotations shown. The protein scores for each replicate are shown, rounded to 2 d.p. Theses scores were provided by the Bristol Proteomics facility, and are an overall measure of the quality of a protein hit (see text for details).
(B) and (C) are graphs demonstrating the representation of different GO terms within the Sec8-interacting proteins. The number of proteins detected is shown as a percentage of the total number of proteins in the genome with that annotation. (B) is examining different mitochondrial structures, and (C) is examining BP_GO annotations regarding mitochondrial fission and fusion.

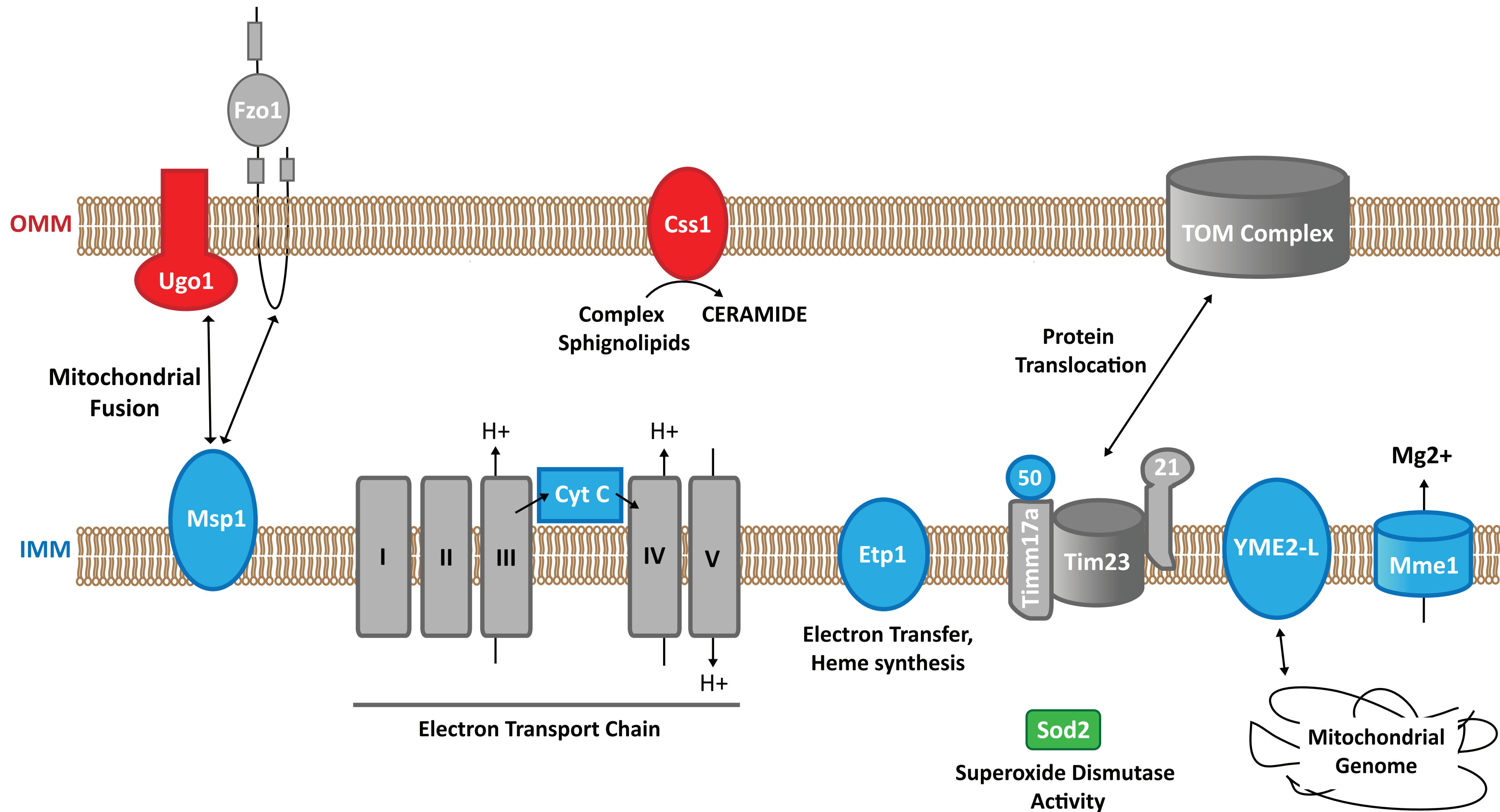


Figure 13: Illustration of the mitochondrial envelope proteins captured by the Sec8 GFP-Trap
Double-positive Sec8-interacting proteins were sorted according to gene ontology terms using the DAVID software. Proteins with the GO annotation "CC_Mitochondrial Envelope" are diagrammed above. Annotations have been added to give some indication of protein function, where known. Outer mitochondrial membrane (OMM) proteins detected are shown in red, and inner mitochondrial membrane (IMM) proteins detected are shown in blue. Proteins shown in grey were not detected in the GFP-TRAP, but have been added to give context.

proteins were detected. This showed that the mitochondrial outer membrane was the best represented cluster, and suggests that this is perhaps where Sec8's interaction is focused. Another possibility is that insufficient permeabilisation during the GFP-TRAP resulted in the entire mitochondrial membrane being pulled down (see discussion for further consideration of these two possibilities).

I further noticed that several of the putative mitochondrial Sec8-interacting proteins were involved in mitochondrial organisation, so I retrieved all the interactors with the BP GO annotations: 'mitochondrial fission' and 'mitochondrial fusion'. Again, I calculated the percentage representation of each cluster as described above (Figure 12C). This showed that none of the proteins required for mitochondrial fission were detected, but 2/3 of the proteins required for mitochondrial fusion were high-confidence Sec8 interactors – Ugo1 and Msp1/Yta4. I therefore retrieved interactors with the annotations 'mitochondrial outer membrane fusion' and 'mitochondrial inner membrane fusion'. Surprisingly, 100% of the proteins in each cluster were detected. Ugo1 and Msp1 are responsible for fusion of the outer and inner membranes respectively. This provides the first evidence for a possible mitochondrial role for Sec8, more specifically in the context of mitochondrial fusion.

3.5. Perturbation of Sec8 Causes a Change in Mitochondrial Morphology

As the proteomics data suggests an interaction between Sec8 and mitochondria, to explore this further I examined mitochondrial morphology upon perturbation of Sec8.

I first attempted to visualise mitochondria in live cells using the Mitotracker dye (data not shown). The mitochondria labelled well in the WT cells, but the staining was very uneven among *sec8-1* cells, even in several replicates. The reason for this was not clear but possibly indicates a functional mitochondrial defect. Nevertheless, this complicated the live-imaging of the mitochondria. To overcome this issue, I instead observed WT and *sec8-1* cells that expressed CoxIV-dsRFP – the *S. cerevisiae* N-termical targeting sequence of cytochrome oxidase subunit IV fused to RFP, which serves as a mitochondrial marker (Yaffe *et al.*, 2003; Chiron *et al.*, 2008) . These cells were live-imaged at the semi-permissive

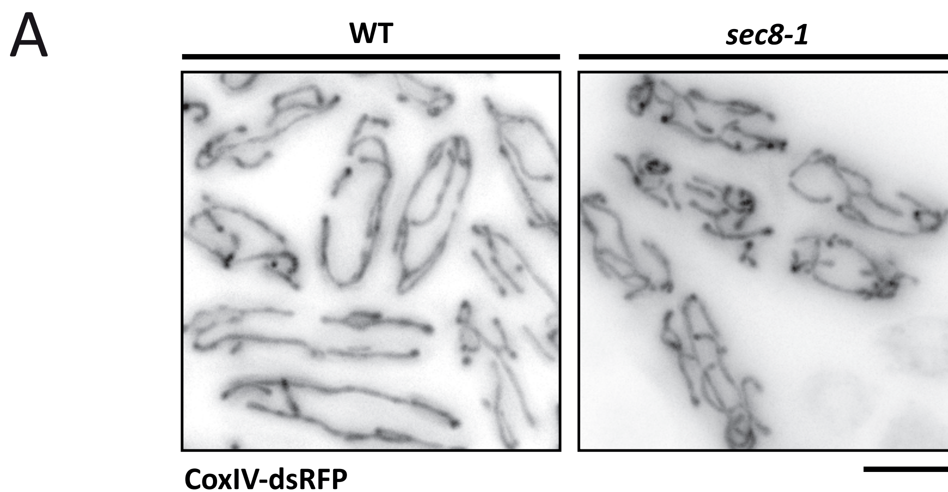
temperature. As described previously (Yaffe *et al.*, 1996), WT cells contain 2-5 long tubular mitochondria that span the length of the cell (Figure 14A). In contrast, in *sec8-1* cells mitochondria appear to be more fragmented, and often loop several times inside the cell. To quantify this phenotype, the images were used to manually assign cells to one of six morphology categories from ‘normal’ to ‘highly fragmented’. The distribution of cells between these morphology categories (Figure 14B) was significantly different in the *sec8-1* population compared to the wildtype (Χ-squared test, p=<0.0001). The proportion of category 1 (normal) cells reduced from 57% to 22%. The percentage of category 2 cells with looped mitochondria increased from 13% to 37%, and the percentage of category 3 cells with short mitochondria increased from 18% to 24%. The percentage of cells with mitochondria in the final three categories, each increasingly more fragmented, were also all increased in the *sec8-1* population compared to the wildtype.

This indicates that perturbation of Sec8 clearly affects mitochondrial morphology, resulting in a decrease in normal morphology and an increase in the presence of both looped mitochondria and shortened fragments.

