# Contribution of the schizophrenia associated protein DISC1 to Mitochondrial Dynamics and Dendritic Development

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I, Rosalind Norkett, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## Abstract

Correct neuronal function is an energy costly process, highly dependent upon the ATP production and calcium buffering capabilities of mitochondria. Further, it is crucial that these organelles are correctly distributed throughout the neuron, matching local energy demand. To achieve this, mitochondrial fusion and transport dynamics must be precisely regulated. Trafficking is dependent upon microtubule based transport of these organelles, mediated by kinesin motors, TRAK adaptors and the mitochondrial anchor, Miro. Mitofusins orchestrate mitochondrial fusion at the outer membrane. Disrupted in schizophrenia 1 (DISC1) – a major candidate susceptibility factor for psychiatric disease – also plays a role in mitochondrial transport. However, the mechanisms of this regulation, and subsequent roles in neuronal development, are poorly understood.

In this study, the interactions between DISC1 and mitochondrial trafficking proteins Miro and TRAKs are investigated. DISC1 is shown to couple to the mitochondrial transport and fusion machinery in brain. Live cell imaging demonstrates the importance of these interactions for normal mitochondrial transport. A schizophrenia associated mutation in DISC1 – the DISC1-Boymaw fusion protein – is shown to disrupt these transport dynamics and similarly impair mitochondrial fusion. Further, this mutation alters contact area between the endoplasmic reticulum and the mitochondria. Moreover, disruption of mitochondrial dynamics by targeting the DISC1-Miro/TRAK complex or upon expression of the DISC1-Boymaw fusion protein impairs the correct development of neuronal dendrites. Finally, mitochondrial dynamics in human neurons, differentiated from induced pluripotent stem cells, are explored. This innovative system is used to investigate impact of pathogenic mutations associated with schizophrenia and Alzheimer's disease upon mitochondrial dynamics. This work highlights DISC1 as an important regulator of mitochondrial dynamics in neurons to mediate transport, fusion and cross-talk of these organelles. Moreover, pathological DISC1 isoforms disrupt this critical function, leading to abnormal neuronal development. These findings implicate regulation of mitochondrial dynamics in aetiology of psychiatric disease.

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## Publications arising from this work:

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## Other publications:

Birsa, N., **Norkett, R**., Higgs, N., Lopez-Domenech, G., and Kittler, J.T. (2013). Mitochondrial trafficking in neurons and the role of the Miro family of GTPase proteins. Biochem Soc Trans *41*, 1525-1531.

Birsa, N., **Norkett, R**., Wauer, T., Mevissen, T.E., Wu, H.C., Foltynie, T., Bhatia, K., Hirst, W.D., Komander, D., Plun-Favreau, H., *et al.* (2014). Lysine 27 ubiquitination of the mitochondrial transport protein miro is dependent on serine 65 of the Parkin ubiquitin ligase. J Biol Chem *289*, 14569-14582.

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## Abbreviations

| | |
|---|---|
| AD | Alzheimer's disease |
| ADP | Adenosine Diphosphate |
| AMP | Adenosine Monophosphate |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| APP | amyloid Precursor Protein |
| ATP | Adenosine Triphosphate |
| BBS | Bardet-Biedl syndrome |
| BDNF | Brain Derived Neurotrophic Factor |
| cAMP | Cyclic Adenosine Monophosphate |
| DISC1 | Disrupted in schizophrenia 1 |
| DIV | Days *in vitro* |
| DN | Dominant Negative |
| Drp1 | Dynamin related protein 1 |
| ER | Endoplasmic reticulum |
| FEZ1 | Fasciculation and elongation protein zeta 1 |

| | |
|---|---|
| FGF | Fibroblast growth factor |
| GAP | GTPase activating protein |
| GDNF | Glial-cell derived neurotrophic factor |
| GEF | Guanine nucleotide exchange factor |
| GFP | Green Fluorescent Protein |
| GSK3 beta | Glycogen synthase kinase 3 beta |
| GST | Glutathione Sepharose transferase |
| GTP | Guanosine Triphosphate |
| IF | Immunofluorescence |
| IMM | Inner mitochondrial membrane |
| IP | Immunoprecipitation |
| $IP_3$ | Inositol trisphosphate |
| iPSCs | induced Pluripotent Stem Cells |
| KIF | Kinesin Superfamily protein |
| KLC | Kinesin light chain |
| MAM | Mitochondria associated membrane |
| MCU | Mitochondrial calcium uniporter |
| MEFs | Murine Embryonic Fibroblasts |

| | |
|---|---|
| Mfn | Mitofusin |
| Nde1 | Nuclear distribution protein E |
| Ndel1 | Nuclear distribution protein E like1 |
| NGF | Nerve growth factor |
| NMDA | N-methyl-D-aspartate |
| NPCs | Neural progenitor cells |
| NudCL | Nuclear distribution protein C like |
| OGT | O-linked N-acetyl glucosamine transferase |
| OMM | Outer mitochondrial membrane |
| OPA1 | Optic Atrophy 1 |
| PD | Parkinson’s disease |
| PDE | Phosphodiesterase |
| PKA | Protein kinase A |
| TRAK | Trafficking kinesin protein |
| WB | Western Blot |

# Chapter 1

# Introduction

## 1.1 Mitochondrial structure and function

First noted in the late 19th century, mitochondria are essential to the cell for aerobic respiration, calcium buffering, apoptotic signalling and reactive oxygen species generation (Alberts, 2008), and thus, are intracellular signalling hubs. Mitochondria are endosymbionts, the result of eubacterial invasion into archaea type hosts and evolution. Each organism then became dependent on the other for genome maintenance or energy production respectively (Balaban et al., 2005). This compartmentalisation and specialisation gave rise to the ubiquitous organelles, mitochondria, and the aerobically respiring cells of higher organisms. Mitochondria are comprised of an inner and outer membrane, enclosing an inter membrane space and, within the inner membrane, a dense matrix. The outer membrane has been described as a sieve, permeable to ions and small molecules, including proteins and pyruvate, of 5kDa or less. This is achieved by the enrichment of porin proteins – beta barrel proteins forming membrane pores large enough for passive diffusion. The outer membrane encloses the inter membrane space – of similar composition to the cytosol. The inner mitochondrial membrane is the site of oxidative phosphorylation (to be discussed in 1.1.1). To aid this process, the inner membrane has a large surface area, achieved by the presence of convoluted invaginations called cristae. It is a far more selective membrane than the outer mitochondrial membrane, lacking porins and instead rich in cardiolipins – dense lipids which may contribute to this selectivity. Thus, proteins must be imported into mitochondria via the translocase machinery – protein complexes at the outer and inner membranes which can sort and fold nuclearly encoded proteins with the aid of chaperones (Endo and Yamano, 2009). The matrix is the site of the mitochondrial genome, encoding a handful of the proteins required for oxidative phosphorylation and the mitochondrial ribosome. The matrix is also the site of tricarboxylic acid cycle (TCA) activity. This process generates high energy

electrons in the form of reduced carriers NADH and $FADH_2$, using pyruvate or fatty acids as a source. These carriers feed directly into the electron transport chain (Alberts, 2008). Please see Figure 1-1 for a schematic of mitochondrial structure.

### 1.1.1 ATP production

The major source of cellular energy is ATP. This is generated by glycolysis, the TCA cycle and via the electron transport chain at the inner mitochondrial membrane. Glycolysis is an anaerobic process, occurring in the cyotosol, which generates two pyruvate molecules per glucose molecule and two NADH molecules in addition to two ATP molecules net. These pyruvate molecules are decarboxylated within the mitochondrial matrix, producing $CO_2$, one NADH molecule and acetyl CoA. It is this compound that enters the TCA cycle, also within the matrix, to allow reduction of three $NAD^+$ molecules and one FAD molecule to generate more high energy electron carriers. The chemical energy from these molecules is harnessed by the electron transport chain. This is a series of five protein complexes. The electrons from NADH or $FADH_2$ lose energy as they pass through this chain, which is utilised to pump protons from the matrix into the intermembrane space at complexes I, III and IV. This generates an electrochemical gradient for protons across the inner mitochondrial membrane. This potential energy is converted to chemical energy in the form of ATP by the flowing of protons through the ATP synthase complex. This complex is anchored into the inner membrane by a 'stator' or stalk, associated with a rotor. Protons flow through a small channel between these two subunits, causing the rotor to spin against a stationary ring on the matrix side of the membrane, allowing phosphorylation of ADP to ATP. Please refer to Figure 1-1 for a schematic. This action of a proton gradient being dissipated to power ATP synthesis was first proposed as the chemiosmotic theory in the 1960's (Mitchell and Moyle, 1965). Each glucose molecule provides sufficient energy to phosphorylate a maximum of 38 ADP molecules, although the yield is typically calculated to be around 30 molecules due to inefficiencies throughout the process (Alberts, 2008).