3.6. Perturbation of Sec8 Causes a Reduction in Mitochondrial Fusion

Mitochondrial morphology is the result of a balance between fusion and fission (aka fragmentation) events (Sesaki *et al.*, 1999). The over-fragmentation phenotype that I have observed in the *sec8-1* mutant could therefore be the result of an excess fission, or defective fusion. To test this, I monitored mitochondria dynamics by live cell imaging in both *sec8-1* and wildtype strains expressing CoxIV-dsRFP at the semi-permissive temperature (Figure 15A). The resulting time-lapses were used to quantify the average number of fission and fusion events per minute in each cell.

Mitochondrial fission does not appear to be statistically different between WT and *sec8-1* cells (WT= 0.49 ± 0.035; *sec8-1* = 0.36 ± 0.051 fission events per minute per cell, unpaired t-test, p = 0.1266, n=10 cells and 13 cells for WT and *sec8-1* respectively, Figure 15B).



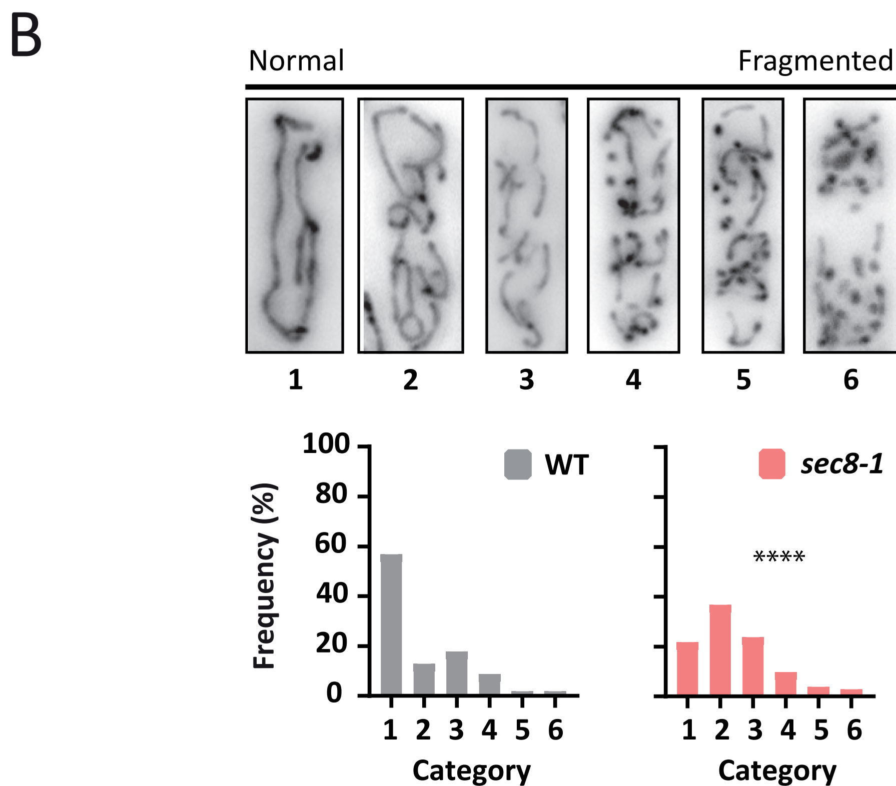
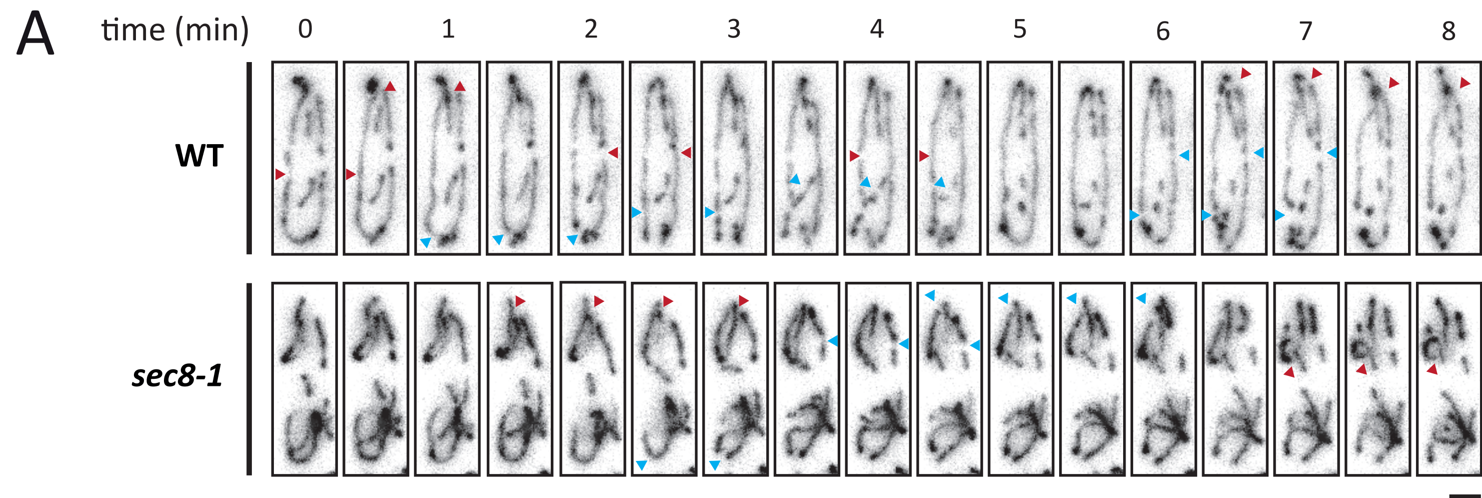
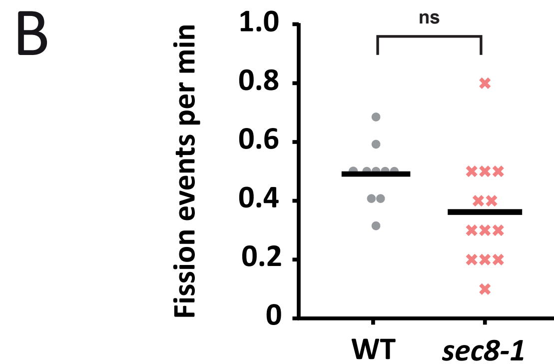


Figure 14: Mutation of Sec8 causes an increase in looped and short mitochondrial tubules

(A) Images of both wildtype and *sec8-1* cells expressing the mitochondrial marker CoxIV-dsRFP. Scale bar represents 5 µm. (B) Analysis of mitochondria morphology. Cells were imaged in 0.5 µm slices, and maximum intensity projections were created from the resulting z-stacks. Mitochondria were scored manually, according to the representative scale shown at the top of the panel. Briefly, 1; Considered 'normal'. Long mitochondria that run the length of the cell. 2; Mitochondria are mixture of short and long tubules that appear looped. 3; Mitochondria are short tubules, but there are no small fragments. 4; Mitochondria are short tubules, and there are some small small fragments. 5; Mitochondria are short tubules, and there are many small fragments. 6; Mitochondria are virtually all small fragments. On the bottom of the panel, the graphs show frequency distributions of different morphology scores in both wildtype and *sec8-1* populations (n=70 and 91 cells respectively. Chi-Squared test, $p<0.0001$).





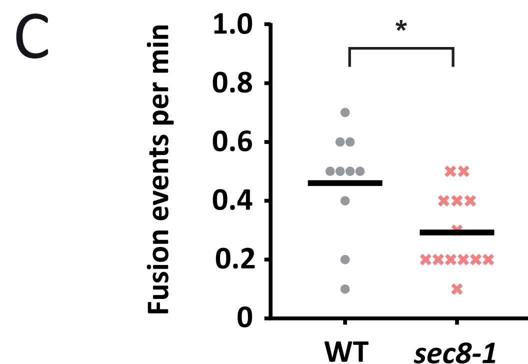


Figure 15: Mutation of Sec8 results in decreased mitochondrial fusion

(A) A z-stack was taken of cells expressing CoxIV-dsRFP every thirty seconds for twenty timepoints. The maximum intensity projection of a z-stack taken at each timepoint is shown for a representative cell from both wildtype and *sec8-1* strains. Fusion events are annotated with red arrows, and fission events with blue arrows. Scale bar represents 2μm. (B,C) Graphs to show the number of (B) mitochondrial fisson and (C) mitochondrial fusion events per minute, in both the wildtype and *sec8-1* strains. In each cell, the number of fission and fusion events at each timepoint was measured (n=10 cells for wildtype, n=13 cells for *sec8-1*). This was used to calculate an average number of events per minute, per cell. These values were plotted on the graphs shown, and the mean is denoted by a bold line. (B: unpaired t-test, p=0.1226. C: Mann-Whitney, p<0.05)

In contrast, and in line with the proteomic data, there was a ~50% reduction in fusion events in *sec8-1* cells compared to the WT (WT= 0.46 ±0.058; *sec8-1* =0.29 ± 0.037 fusion events per minute per cell, Mann-Whitney test, $p<0.05$, $n=10$ cells and 13 cells for WT and *sec8-1* respectively, Figure 15C). This therefore indicates that whilst fission is unaffected, mitochondrial fusion is greatly reduced upon mutation of Sec8.

Discussion

Together, the results of this study indicate two novel putative interactions for Sec8; firstly with constituents of the nuclear envelope (but this was not investigated further due to time constraints). Secondly, both the proteomics data and initial experiments *in vitro* indicate a possible interaction with the mitochondrial fusion machinery. The author acknowledges that it is highly likely that the rest of the exocyst components are involved in these associations (as discussed in Section 1.2.2), but for simplicity I will refer to Sec8 alone in this discussion.

4.1. <u>Evaluating the Success of the GFP-Trap</u>

Whilst the GFP-Trap has seemingly unearthed multiple novel putative interactors of Sec8, it is worth discussing the potential caveats of this particular technique. There was no perfect control for this experiment. Ideally it would have been desirable to have a strain expressing just GFP, but this raises several problems. Which promoter do you express it under? How can you get the same level of expression as endogenous Sec8? Inserting the *gfp* cassette in the place of a different gene could cause potential off-target effects and skew the results. I therefore went with the most straightforward control, which was to use a wildtype strain. The GFP-Trap technique has been used multiple times in the lab, so if any proteins were being pulled down by GFP specifically then we would have consistently detected them across experiments with different fusion proteins. As this is not the case, then I can be fairly confident that the wildtype strain acts as a sufficient control.

By nature, this technique cannot give any indication as to whether the detected proteins are interacting directly with Sec8, and the results are not quantitative. For example, since I pulled down the other members of the exocyst complex as expected, it is possible that many of the detected interacting proteins are interacting with other members of the exocyst complex, and not Sec8 itself. Furthermore, the proteins that do interact with Sec8 are likely pulling their binding partners down too. For example, it is possible that Sec8 directly interacts with chromatin-associated proteins, and that when these were pulled down during the GFP-Trap, components of the inner nuclear membrane (which interact) came

with it, along with NPC components. For this reason the results of the proteomics data required careful interpretation, and were regarded more as ‘hints’ or ‘suggestions’ at interactions (with scores giving an indication of the most significant interactors), which were in turn used to guide the *in vitro* studies. A technique such as reciprocal co-immunoprecipitation or yeast two-hybrid would be required to verify whether any of these interactors directly bind to Sec8, and to determine the strength of such an interaction.

In addition, as discussed in Section 1.4.1, the exocyst appears to exist in multiple conformations in different subcellular locations, likely performing different roles. In this study, it is perhaps possible that the Sec8-GFP fusion protein used in the GFP-Trap may only have been able to assemble with the rest of the complex in certain conformations due to the GFP tag. This would mean that the Sec8-interacting proteins would only be detected for a subset of the cellular functions which the exocyst is performing.

Nonetheless, GFP-Trap has provided several useful insights in this study, which I will henceforth discuss.

4.2. Novel nuclear roles for Sec8

Prior to this study, the only described nuclear role of Sec8 was its involvement in the DNA repair pathway (outlined in Section 1.3.4), where it acts as a physical platform and acts to influence the expression of several signalling and histone modifying proteins (Torres *et al.*, 2015). In addition, subpopulations of Sec8 co-localise with members of the DNA repair pathway inside the nucleoplasm. However, to the author’s knowledge no association with the nuclear envelope has been described, despite Sec8 being observed to aggregate around the nucleus in several studies (Fölsch *et al.*, 2003; Chen *et al.*, 2006; Pohl *et al.*, 2008). This GFP-Trap in this study revealed multiple constituents of the nuclear envelope to be putative Sec8-interacting proteins. Furthermore, the DAVID analysis showed that 70% of the Sec8-interacting proteins in the ‘nuclear envelope’ cluster localise to the nuclear pore, which indicates that this is where Sec8’s role is focused. Interestingly in yeast, a functional link between nuclear pores and the DNA damage repair has been demonstrated; persistently unrepaired DSBs migrate to and stably associate with nuclear pores, where it is thought that repair systems may be coordinated (Nagai *et al.*, 2008). Given Sec8’s demonstrated role in

coordinating DNA repair systems in mammalian cells, it is an intriguing possibility that it is also performing this role in yeast, specifically at the nuclear pore complex.

Another possibility is that Sec8 aids in the formation of NPCs. In order to build an NPC, a fusion event between the inner and outer nuclear membranes is first required to form the ‘pore’ into which NPC proteins are then inserted (Reviewed in Rothballer *et al.*, 2013). This fusion event is analogous to the fusion of a secretory vesicle to an accepting membrane, so it is exciting to speculate that Sec8 may perform a similar role in helping to tether the two membranes. However, further examination of the specific NPC components detected in the GFP-Trap makes this seem unlikely. The fact that ‘cytoplasmic filaments’ is the most represented structural group of the NPC implies that Sec8 is interacting with the cytoplasmic face of the pore. However, there are only a total of 2 proteins in ‘cytoplasmic filaments’, which makes it easier to detect a high percentage compared to say ‘outer ring’, which has 7 proteins in total. Yet the fact that none of the membrane-bound nucleoporins were detected (see Figure 10) supports this deduction, and further indicates that Sec8 is likely interacting with NPCs when they are already formed, not during membrane fusion or nucleoporin recruitment.

So if Sec8 is not functioning during NPC formation, perhaps it may perform a transport role instead. Two of the other proteins in the ‘nuclear envelope’ cluster, Imp1 and Mtr10, are importins - these help to deliver cargo into the nucleus. Importin-αs bind cargo in the cytoplasm, whilst importin-βs bind to the importin-αs and transport the cargo into the nucleus. As discussed in Section 1.1.3, many of Sec8’s emerging non-conventional roles show that Sec8 can modify protein expression in several cellular contexts, but the mechanism behind this is not known. If Sec8 is involved in nucleocytoplasmic transport then this could provide a potential answer, but this requires further investigation.

I observed that mutation of Sec8 causes a mild increase in nuclear volume. Given that the factors that govern nuclear volume are not well understood (Reviewed in Webster *et al.*, 2009), it is difficult to speculate how Sec8 may be involved. The consensus in yeast seems to be that it is heavily influenced by both the cytoplasmic volume (Jorgensen *et al.*, 2007; Neumann *et al.*, 2007) and the

the cytoplasmic volume (Jorgensen *et al.*, 2007; Neumann *et al.*, 2007) and the stage of the cell cycle (Kume *et al.*, 2017). Given that the *sec8-1* mutant is blocked at septation, the increase in the N:C ratio may be an artefact of this, so would need to be investigated further (Kume *et al.*, 2017).

However, the nuclear import of proteins has also been implicated in controlling nuclear size (Newport *et al.*, 1990; Brandt *et al.*, 2006; D'Angelo *et al.*, 2006; Dittmer *et al.*, 2007; Kume *et al.*, 2017). It is possible that Sec8 somehow regulates the transport of certain proteins through the nuclear pore, perhaps by influencing importins as discussed above. In its absence, certain factors may be over or under imported, resulting in the mild change in nuclear volume I observed.

4.3. Novel Mitochondrial Roles for Sec8

The only notable publication examining both the exocyst and mitochondria is a 2014 study by Luo *et al.* who demonstrated that by fusing exocyst components with portions of mitochondrial outer membrane protein Tom20, the exocyst complex and vesicle trafficking could be ectopically targeted to the mitochondria. It demonstrated that ectopically-localised Sec3 could recruit the rest of the complex and reroute vesicular traffic to the mitochondria. This serves as a proof-of-principle that exocyst members are still able to recruit their interactors, no matter their cellular location. If a subset of the exocyst is interacting with the mitochondrial envelope, then it is possible that it can target other proteins there too. The study points out that secretory vesicles cannot fuse with the mitochondria due to the lack of complementary tSNAREs. This, in addition to the fact that cytoplasmic transport of proteins to the mitochondria appears to be dependent on cytoplasmic chaperones such as Hsp70 (Young *et al.*, 2003), and also the hydrophobicity and charge of the protein (Costello *et al.*, 2017), makes it unlikely that the exocyst is involved in trafficking proteins to the mitochondria. That being said, chaperones like Hsp70 have been shown to localise at both the plasma membrane and in cytoplasmic vesicles (VanBuskirk *et al.*, 1991; Singh *et al.*, 1997), so whilst unlikely the possibility still exists.

An obvious challenge to this putative association with mitochondria is that Sec8 has never been demonstrated to localise to mitochondria. It may be that the subpopulation of Sec8 there is so small that the signal it is not detected by immunofluorescence, as it is so abundant in other cellular locations. Furthermore,

ectopic targeting of Sec3 to mitochondria in no way affected mitochondrial morphology (Luo *et al.*, 2014), which challenges the clear phenotype I have observed upon disruption of Sec8. I cannot explain this discrepancy at present, especially as a further GFP-Trap performed in our lab using Sec3 detected even more mitochondrial interacting partners (unpublished lab data) and seems to support this association.

An initial examination of mitochondrial morphology in the *sec8-1* mutant showed that perturbation of Sec8 clearly affects mitochondrial morphology. The category with the highest frequency was 'short looped' mitochondria (37%), followed by 'short tubular' mitochondria (24%). So how might mutation of Sec8 cause an increase in short mitochondria? The simplest explanation could be the health of the cells. As mentioned previously, the *sec8-1* mutant exhibits a severe septation defect, even at lower temperatures. Mitochondria are very sensitive to the health of the cell, and it could be that the mitochondria are fragmented because these cells are very stressed and sick. That being said, unhappy cells tend to produce mitochondrial fragments rather than short tubules (unpublished lab observation). The *sec8-1* cells survive for several days, and even when the septum is not broken down, the individual cell compartments still appear to progress normally through the cell cycle. Therefore, if the excess fragmentation is not due to cell stress, Sec8 is potentially influencing mitochondrial morphology itself, either directly or indirectly.