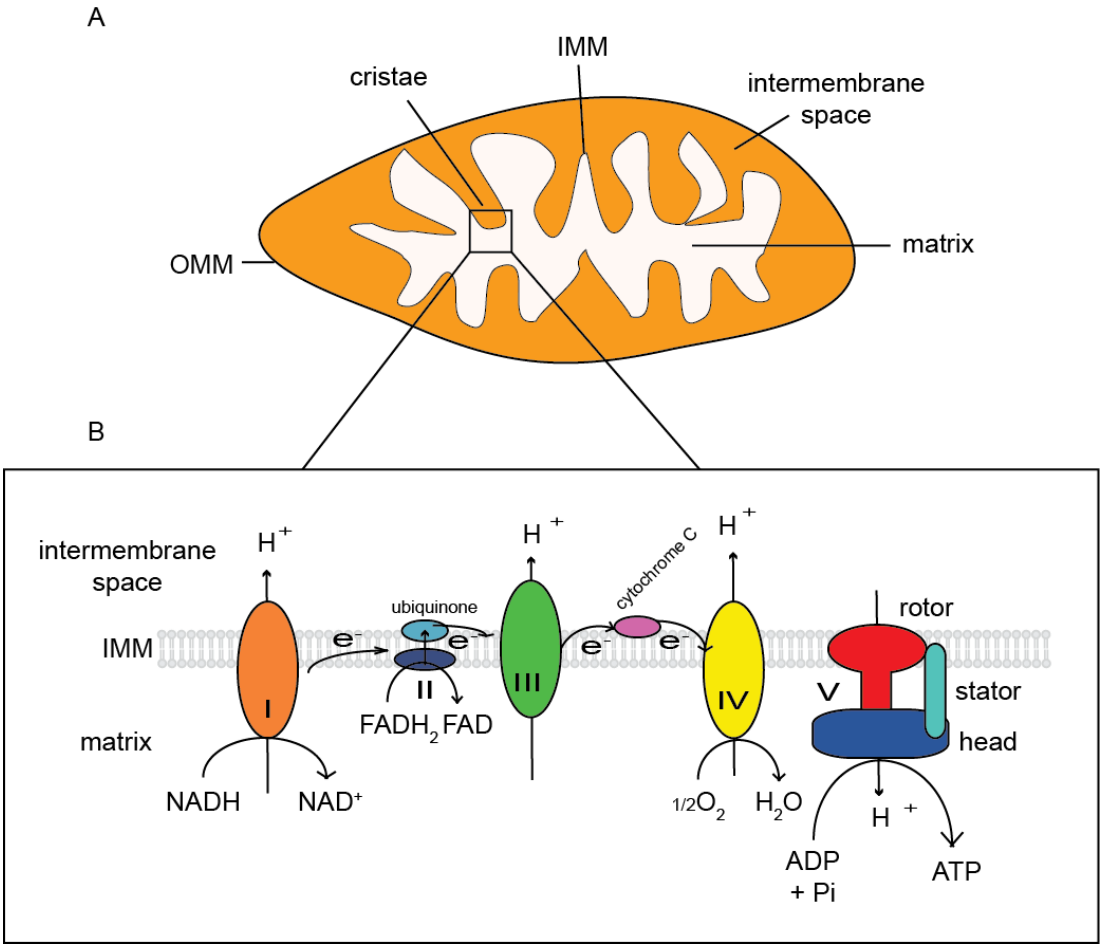



**Figure 1-1 Mitochondrial structure and function**

(A) Shows mitochondrial compartments; the outer mitochondrial membrane (OMM), the cristae – infoldings of the inner mitochondrial membrane (IMM), the intermembrane space and the matrix. (B) Shows a schematic of the electron transport chain, converting high energy electrons into ATP via generation of a proton gradient across the IMM.