The proteomics data was able to provide some clues in this respect. The results of the GFP-Trap indicate that whilst two out of three members of the mitochondrial fusion machinery are putative Sec8 interactors, none of the three members of the fission machinery are. This was supported by examination of mitochondrial dynamics, which showed that whilst the rate of fission was unaffected by mutation of Sec8, the rate of fusion was significantly reduced. This reduction in fusion could explain the increased proportion of cells with shorter mitochondria tubules and increased fragments. Mitochondria naturally fragment during mitosis, and then recover their longer morphology after cytokinesis (Jourdain *et al.*, 2009). In the *sec8-1* cells, with fission unaffected the mitochondria should still be able to fragment during mitosis, but with a reduced rate of fusion they may take longer to recover after cytokinesis. This could even be exacerbated by the previously described cytokinesis defect.

So Sec8 is somehow required for the efficient functioning of the mitochondrial fusion machinery. How might this interaction work? Mitochondrial fusion (Reviewed in Westermann, 2008) is thought to consist of four main steps, which have been better elucidated in *S. cerevisiae*. Briefly, the two outer membranes of opposing mitochondria must first dock. The large outer-membrane GTPase Fzo1 (homolog of the mammalian mitofusins) plays a key role in forming *trans*-complexes at this point. Next, lipid bilayer mixing occurs, merging the two membranes. Docking must then occur between the two inner membranes; the inner membrane GTPase Mgm1 (the orthologue of Msp1 in *S. pombe* and Opa1 in mammalian cells) can form *trans*-complexes and has been shown to be required for this step. Again, lipid bilayer mixing must then occur, thus creating a single double-membrane bound mitochondria. Ugo1 binds to both Fzo1 and Mgm1, so is thought to coordinate both the interaction between these two proteins, and the fusion of the two membranes.

Given the exocyst's role in the fission/fusion of secretory vesicle and target membranes, it is possible that it may perform a similar function in helping these proteins to fuse mitochondrial membranes (Figure 16A). It is interesting that only Msp1 and Ugo1 were detected as double positive Sec8-interacting proteins, and not Fzo1. Fzo1 was detected as a true positive, but only in one replicate. However, as several other mitochondrial inner membrane proteins were also detected, for the sake of discussion I will entertain that the interaction with Ugo1 and Msp1 is more significant. It would appear more plausible that the exocyst could help to tether two adjacent mitochondria in sufficient proximity for Fzo1 complexes to occur. However given the proteomics, it seems that the exocyst could possibly help to tether the inner mitochondrial membranes instead in order to allow *trans*-complexes of Msp1 to form. Whilst it has been shown that Msp1 and Fzo1 can form *trans*-complexes and that they are key mediators of fusion, and that as GTPases they have the potential to generate the energy required, it has not yet been experimentally demonstrated that they can function as fusogens. Thus, the exocyst could be a plausible candidate for assisting in mitochondrial fusion by tethering membranes. It may also regulate Ugo1 similarly to how it mediates SNARE regulating proteins, as Ugo1 is thought to be involved in mediating the formation of Msp1 or Fzo1 *trans*-complexes.

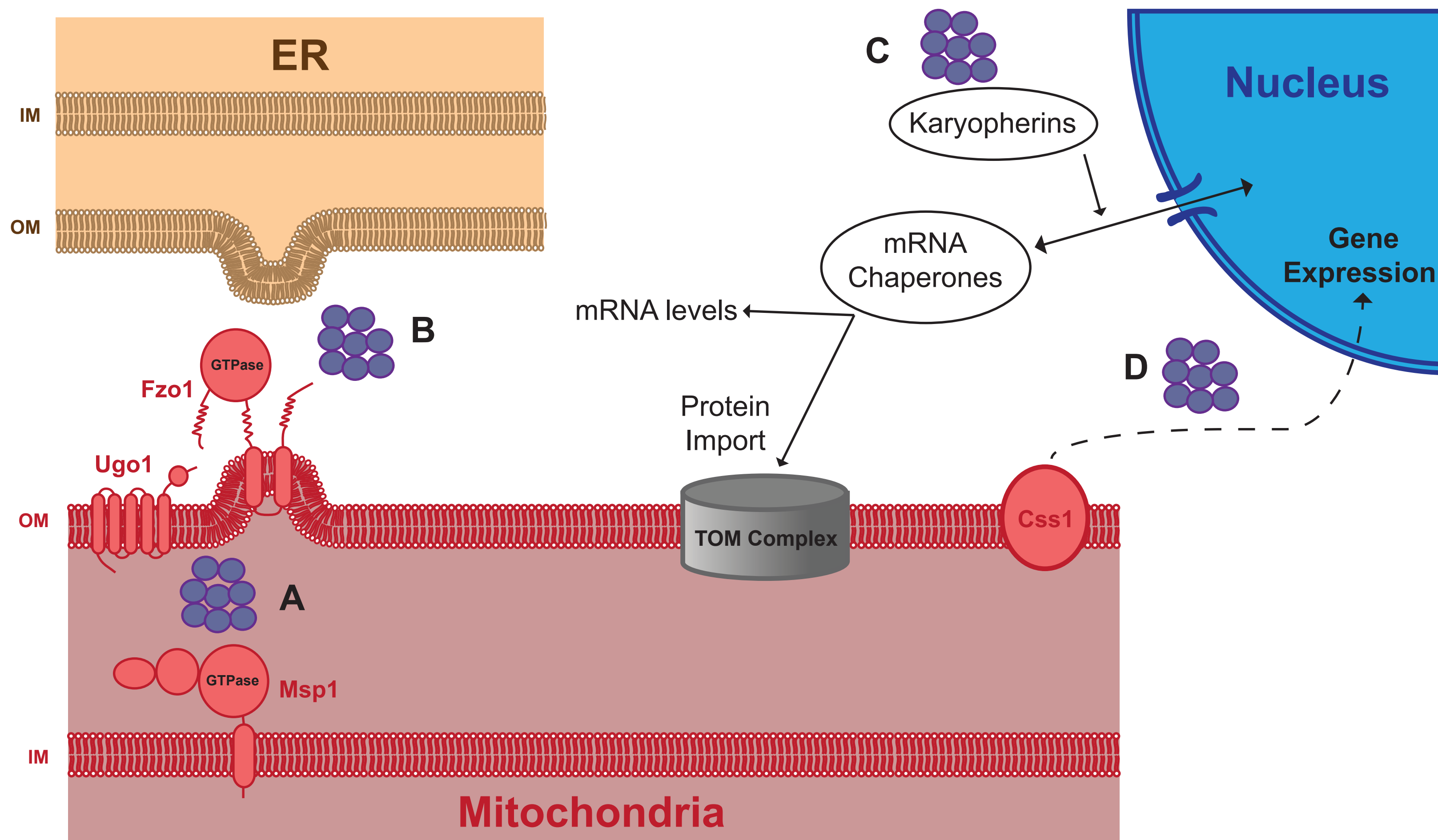


Figure 16: Hypothetical models of Sec8's putative association with mitochondria
Potential mechanisms by which Sec8 may interact with mitochondria: (A) Tethering membranes or interacting with proteins during mitochondrial fusion (B) ER/Mitochondrial tethering (C) Indirectly influencing the levels of cytoplasmic chaperones by interacting with karyopherins (D) Participating in Css1's ability to modulate nuclear gene expression. ER = Endoplasmic Reticulum, IM = Inner membrane, OM = Outer Membrane.

Another possibility is that Sec8 is somehow participating in the tethering between the ER and mitochondria (Figure 16B). This is one of the most extensively described organelle contact sites (Reviwed in Rowland *et al.*, 2012) and is known to regulate mitochondrial distribution and dynamics. Clustering of the Sec8-interacting partners by cellular compartment revealed that as well as mitochondrial envelope proteins, there was a cluster of ER proteins which was the third-most significant (Figure 8). The gap between the opposing membranes at ER/mitochondria contact sites is approximately 10-30nm (Csordás *et al.*, 2006; Friedman *et al.*, 2011), which is close enough to suppose that proteins on opposing membranes interact to form a tether (Rowland *et al.*, 2012).Whilst an important role for such contact sites has been predominantly described for mitochondrial fission and distribution, an interplay with the mitochondrial fusion machinery has also been implicated. The mitofusins (mammalian orthologues of Fzo1) have been long thought to be involved in ER-mitochondria tethering (Kornmann, 2013; Raturi *et al.*, 2013; Klecker *et al.*, 2014; Prinz, 2014), but more recent studies indicate that they are actually antagonists of such tethering, as knockdown appears to promote an excessive and potentially harmful amount of tethering between the ER and mitochondria (Cosson *et al.*, 2012; Filadi *et al.*, 2015). However, Fzo1 was a weak candidate as a Sec8-interacting partner, and no components of the ER-mitochondria tethering complex were detected. So it is possible that the exocyst is not interacting directly, instead serving a separate function. Perhaps it tethers the membranes in close enough proximity for complexes to form at contact sites? Perhaps it stabilises Ugo1 and/or Msp1's associations with Fzo1 to maintain the integrity of inner/outer membrane contact sites?

The inefficiency of the Mitotracker stain on these cells is also worth noting, as this indicates that the mitochondria may also have a functional defect. Perhaps Sec8 may be responsible for the delivery of other mitochondrial components, and that its absence therefore affects processes beyond just fusion. Whilst it was not possible to examine mitochondrial function within the time constraints of this study, it leaves exciting potential for further work.

It is intriguing to speculate as to whether this putative association is conserved in mammalian cells. As discussed in Section 1.1.3, Sec8 can influence the expression levels of Pirh2 (an E3 ubiquitin ligase) which in turn affects the

organisation of intermediate filament protein cytokeratin 8 (Tanaka *et al.*, 2015). Disruption of the interaction between Pirh2 and cytokeratin 8 leads to the aggregation of cytokeratin 8 filaments and mitochondrial redistribution, likely by affecting a microtubule-dependent process (Duan *et al.*, 2009). It has therefore been previously speculated (Tanaka *et al.*, 2015) that regulation of the Pirh2-Cytokeratin 8 interaction by Sec8 could potentially influence the distribution of mitochondria. This would be an intriguing possibility to test in future.

4.4. Links Between the Exocyst, the Nucleus and Mitochondria?

One of the karyopherins detected in the GFP-Trap - Mtr10 - is an importin β-like protein, whose function has not been characterised in *S. pombe.* However in *S. cerevisiae* it has been shown to be required for nucleocytoplasmic transport of Npl3. Npl3 is a chaperone essential for mRNA export, that has also been shown to be responsible for mitochondrial protein targeting (Ellis *et al.*, 1993). Mutation of Npl3 increased its residency in the cytoplasm, increased localisation to polysomes (clusters of cytoplasmic ribosomes translating an mRNA), and stabilised multiple cytoplasmic mRNAs, including those encoding mitochondrial precursor proteins (Gratzer *et al.*, 2000). Overexpression of other karyopherins known to transport Npl3 suppresses defects in mitochondrial protein import (Belgareh *et al.*, 1999). It was therefore suggested that Npl3 may indirectly influence co-translational protein import into mitochondria. As discussed previously, the mechanism by which Sec8 is able to influence protein expression in multiple cellular contexts is not well understood. If it is interacting with karyopherins, and subsequently somehow affecting the levels of cytoplasmic chaperones (Figure 16C), then this could be affecting mRNA levels and subsequent protein expression of mitochondrial proteins (and many others). Npl3 shows 32% coverage with mRNA export factor Srp2 in *S. pombe,* so it would be interesting to investigate whether the expression or cytoplasmic residency time of Srp2 (or even other mRNA chaperones) is affected in the *sec8-1* mutant.

Another interesting result from the proteomics was the detection of Css1 as a putative Sec8-interacting partner. It had the highest scores of all the mitochondrial proteins detected. This enzyme is the ortholog of Isc1, the *S. cerevisiae* inositol sphingolipid phospholipase C. Sphingolipids are both key structural components

of the plasma membrane and essential signalling molecules. Isc1 catalyses the hydrolysis of complex sphingolipids to produce ceramide: the enzyme at the centre of the sphingolipid pathway. Ceramide can influence several different processes including apoptosis, cell cycle progression, DNA damage response and differentiation (Reviewed in Tripathi *et al.*, 2015). Isc1 sits in the mitochondrial outer membrane in the post-diauxic phase (De Avalos *et al.*, 2004; Kitagaki *et al.*, 2007), and as well as an involvement in apoptosis (Almeida *et al.*, 2008) it has also interestingly been shown to somehow regulate nuclear gene expression (Kitagaki *et al.*, 2009). When *S. cerevisiae* encounters low levels of environmental glucose, extensive metabolic adaptation and changes in gene expression occur, which is known as the 'diauxic shift'. Many genes whose expression is induced during diauxic shift are not induced in Isc1-deficient cells (Kitagaki *et al.*, 2009). The authors speculate that Isc1 may function in signalling the health of the mitochondria to the rest of the cell, and thus affect the import of respiratory substrates into mitochondria. However the mechanism by which this is achieved is not fully clear, and how Sec8 may be involved is interesting to speculate. Sec8 is already known to be involved in several signalling pathways, so this may suggest an additional role in mitochondrial signalling (Figure 16D).

4.5. Conclusions

GFP-Trap has proved an extremely useful technique in providing hints at novel cellular interactions. I have unearthed putative associations between Sec8 and the nuclear envelope, the mitochondrial envelope and the mitochondrial fusion machinery, none of which have been previously described.

I have shown that Sec8 appears to be involved in regulating mitochondrial morphology, likely through mitochondrial fusion. In order to further elucidate the nature of Sec8's interaction with mitochondria, there are several important questions that will need to be answered. The putative Sec8-interacting partners need to be validated by a technique such as reciprocal co-immunoprecipitation, yeast two hybrid, or by a study of genetic interactions. This would help confirm whether Sec8 does indeed bind to these proteins. If so, then the next question that would need to be answered - does Sec8 localise to the mitochondrial envelope? If so, then is it on the outer membrane or in the intermembrane space? How does it get there? If it does not localise to mitochondria, then where is it interacting with these proteins? The fact that two GFP-Traps performed in our lab (one on Sec8 and one on Sec3) both detected mitochondrial interacting proteins suggests that either this mitochondrial association is not Sec8 specific, or that this is an artefact of the method i.e. incomplete permeabilisation resulted in the pulling down of the entire mitochondrial envelope. However, given that thee functional analyses seem to confirm an involvement with mitochondria, the latter seems less likely. In addition, it would be interesting to see if this involvement with mitochondria is conserved in mammalian cells. Unpublished preliminary data I have collected (not shown) does appear to support this. This potentially links in with one of Sec8's already described roles in regulating the Pirh2/cytokeratin 8 interaction. Furthermore, abnormal mitochondrial morphology has been described in several human diseases (Trimmer *et al.*, 2000; Chen *et al.*, 2009), which highlights the need to better characterise Sec8's (or most likely the exocyst's) involvement.

Functional experiments also demonstrated that mutation of Sec8 mildly affects nuclear volume. As that the mechanisms which regulate nuclear volume are still not fully understood, it will require extensive further investigation to determine how Sec8 is involved. However, given that some existing evidence seems to

suggest that Sec8 may be able to regulate protein expression, it is perhaps worth pursuing.

Appendices

Appendix Table 1: *Schizosaccharomyces pombe* strains used in this study

Strain Name	Inventory Number	Genotype	Source
Wild-type	IJ1209	*972* h$^-$	Lab stock
Wild-type	IJ137	*ade6-M216 leu1-32 ura4-D18* h$^-$	Lab stock
sec8-1	IJ846	*sec8-1 leu1-32 ura4-D18* h$^+$	Balasubramanian Lab[a] (Wang *et al.*, 2002)
sec8-1	IJ881	*sec8-1 ade6-M216 leu1-32* ura4-D18 h$^+$	Lab Stock
sec8-gfp	IJ869	*sec8-gfp-ura4 leu1-32* h$^-$	Balasubramanian Lab[a] (Wang *et al.*, 2002)
pom152-gfp	IJ489	*pom152-gfp-kanMX6 leu1-32 ura4-D18* h$^-$	Toda Lab[b]
sec8-1 pom152-gfp	IJ1555	sec8-1 *pom152-gfp-kanMX6 leu1-32 ura4-D18*	This study
coxIV-dsrfp	IJ426	*ade6-M210 leu1-32::nmt1::coxIVdsrfp:leu1+ ura4-D18 h*$^-$	Yaffe Lab[c] (Chiron *et al.*, 2008)
sec8-1 coxIV-dsrfp	IJ1553	*sec8-1 leu1-32::nmt1::coxIVdsrfp:leu1+*	This study

[a] - The Cancer Research UK Cambridge Institute, UK.
[b] - Hiroshima University, Japan.
[c] - Koch Institute for Integrative Cancer Research at MIT, Cambridge, MA.

Appendix Table 2: Recipe for rich medium (YE5S). Solution was made up in water, and the pH adjusted to 5.6 using HCL.

Ingredient	Manufacturer	Concentration
Yeast Extract	VWR Chemicals	5 g/L
Glucose	Fisher Scientific	30 g/L
Uracil	Sigma Aldrich	250 mg/L
Adenine	Sigma Aldrich	250 mg/L
Histidine	Sigma Aldrich	250 mg/L
Lysine	Sigma Aldrich	250 mg/L
Leucine	Sigma Aldrich	250 mg/L

Appendix Table 3: Recipe for mating medium (ME4S). Solution was prepared in water, and the pH adjusted to 7.6 using HCL.

Ingredient	Manufacturer	Concentration
Difco Malt Extract	Oxoid	30 g/L
Histidine	Sigma Aldrich	75 mg/L
Adenine	Sigma Aldrich	75 mg/L
Uracil	Sigma Aldrich	75 mg/L
Leucine	Sigma Aldrich	75 mg/L

Appendix Table 4: Recipe for freezing medium (YFM). Solution was prepared in water.

Ingredient	Manufacturer	Concentration
Yeast Extract	VWR Chemicals	10 g/L
Bacto tryptone	BD Biosciences	10 g/L
Glucose	Fisher Scientific	20 g/L
Glycerol	Fisher Scientific	25%

Appendix Table 5: Recipe for minimal medium (EMM) supplemented with amino acids. Solution was prepared in water, and the pH adjusted to 5.6 using HCL.