### 1.1.2 Calcium buffering

Mitochondrial calcium buffering is crucial to shape cellular responses such as apoptotic signalling and neuronal activity (Szabadkai and Duchen, 2008). Additionally, mitochondrial calcium uptake increases ATP production (Jouaville et al., 1999). Within the mitochondrion, calcium upregulates activity of pyruvate, NAD(+) isocitrate and 2-oxoglutarate dehygrogenases in the TCA cycle (McCormack et al., 1990). The outer mitochondrial membrane is freely permeable to calcium, as previously mentioned. However, the mechanism by which calcium could cross the inner membrane has been more recently described, both by Baughman and colleagues and De Stefani and colleagues (Baughman et al., 2011; De Stefani et al., 2011). Calcium crosses IMM via the mitochondrial calcium uniporter (MCU) complex. This is comprised of a 40kDa protein with two transmembrane domains and two coiled-coil regions known as MCU, and a similar protein MCUb. These proteins associate as a tetramer, forming a membrane spanning pore. MCU activity is regulated by associated proteins MICU1 and 2 in the intermembrane space – EF hand domain containing proteins able to sense calcium levels. These proteins are tethered to the MCU via another MCU component EMRE (De Stefani et al., 2015). Once inside the mitochondria, calcium is likely to encounter phosphate groups and form calcium phosphate precipitates (Szabadkai and Duchen, 2008).

## 1.2 Mitochondrial fission and fusion

Mitochondrial morphology is dependent on the balance of fission and fusion, at both the outer and inner mitochondrial membranes. Fusion events are necessary to exchange mitochondrial contents, e.g. mitochondrial DNA and metabolites. This maintains mitochondrial function. Some components of the electron transport chain are encoded by the mitochondrial genome (Chen et al., 2005). However, the majority of these components is nuclearly encoded and must be imported to the mitochondria. Therefore, mitochondrial fusion also acts to maintain a homogenous population in terms of nuclear encoded protein content (Mishra and Chan, 2014; Westermann, 2010). Mutations in components of the fusion machinery are linked to neuropathies such as Charcot Marie Tooth 2A (CMT2A) (Detmer and Chan, 2007), highlighting the importance of this process for correct neuronal function. Fission is then required to

maintain numbers of mitochondria (Mishra and Chan, 2014). Both of these processes are regulated by large GTPases to be discussed in the following sections.

### 1.2.1 Mitochondrial fusion

Mitochondrial fusion is regulated in two separate steps. Initially the outer membranes are fused, regulated by Mitofusins (Mfn1 and 2). These proteins contain an N-terminal GTPase domain and two heptad repeats flanking two transmembrane domains which tether the proteins into the mitochondrial outer membrane (Koshiba et al., 2004). Please see Figure 1-2 part A for a schematic of outer membrane fusion. It has been demonstrated that these enzymes are essential for mitochondrial fusion by cell hybrid assays between wild-type and Mfn double knockout cells (Chen et al., 2003; Koshiba et al., 2004). Mitochondria lacking both of these proteins cannot fuse, even though mitochondria from the wild-type cell are positive for Mitofusins. Thus the Mitofusins are trans acting factors and can associate to mediate fusion. The interaction between Mitofusins can be homotypic or heteroptypic and occurs via the heptad repeat domains, as determined by crystallographic studies. Point mutations at key leucine residues in these domains drastically decrease fusion, indicating the necessity of these domains (Koshiba et al., 2004). In order to investigate the contribution of the N-terminal GTPase domain, a construct lacking this region was expressed in Mfn double knock-out MEFs. Fluorescence microscopy revealed that the mitochondrial network became aggregated, and via electron microscopy, it was notable that the mitochondria were an even distance apart in this condition. This might imply tethering without full fusion, and so the GTPase domain is necessary to complete the fusion process (Ishihara et al., 2004; Koshiba et al., 2004). Upon further study of the GTPase activity of each protein, it was notable that Mfn1 exhibited greater GTP hydrolysis than Mfn2. This suggests that Mfn1 may be more effective in fusing mitochondria, and that Mfn2 might have other roles beyond mitochondrial fusion (Ishihara et al., 2004) as will be discussed in 1.3.5 and Chapter 4.

After docking, and tethering of two mitochondria, and fusion of the outer membrane, fusion of the inner mitochondrial membrane must occur. This is regulated by dynamin related GTPase, OPA1. OPA1 is known to localise to the inner mitochondrial membrane, as demonstrated by electron microscopy and fractionation studies (Olichon

et al., 2002). The role of this protein in inner mitochondrial membrane fusion was demonstrated by knockdown of this protein, and a decrease in mitochondrial fusion as determined by cell hybrid assays. OPA1 activity is dependent upon Mfn1, but not Mfn2 – perhaps dependent upon the higher GTPase activity of Mfn1 (Cipolat et al., 2004).

### 1.2.2 Mitochondrial fission

In contrast to the mitochondrial proteins that regulate fusion, fission is regulated by cytsolic GTPase Drp1, which must translocate to the mitochondria (MacAskill and Kittler, 2010). Once at the OMM, Drp1 oligomerises to divide mitochondria in a GTPase dependent manner - upon GTP hydrolysis, the Drp1 helices constrict, causing membrane fission (Mears et al., 2011). Recruitment of Drp1 to the mitochondria is dependent on its interaction with OMM proteins including Fis1, Mff, and MiD49 and 51 which can act as Drp1 docking sites (Kasahara and Scorrano, 2014). Please see Figure 1-2 part B. Fis1 plays an important, but not essential, role in recruiting Drp1. This was demonstrated in Fis1 knock-out cells where the mitochondrial network is elongated, and mitochondrial Drp1 is decreased but not ablated. Knockout of Mff and knock down of MiD49 and 51 (mitochondrial dynamics proteins of 49 and 51 kDa respectively) give similar phenotypes. Additionally, Mff and Fis1 may be involved in oligomerisation – Drp1 puncta on mitochondria are smaller in cells lacking these factors (Loson et al., 2013). Further, the recruitment and activity of Drp1 can be regulated by multiple post translational modifications including SUMOylation, ubiquitination, phosphorylation and S-nitrosylation (Chang and Blackstone, 2010). S-nitrosylation is a pro-fission event, enhancing Drp1 dimerisation (Cho et al., 2009). Phosphorylation can inhibit or promote fission dependent on the Drp1 residue. For example, phosphorylation by PKA (protein kinase A) prevents Drp1 GTPase activity, whereas phosphorylation by cyclin B dependent kinase promotes GTPase activity (Kasahara and Scorrano, 2014). Likewise, SUMOylation (addition of a Small Ubiquitin-like Modifier) can have dual effects on Drp1 activity and localisation. The E3 ligase, MAPL, is known to SUMOylate Drp1 to promote fission (Braschi et al., 2009), yet de-SUMOylation by SENP3 has the same effect (Guo et al., 2013). It is possible that the outcome of SUMOylation depends on stage in Drp1 activity cycle, or acts in concert with other signals such as phosphorylation. Regulation of mitochondrial

fission can also be achieved via augmenting Drp1 protein levels. Drp1 can be ubiquitinated by the E3 ligase, Parkin, – a component of the mitochondrial clearance machinery. It is subsequently proteasomally degraded, yet this process can be impaired upon loss of Parkin function (such as by mutation in Parkinson's disease), promoting mitochondrial fission (Wang et al., 2011a).