Ingredient		Manufacturer	Concentration
Potassium hydrogen phtalate		Sigma Aldrich	3 g/L
Na_2HPO_4		Alfa Aesar	2.2 g/L
NH_4Cl		Fisher Scientific	5 g/L
Adenine		Sigma Aldrich	250 mg/L
Uracil		Sigma Aldrich	250 mg/L
Leucine		Sigma Aldrich	250 mg/L
Glucose			5%
Salts Solution			2%
$MgCl_2\ _6H_2O$	5.2 mM	Fisher Scientific	
$CaCl_2\ _2H_2O$	0.1 mM	Alfa Aesar	
KCl	13.4 mM	Fisher Scientific	
Na_2SO_4	0.28 mM	Fisher Scientific	
Vitamins Solution			0.01%
Pantothemic Acid	4.2 µM	Sigma Aldrich	
Nicotinic Acid	81.2 µM	Sigma Aldrich	
Myo-inositol	55.5 µM	Sigma Aldrich	
Biotin 100X	40.8 µM	Sigma Aldrich	
Minerals Solution			0.001%
Boric acid	8.1 µM	VWR Chemicals	
$MnSO_4$	2.37 µM	Sigma Aldrich	
$ZnSO_4\ _7H_2O$	1.39 µM	Alfa Aesar	
$FeCl_2\ _6H_2O$	0.74 µM	Sigma Aldrich	
Molybdic acid	0.25 µM	VWR Chemicals	
KI	0.6 µM	Sigma Aldrich	
$CuSO_4\ _5H_2O$	0.16 µM	Sigma Aldrich	
Citric acid	4.76 µM	Alfa Aesar	

Appendix Table 6: Recipe for Extraction Buffer (EB). Solution was prepared in water, used at 4°C and stored at 20°C.

Ingredient	Manufacturer	Concentration
EGTA	Sigma Aldrich	5mM
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	5mM
HEPES	Sigma Aldrich	50mM
NaF	Sigma Aldrich	50mM
Na-β-glycerophosphate	Sigma Aldrich	50mM
Phenylmethylsulphonyl fluoride (PMSF)	Sigma Aldrich	1mM
Protease Inhibitor Cocktail	Sigma Aldrich	1x
Triton X100	Sigma Aldrich	0.2%

Appendix Table 7: Recipe for 10% Polyacrylamide Running Gel. Solution was prepared in water.

Ingredient	Manufacturer	Concentration
Tris-HCl (pH=8.8)	Sigma	0.375 mM
SDS	Sigma	0.0002%
Protogel Runing Gel	National diagnostics	0.0801%
APS	Sigma	0.0005%
TEMED	Sigma	0.001%

Appendix Table 8: The double-positive Sec8-interacting proteins

Uniprot Acc.	Gene Name	Description	WT (1) Score	Sec8 (1) Score	Unique Peptides	Score Change	Fold Increase	WT (2) Score	Sec8 (2) Score	Unique Peptides	Score Change	Fold Increase
O74562	***sec8***	Exocyst complex component sec8 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sec8 PE=1 SV=2 - [SEC8_S	11.58	790.59	85	779.01	68.27	0.00	435.43	63	435.43	
O74846	***sec6***	Exocyst complex component sec6 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sec6 PE=1 SV=2 - [SEC6_S	2.86	280.85	33	278.00	98.22	0.00	146.99	26	146.99	
O75006	***sec15***	Exocyst complex component sec15 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sec15 PE=3 SV=2 - [SEC1	0.00	259.09	35	259.09		0.00	102.12	26	102.12	
O13705	***sec10***	Exocyst complex component sec10 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sec10 PE=1 SV=2 - [SEC1	0.00	255.99	51	255.99		0.00	104.84	33	104.84	
O94598	***sec5***	Exocyst complex component sec5 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sec5 PE=1 SV=1 - [SEC5_S	1.84	170.08	32	168.24	92.52	0.00	71.32	24	71.32	
O14226	***exo84***	Exocyst complex component exo84 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=exo84 PE=3 SV=2 - [EXO8	0.00	168.42	26	168.42		0.00	60.52	16	60.52	
Q10339	***exo70***	Exocyst complex component exo70 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=exo70 PE=1 SV=1 - [EXO7	0.00	130.21	28	130.21		0.00	41.36	14	41.36	
Q10411	***spo15***	Sporulation-specific protein 15 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=spo15 PE=1 SV=1 - [SPO15_S	29.98	103.30	33	73.32	3.45	17.55	47.89	13	30.34	2.73
O14065	***tcb1***	Tricalbin, C2 domain protein (phospholipid binding) ER-plasma membrane tethering protein Tcb1 (predicted)	33.58	91.05	22	57.47	2.71	14.36	55.55	18	41.19	3.87
Q10324	***sec3***	Uncharacterized protein C17G8.12 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC17G8.12 PE=4 SV=1	0.00	100.29	25	100.29		0.00	29.17	11	29.17	
Q9UUE2	***csx2; cnt5***	Protein csx2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=csx2 PE=1 SV=1 - [CSX2_SCHPO]	6.80	32.78	11	25.98	4.82	13.11	35.84	9	22.72	2.73
Q1MTR3	***vid27***	Vacuolar import and degradation protein 27 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=vid27 PE=1 SV=1 -	11.81	35.55	10	23.74	3.01	6.80	22.02	6	15.22	3.24
O14072	***cta4***	Manganese-transporting ATPase 4 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=cta4 PE=3 SV=1 - [ATC4_S	14.08	35.80	12	21.72	2.54	7.46	20.64	9	13.18	2.77
Q09891	**Unassigned**	Putative phospholipid-transporting ATPase C24B11.12c OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC	8.09	36.65	13	28.57	4.53	0.00	13.06	5	13.06	
Q09857	***uso1***	Intracellular protein transport protein uso1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=uso1 PE=3 SV=3 - [	8.04	35.30	13	27.26	4.39	2.43	13.69	7	11.25	5.62
O74319	***taf73***	Transcription initiation factor TFIID subunit taf73 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=taf73 PE=1 SV	8.36	23.66	8	15.30	2.83	5.50	22.98	9	17.47	4.18
O13791	***slt1***	Protein slt1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=slt1 PE=1 SV=3 - [SLT1_SCHPO]	4.67	33.62	9	28.95	7.20	1.77	12.18	5	10.41	6.90
Q9UT35	***gdp1***	Guanosine-diphosphatase OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=gdp1 PE=3 SV=1 - [GDA1_SCHPO]	9.40	25.71	8	16.31	2.73	6.65	17.13	7	10.48	2.58
O74369	***css1***	Inositol phosphosphingolipids phospholipase C OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=css1 PE=1 SV=	7.14	24.89	11	17.75	3.49	1.92	17.47	9	15.55	9.08
Q10064	***tra2***	NuA4 complex phosphatidylinositol pseudokinase complex subunit Tra2	3.36	35.29	16	31.92	10.49	0.00	6.65	9	6.65	
O14283	***prr1***	Transcription factor prr1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=prr1 PE=1 SV=2 - [PRR1_SCHPO]	10.87	27.96	8	17.09	2.57	2.41	13.57	5	11.16	5.63
P78847	***nup186***	Nucleoporin nup186 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nup186 PE=2 SV=2 - [NU186_SCHPO]	6.77	18.81	9	12.03	2.78	5.96	20.45	7	14.49	3.43
O14290	**Unassigned**	Unassigned BCAP family homolog C9E9.04 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC9E9.04 PE=	5.35	14.19	5	8.84	2.65	8.80	23.85	6	15.04	2.71
O74349	***rkr1***	E3 ubiquitin-protein ligase listerin OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rkr1 PE=3 SV=1 - [LTN1_SC	6.22	22.84	8	16.62	3.67	0.00	14.43	6	14.43	
Q10435	**Unassigned**	Unassigned hect-type ubiquitin-protein ligase E3 C12B10.01c OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=S	6.35	25.57	12	19.21	4.02	3.03	7.85	5	4.83	2.60
Q7Z9H9	***fig4***	Polyphosphoinositide phosphatase OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC1093.03 PE=3 SV=3	0.00	22.52	8	22.52		0.00	10.46	3	10.46	
O42901	***orm1***	ORMDL family protein Orm1 (predicted)	3.81	17.91	4	14.10	4.70	3.01	13.16	4	10.15	4.37
Q10243	***vps26***	Vacuolar protein sorting-associated protein 26 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=vps26 PE=3 SV	2.96	14.95	2	11.99	5.06	0.00	15.95	4	15.95	
O13282	***taf5***	Transcription initiation factor TFIID subunit 5 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=taf5 PE=1 SV=1 -	7.65	22.31	6	14.67	2.92	0.00	7.87	4	7.87	
Q10496	***gyp51***	GTPase activating protein Gyp51 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=gyp51 PE=3 SV=1 - [GYP51_	2.08	27.37	10	25.29	13.17	0.00	2.67	2	2.67	
O14197	**Unassigned**	Uncharacterized auxin family transmembrane transporter (predicted) C5D6.04 OS=Schizosaccharomyces pombe (strain 972 / ATC	6.65	18.47	3	11.82	2.78	3.66	10.17	2	6.50	2.77
Q9P782	***aha1***	Chaperone activator Aha1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC1711.08 PE=2 SV=1 - [YNY8_	5.01	18.32	10	13.31	3.65	2.17	10.17	9	8.00	4.69
O74534	***sly1***	Protein sly1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sly1 PE=3 SV=1 - [SLY1_SCHPO]	5.73	18.27	8	12.54	3.19	0.00	9.22	6	9.22	
O60081	***trm72***	tRNA 2'-O-methylase subunit Trm72 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC1494.07 PE=3 SV=	4.25	13.37	7	9.12	3.15	3.40	13.17	6	9.77	3.87
P50524	***rpn12***	26S proteasome regulatory subunit rpn12 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rpn12 PE=1 SV=1 - [	7.46	20.41	6	12.95	2.74	1.72	5.47	4	3.75	3.18
Q9P7W8	***rsc9***	Chromatin structure-remodeling complex subunit rsc9 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rsc9 PE=	6.42	21.62	6	15.20	3.37	0.00	4.21	3	4.21	
Q9Y7K2	***tor2***	Serine/threonine-protein kinase tor2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=tor2 PE=1 SV=2 - [TOR2_	2.79	16.58	11	13.79	5.93	0.00	8.57	5	8.57	
O14301	***aip1***	Actin cortical patch component Aip1 C9G1.05 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC9G1.05 PE	1.96	4.94	2	2.98	2.52	0.00	19.60	5	19.60	
O74925	***pep3***	Vacuolar membrane protein pep3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=pep3 PE=3 SV=1 - [PEP3_S	4.65	15.42	7	10.77	3.31	0.00	8.53	5	8.53	
Q9UTH0	***nup132***	Nucleoporin nup132 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nup132 PE=1 SV=1 - [NU132_SCHPO]	1.90	21.17	3	19.27	11.12	0.00	2.35	2	2.35	
O42954	***mbx1***	MADS-box transcription factor 1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=mbx1 PE=1 SV=2 - [MBX1_S	2.97	14.76	3	11.79	4.97	0.00	8.18	4	8.18	
Q9Y7J8	***psy2***	Protein phosphatase PP4 complex subunit Psy2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC216.01	2.11	16.01	7	13.91	7.61	0.00	6.38	4	6.38	
Q9P7X5	***ppk32***	Protein kinase domain-containing protein ppk32 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=ppk32 PE=1 S	0.00	13.62	4	13.62		0.00	8.74	3	8.74	
O14340	**Unassigned**	Sterol binding ankyrin repeat protein (predicted) C2F12.05c OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SF	3.10	9.03	1	5.94	2.92	0.00	13.03	3	13.03	
O94374	***imp1***	Importin subunit alpha-2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=imp1 PE=1 SV=1 - [IMA2_SCHPO]	5.13	16.62	5	11.49	3.24	0.00	5.12	2	5.12	
Q9UTK7	***dsc2***	DSC E3 ubiquitin ligase complex subunit 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=dsc2 PE=1 SV=1 - [	0.00	12.90	2	12.90		0.00	8.34	2	8.34	
Q9UUH0	**Unassigned**	Mannose-ethanolamine phosphate phosphodiesterase (predicted) C630.12 OS=Schizosaccharomyces pombe (strain 972 / ATCC	3.17	15.03	6	11.85	4.74	0.00	6.15	1	6.15	
Q10331	***nup107***	OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nup107 PE=1 SV=4 - [NU107_SCHPO]	5.40	19.10	8	13.70	3.54	0.00	1.67	1	1.67	
O94712	***gid1; vid30***	GID complex subunit, Ran GTPase binding protein Gid1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPC	1.74	12.24	5	10.50	7.02	0.00	7.60	3	7.60	
O13897	***fsh2***	Serine hydrolase-like OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC22A12.06c PE=3 SV=1 - [YF36_S	4.58	11.49	3	6.90	2.51	0.00	7.96	3	7.96	
Q09849	***arp42***	SWI/SNF and RSC complexes subunit arp42 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=arp42 PE=1 SV=	0.00	8.55	3	8.55		2.92	10.60	3	7.68	3.63
Q9P7R8	***gea1***	Guanyl-nucleotide exchange factor OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC211.03c PE=1 SV=1	0.00	14.71	7	14.71		0.00	3.84	2	3.84	
O74773	***msh2***	DNA mismatch repair protein msh2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=msh2 PE=3 SV=2 - [MSH2	4.40	14.22	6	9.81	3.23	0.00	4.09	3	4.09	
Q10429	***cnd3***	Condensin complex subunit 3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=cnd3 PE=1 SV=1 - [CND3_SCH	3.86	13.03	5	9.17	3.38	0.00	4.98	2	4.98	
Q09779	***tho2***	THO complex subunit 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=tho2 PE=1 SV=3 - [THO2_SCHPO]	5.00	15.64	7	10.64	3.13	0.00	2.16	3	2.16	
Q9C100	***ogm2***	Dolichyl-phosphate-mannose--protein mannosyltransferase 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=o	2.19	12.32	4	10.13	5.63	0.00	5.30	5	5.30	
Q10108	***edc1***	Dcp2-Dcp1 mRNA-decapping complex subunit Edc1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC18G	2.13	5.85	2	3.72	2.75	0.00	11.34	2	11.34	
P78953	***mid1***	Division mal foutue 1 protein OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=mid1 PE=1 SV=1 - [MID1_SCHP	0.00	12.93	4	12.93		0.00	4.10	1	4.10	
O74504	**Unassigned**	DUF1769 famil protein C594.01 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC594.01 PE=1 SV=2 - [Y.	3.22	13.52	4	10.30	4.20	0.00	3.21	2	3.21	
O13636	***tim50***	Mitochondrial import inner membrane translocase subunit tim50 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN	0.00	8.94	3	8.94		0.00	7.31	1	7.31	
Q09743	***ste20***	Target of rapamycin complex 2 subunit ste20 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=ste20 PE=1 SV=	0.00	12.27	2	12.27		0.00	3.91	2	3.91	