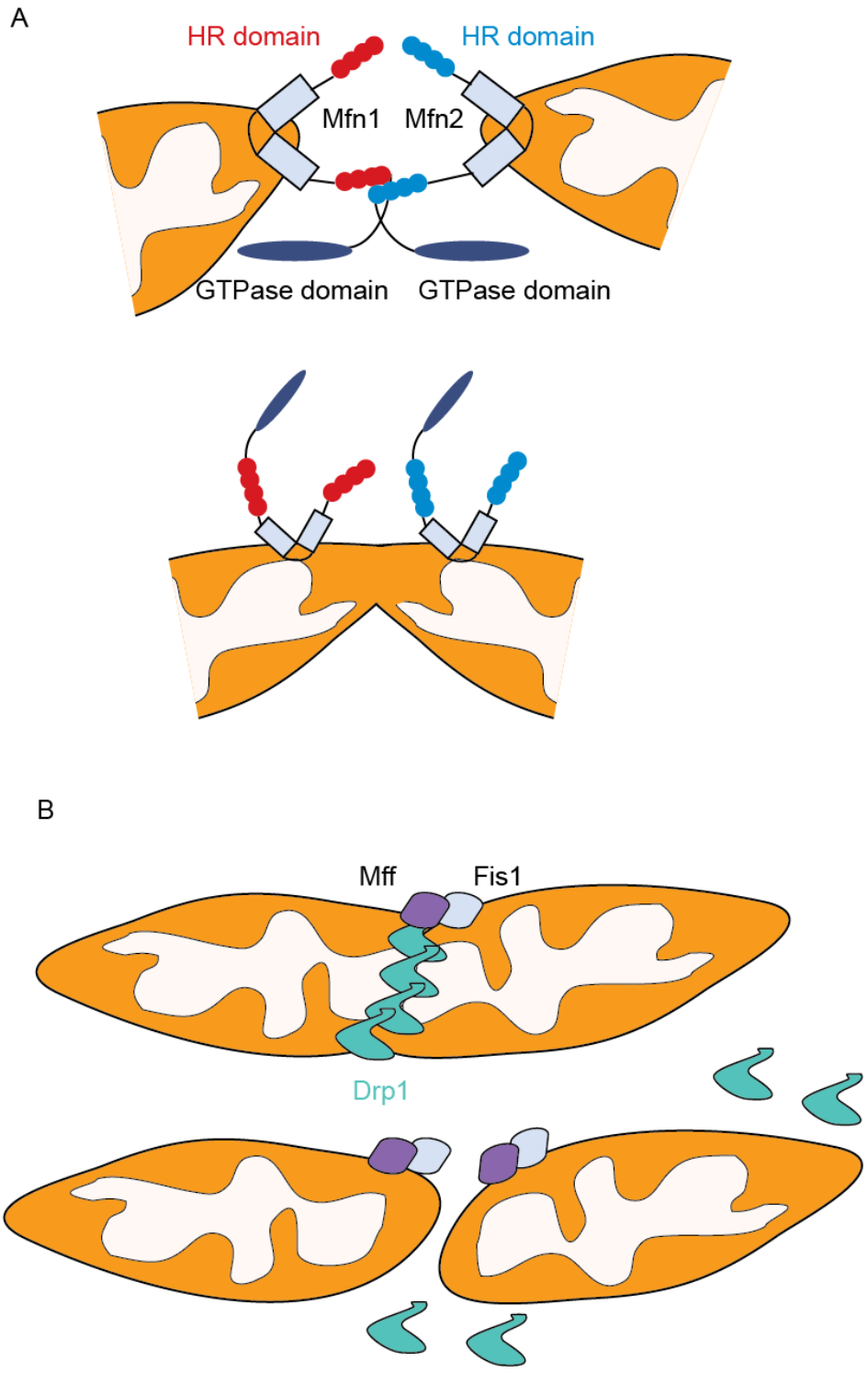



**Figure 1-2 Mitochondrial fusion and fission**

(A) Shows a schematic of Mfn Heptad repeat domains (HR) oligomerising in trans to cause outer membrane fusion. (B) Shows Drp1, recruited to the OMM by Fis1 and Mff, oligomerising to cause fission