Q9P7W0	**Unassigned**	Optic atrophy 3 family protein OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC1703.11 PE=3 SV=1 - [OP	3.88	12.41	1	8.54	3.20	0.00	3.53	1	3.53	
Q10165	***cnt6***	Probable ribosylation factor GTPase-activating protein cnt6 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=cnt	0.00	8.47	4	8.47		0.00	7.21	2	7.21	
Q10268	***mac1***	Membrane-anchored protein 1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=mac1 PE=1 SV=1 - [MAC1_SC	3.28	12.24	2	8.97	3.73	0.00	3.35	1	3.35	
P00046	***cyc1***	Cytochrome c OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=cyc1 PE=1 SV=3 - [CYC_SCHPO]	0.00	2.07	1	2.07		2.13	13.49	4	11.36	6.33
O14188	***rng2***	Ras GTPase-activating-like protein rng2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rng2 PE=1 SV=1 - [RN	1.79	6.46	6	4.67	3.61	0.00	8.83	6	8.83	
O59712	**Unassigned**	Sodium ion transmembrane transporter (predicted) C3B8.04c OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=	0.00	8.38	4	8.38		0.00	6.72	3	6.72	
Q09682	***pre9***	Probable proteasome subunit alpha type-3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC13C5.01c PE	2.20	8.43	2	6.22	3.82	1.97	6.23	2	4.26	3.16
Q9USJ4	***nte1***	Lysophospholipase NTE1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nte1 PE=3 SV=1 - [NTE1_SCHPO]	0.00	12.55	6	12.55		0.00	1.90	1	1.90	
Q9USZ2	***mtr10***	Karyopherin, nuclear import receptor Mtr10 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC11G11.07 P	0.00	10.57	6	10.57		0.00	2.81	2	2.81	
O42963	***nup44***	OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nup44 PE=2 SV=1 - [NUP44_SCHPO]	2.23	10.56	4	8.33	4.74	0.00	2.65	2	2.65	
P33277	***gap1***	GTPase-activating protein OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=gap1 PE=3 SV=1 - [GAP1_SCHPO]	3.96	10.35	5	6.39	2.62	0.00	2.75	2	2.75	
O42854	***bbc1***	SH3 domain-containing protein C23A1.17 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC23A1.17 PE=1	0.00	6.55	3	6.55		2.34	6.51	3	4.17	2.78
Q9P792	***nbr1***	Cargo receptor for selective autophagy pathway OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBP35G2.11	2.68	11.20	4	8.52	4.18	0.00	1.65	1	1.65	
Q9P7H8	***ecm29***	Proteasome component ecm29 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=ecm29 PE=1 SV=1 - [ECM29_	2.38	8.97	8	6.60	3.77	0.00	3.51	6	3.51	
Q10222	***pta1***	mRNA cleavage and polyadenylation specificity factor complex subunit pta1 OS=Schizosaccharomyces pombe (strain 972 / ATCC	1.73	7.96	4	6.24	4.61	0.00	4.26	3	4.26	
O42998	***sip1***	Pof6 interactor protein 1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sip1 PE=1 SV=1 - [SIP1_SCHPO]	0.00	6.00	5	6.00		2.11	6.10	3	4.00	2.90
Q1MTQ5	***mug89***	Meiotically up-regulated gene 89 protein OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=mug89 PE=1 SV=1 - [	0.00	8.82	4	8.82		0.00	2.31	1	2.31	
O94548	**Unassigned**	Conserved fungal proteins OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC1322.09 PE=3 SV=1 - [MTC1	0.00	7.79	3	7.79		0.00	3.12	2	3.12	
Q1MTN8	**Unassigned**	Conserved fungal protein OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC16D10.01c PE=3 SV=2 - [ACL	1.60	5.81	2	4.21	3.63	0.00	5.07	2	5.07	
Q9UTF8	***ugo1***	Mitochondrial fusion and transport protein ugo1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=ugo1 PE=3 SV	0.00	8.08	3	8.08		0.00	2.56	1	2.56	
O74738	***set10***	Ribosomal lysine N-methyltransferase set10 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=set10 PE=3 SV=1	0.00	8.79	3	8.79		0.00	1.84	1	1.84	
O74547	***bit61***	Target of rapamycin complex 2 subunit bit61 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=bit61 PE=1 SV=1	0.00	6.90	2	6.90		0.00	3.47	1	3.47	
O74541	**Unassigned**	Uncharacterized transcriptional regulatory protein C777.02 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPC	0.00	7.86	1	7.86		0.00	2.45	1	2.45	
O14130	**Unassigned**	Uncharacterized transcriptional regulatory protein C3C7.04 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SP	1.95	7.37	3	5.42	3.78	0.00	2.74	2	2.74	
Q9P382	***nup82***	OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nup82 PE=3 SV=1 - [NUP82_SCHPO]	0.00	8.12	4	8.12		0.00	1.70	2	1.70	
Q9UT67	***imt1***	Inositol phosphoceramide mannosyltransferase 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC4F11.0	0.00	5.89	2	5.89		0.00	3.58	1	3.58	
Q9UQX0	***sod2***	Superoxide dismutase [Mn], mitochondrial OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sod2 PE=1 SV=1 - [	0.00	2.49	2	2.49		2.39	6.64	4	4.24	2.78
P87114	***fft1***	ATP-dependent helicase fft1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=fft1 PE=3 SV=1 - [FFT1_SCHPO]	0.00	7.30	4	7.30		0.00	1.73	2	1.73	
O42930	***vps10***	Vacuolar protein sorting/targeting protein 10 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=vps10 PE=1 SV=1	2.17	6.21	2	4.04	2.87	0.00	2.73	2	2.73	
O74481	***pdi5***	Protein disulfide isomerase OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC1840.08c PE=3 SV=1 - [YQJ	2.04	6.27	4	4.23	3.07	0.00	2.27	1	2.27	
O94516	***pex16***	Peroxisomal membrane protein PEX16 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=pex16 PE=1 SV=2 - [P	0.00	6.07	2	6.07		0.00	2.32	1	2.32	
O94520	***emc4***	ER membrane protein complex subunit 4 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC1281.03c PE=3	1.62	4.34	2	2.71	2.67	0.00	4.01	1	4.01	
P78871	***rst2***	Zinc finger protein rst2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rst2 PE=2 SV=2 - [RST2_SCHPO]	1.99	5.41	1	3.42	2.72	0.00	2.50	2	2.50	
O14068	**Unassigned**	Conserved fungal protein C1687.07 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC1687.07 PE=3 SV=1	0.00	4.54	2	4.54		0.00	3.14	1	3.14	
Q9P7J5	***yta4***	Mitochondrial outer membrane ATPase Msp1/Yta4 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC24B	0.00	4.81	2	4.81		0.00	2.72	1	2.72	
Q9UTL2	***klp8***	Kinesin-like protein 8 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=klp8 PE=1 SV=1 - [KLP8_SCHPO]	0.00	4.95	2	4.95		0.00	2.57	2	2.57	
Q10361	***etp1***	Electron transfer protein 1, mitochondrial OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=etp1 PE=1 SV=2 - [E	0.00	5.39	2	5.39		0.00	2.09	2	2.09	
O59747	***pdf1***	Palmitoyl-protein thioesterase-dolichyl pyrophosphate phosphatase fusion 1 OS=Schizosaccharomyces pombe (strain 972 / ATCC	0.00	2.54	1	2.54		0.00	4.74	1	4.74	
Q8WZK2	***its8***	GPI ethanolamine phosphate transferase 1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=its8 PE=1 SV=1 - [	0.00	4.70	3	4.70		0.00	2.05	1	2.05	
Q9UTK6	**Unassigned**	RNA-binding splicing factor (predicted) C1486.03 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC1486.0	0.00	3.77	2	3.77		0.00	2.57	1	2.57	
O94689	***yme2***	Mitochondrial escape protein 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=yme2 PE=3 SV=1 - [YME2_SC	0.00	4.05	3	4.05		0.00	2.20	1	2.20	
Q9Y7N9	**Unassigned**	PXA domain-containing protein C1450.12 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC1450.12 PE=1	0.00	2.92	1	2.92		0.00	3.15	1	3.15	
Q9UUI8	***pet1***	Phosphoenolpyruvate transmembrane transporter Pet1OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC2	0.00	4.21	2	4.21		0.00	1.74	2	1.74	
P27584	***gpa1***	Guanine nucleotide-binding protein alpha-1 subunit OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=gpa1 PE=3	0.00	3.70	1	3.70		0.00	2.21	1	2.21	
Q09922	**Unassigned**	Transcription factor, zf-fungal binuclear cluster type OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC1F7	0.00	3.43	2	3.43		0.00	2.30	3	2.30	
Q09787	**Unassigned**	SSU-rRNA maturation protein Tsr4 homolog 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC13G6.09	0.00	2.39	1	2.39		0.00	3.34	1	3.34	
O42929	***tma22***	Translation machinery-associated protein 22 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=tma22 PE=3 SV=	0.00	3.32	3	3.32		0.00	2.35	1	2.35	
O94691	***jmj3***	Lid2 complex component jmj3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=jmj3 PE=3 SV=1 - [JMJ3_SCHP	0.00	3.81	2	3.81		0.00	1.76	1	1.76	
Q10347	***any2***	Arrestin-related endocytic adaptor Any2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC1F12.05 PE=4	0.00	3.53	2	3.53		0.00	1.88	1	1.88	
Q09830	**Unassigned**	GTPase activating protein (predicted) C4G8.04 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC4G8.04	0.00	3.51	4	3.51		0.00	1.79	2	1.79	
O42973	**Unassigned**	Conserved eukaryotic protein, human IFRD1 ortholog C20F10.03 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) G	0.00	2.52	2	2.52		0.00	2.60	1	2.60	
Q1MTQ1	***tea2***	Kinesin-like protein tea2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=tea2 PE=1 SV=1 - [TEA2_SCHPO]	0.00	2.57	3	2.57		0.00	2.53	1	2.53	
O74308	***gsf1***	Transcription factor, zf-fungal binuclear cluster type Gsf1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPB	0.00	2.15	1	2.15		0.00	2.91	1	2.91	
Q9US60	***klp3***	Kinesin-like protein 3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=klp3 PE=2 SV=1 - [KLP3_SCHPO]	0.00	2.60	2	2.60		0.00	2.41	1	2.41	
O14002	***mak2***	Peroxide stress-activated histidine kinase mak2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=mak2 PE=3 S	0.00	3.31	1	3.31		0.00	1.60	2	1.60	
Q9UTK4	***nup189***	OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=nup189 PE=1 SV=1 - [NU189_SCHPO]	0.00	2.83	2	2.83		0.00	1.85	2	1.85	
Q10245	***ifa38***	Ketoreductase involved in fatty acid elongation OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC4G9.15	0.00	2.99	2	2.99		0.00	1.69	1	1.69	
Q92359	***puf4***	Pumilio family RNA-binding protein Puf4 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC6G9.14 PE=3 S	0.00	2.13	1	2.13		0.00	2.54	1	2.54	
Q9UT38	***vps16***	Probable vacuolar protein sorting-associated protein 16 homolog OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) G	0.00	2.35	1	2.35		0.00	2.28	1	2.28	
Q9UTN3	***cid14***	Poly(A) RNA polymerase cid14 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=cid14 PE=1 SV=2 - [CID14_SC	0.00	1.69	1	1.69		0.00	2.91	1	2.91	
O74823	***red5***	Zinc finger CCCH domain-containing protein C337.12 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC33	0.00	2.27	2	2.27		0.00	2.34	1	2.34	
P78875	***tpp1***	Trehalose-phosphatase OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=tpp1 PE=1 SV=2 - [TPP1_SCHPO]	0.00	2.37	2	2.37		0.00	2.08	3	2.08	
O43092	***oxa102***	Mitochondrial inner membrane protein oxa1-2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=oxa102 PE=2 S	0.00	2.03	1	2.03		0.00	2.32	1	2.32	
Q10093	**Unassigned**	5-oxoprolinase (ATP-hydrolizing) OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC11D3.14c PE=3 SV=1	0.00	2.27	1	2.27		0.00	1.98	1	1.98	
Q10366	***pik1***	Phosphatidylinositol 4-kinase pik1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=pik1 PE=1 SV=1 - [PIK1_SC	0.00	2.35	1	2.35		0.00	1.78	1	1.78	