## 1.3 Mitochondrial transport

Mitochondrial dynamics in culture were first noted in the early 20th century. These organelles were described as 'never at rest, but continually changing position and shape'. Further, in this study, the mitochondria were reported to 'radiate out from the central body as if under the influence of the centrosome' (Lewis and Lewis, 1914). This has become apparent in more recent studies where mitochondria have been observed to undergo rapid and bidirectional transport in culture and *in vivo* (Macaskill et al., 2009b; Marinkovic et al., 2012; Russo et al., 2009). This transport is of exceptional importance within neurons as synaptic communication is an energy costly process; the brain comprises 2% of the body's weight but consumes 20% of the oxygen required by the body at rest (Harris et al., 2012). Further, the long, asymmetric and specialised structure of neurons precludes diffusion of ATP from one end of the cell to the other – for example, the distance between the soma to the distal axon can be up to a metre in human motor neurons. Thus mitochondria must undergo active transport and localisation to specialised sites (Hollenbeck and Saxton, 2005; MacAskill and Kittler, 2010). Indeed, a high level of dynamism in terms of mitochondrial distribution has been noted in dendrites. Mitochondria have been shown to relocate from the dendritic shaft into the spine upon neuronal activity. This activity dependent redistribution is essential for the formation of new synapses, as shown by the inhibition of this process with a dominant negative Drp1 construct (Li et al., 2004). Thus mitochondrial distribution is essential for powering neuronal communication and the trafficking of these organelles must be tightly regulated to achieve this. The following sections will discuss both the proteins and mechanisms involved in this phenomenon. A schematic of the mitochondrial trafficking complex can be seen in Figure 1-3 on page 35. A schematic of proposed regulatory mechanisms can be seen in Figure 1-4 on page 41.

### 1.3.1 Molecular motors and the cytoskeleton

Molecular motors are responsible for active transport and positioning of cargo throughout the cell. Microtubules are a major component of the cytoskeleton and form the tracks for long range intracellular transport. They are comprised of heterodimers of α and β tubulin, associated to form a hollow tube. The orientation of these dimers

determines microtubule polarity, with the β tubulin subunits forming the plus end. In neuronal axons, microtubule polarity is uniform, with plus ends distal to the soma (Kapitein and Hoogenraad, 2015). Polarity is mixed in proximal dendrites, becoming progressively more organised into the distal dendrites (Kwan et al., 2008). Microtubule based transport is essential in neurons to distribute cargo such as organelles, RNA and proteins to distal, specialised sites. These sites, such as growth cones and synapses, can be up to a metre from the soma in human motor neurons, or millimetres in cortical neurons. Therefore, active transport must be tightly regulated. This is achieved by motor proteins and adaptor proteins that contribute to the specificity of assembled complexes (Maday et al., 2014). The adaptor proteins specific to mitochondria will be discussed in 1.3.3 and 1.3.4. The motor proteins involved are kinesins and dynein, which use the microtubules as tracks, and myosins which travel along actin filaments, usually over shorter ranges.

A diverse array of kinesins act to traffic cargo to the microtubule plus ends (anterograde transport, away from the soma in axons) and the dynein complex acts as the retrograde (minus end directed) motor (Maday et al., 2014). There are thought to be 45 mammalian kinesin genes, 38 of which are expressed in the brain. This super family of proteins are grouped and classed according to the location of their motor domain – N or C-terminal, or in the middle of the protein. N kinesins are currently the only group known to traffic mitochondria. Kinesin-1 proteins (or KIF5A-C, key mitochondrial transporters) are comprised of a dimer of kinesin heavy chains (KHC) with N-terminal motor domains and C-terminal cargo/adaptor binding domains. Dimerisation occurs via central coiled-coil regions.

In contrast to the diversity of kinesin motors; the core of the dynein complex is made up of a dimer of dynein heavy chains (DHC) encoded for by just one gene (DYNC1H1). Other factors are assembled on this core to confer specificity for cargo binding. The C-terminus of each dynein heavy chain folds into six ATPase domains and contains the microtubule binding capabilities. The N-terminal tail domain is the binding site for cargoes (e.g. mitochondria, signalling endosomes), adaptors (e.g. HAP1, BICD, dynein light and intermediate chains) and regulatory components (e.g. Lis1 and NDE/Ndel1). Dimerisation occurs via the stalk regions of the heavy chains (Schiavo et al., 2013; Vallee et al., 2012). Dynein processivity is enhanced by the

multi-subunit complex dynactin, an essential complex which binds both to the microtubules and to the dynein heavy chain N-terminus (Vallee et al., 2012). Both kinesin and dynein driven transport is dependent upon ATP hydrolysis. This ATP is provided in the majority by on board glycolysis (Zala et al., 2013) and is necessary for the motor domains to walk 'hand over hand', alternating the motor domains associated with the microtubules (Yildiz et al., 2004).