P87234	***gyp3***	GTPase-activating protein gyp3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=gyp3 PE=1 SV=1 - [GYP3_SC	0.00	2.23	2	2.23	0.00	1.77	2	1.77
O60108	***cbh2***	CENP-B homolog protein 2 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=cbh2 PE=3 SV=1 - [CBH2_SCHPO	0.00	1.78	3	1.78	0.00	2.01	2	2.01
O94733	**Unassigned**	Schizosaccharomyces specific protein OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPCC191.01 PE=4 SV=	0.00	1.66	1	1.66	0.00	2.07	1	2.07
O14076	***mpp6***	Nuclear exosome-associated RNA binding protein Mpp6 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPAC	0.00	1.91	1	1.91	0.00	1.69	2	1.69
Q9HGN7	***sec63***	Translocation protein sec63 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=sec63 PE=1 SV=1 - [SEC63_SCH	0.00	1.61	3	1.61	0.00	1.95	2	1.95
O59741	***prt1***	Transcription factor prt1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=SPBC530.05 PE=3 SV=2 - [YN25_SC	0.00	1.82	2	1.82	0.00	1.72	1	1.72
Q76PC3	***mme1***	Mitochondrial magnesium ion transmembrane transporter Mme1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN	0.00	1.85	1	1.85	0.00	1.68	1	1.68
Q10110	***rrn3***	RNA polymerase I-specific transcription initiation factor rrn3 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rrn3	0.00	1.85	1	1.85	0.00	1.63	1	1.63
Q9Y802	***lsd1***	ysine-specific histone demethylase 1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=lsd1 PE=1 SV=1 - [LSD1_	0.00	1.66	3	1.66	0.00	1.70	1	1.70
O13960	***ecm33***	Cell wall protein ecm33 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=ecm33 PE=1 SV=2 - [ECM33_SCHPO]	0.00	1.61	1	1.61	0.00	1.73	1	1.73

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