Kinesin-1 (KIF5 isoforms), and dynein have been shown to be the major motor proteins involved in mitochondrial trafficking (Pilling et al., 2006; Tanaka et al., 1998). Knockout of kinesin heavy chain causes mitochondria to cluster in the perinuclear region of extraembryonic cells (Tanaka et al., 1998). In Drosophila studies, kinesin heavy chain, and dynein heavy chain and dynactin components p50 and p150 were enriched in the mitochondrial fraction. Mutations induced in dynein heavy chain to prevent its expression conferred a decrease in mitochondrial motility in the retrograde direction specifically. Comparable mutations in kinesin heavy chain abrogated mitochondrial transport in both directions, likely reflecting a homeostatic effect whereby retrograde transport is dependent upon anterograde transport. These impairments in trafficking corresponded to a decrease in mitochondria in both proximal and distal axons (Pilling et al., 2006). This role for kinesin-1 has been confirmed in cultured neurons from rodents. Expression of the cargo binding domain of kinesin heavy chain depletes mitochondria in neuronal processes, consistent with a decrease in anterograde transport (Cai et al., 2005) and a decrease in mitochondrial trafficking in dendrites is noted upon treatment with a KIF5 function blocking antibody (Macaskill et al., 2009b). Further, knockout of a KIF5 isoform in zebrafish leads to a loss of mitochondria in peripheral axons, and subsequent neurodegeneration (Campbell et al., 2014). However, the role of KIF5 in dendrites is open to question; a recent study showed no effect on mitochondrial trafficking in proximal dendrites upon expression of the KIF5C tail (cargo binding region) (van Spronsen et al., 2013). In addition to kinesin-1 proteins, a kinesin-3 has also been linked to mitochondrial trafficking. KIF1B localises to mitochondria and functions as a monomer (Nangaku et al., 1994). It appears the adaptor for KIF1B may me KBP (kinesin binding protein) which interacts with KIF1B, increasing its motility, and itself localises to mitochondria (Wozniak et al., 2005). Another N kinesin, Klp6 (kinesin like protein 6), has been recently identified to function in mitochondrial motility in differentiated neuronal cells

(Tanaka et al., 2011). To date, these motors are the only ones linked to mitochondrial trafficking of around 45 mammalian kinesin genes (Hirokawa et al., 2009). This is indicative of one level of selectivity in regulating mitochondrial transport.

Whilst the dynein complex is known to be necessary for mitochondrial transport (Pilling et al., 2006), the contributions of dynein components to retrograde mitochondrial trafficking are less well defined than those of kinesin motors. Further, it is of great interest to delineate mechanisms controlling a switch between kinesin and dynein acitivty – achieving a change in direction of transport. Dynein is known to be present on bidirectionally moving organelles, and so its distribution on mitochondria at the distal axon is likely kinesin dependent (Hirokawa et al., 1990). Nuclear distribution family proteins, Lis1, Ndel1 and NudCL, which interact with dynein heavy chain, have been shown to regulate mitochondrial trafficking in hippocampal neurons. Knockdown of Ndel1 and NudCL results in a specific decrease in retrograde transport. Knockdown of Lis1 decreases trafficking in both directions, perhaps reflective of coordination between anterograde and retrograde motor complexes (Shao et al., 2013). Dynein light chain, tctex-1 (t complex testis expressed-1), has been shown to interact with the outer mitochondrial membrane protein, VDAC1, suggesting a potential role for this protein as an adaptor for dynein dependent mitochondrial transport (Schwarzer et al., 2002). Recently, a role for TRAK2 (see 1.3.4) as an adaptor for p150 at the mitochondrion has been proposed (van Spronsen et al., 2013). Similar to Lis1, this finding may indicate TRAK as a molecular link between kinesin and dynein based motility.

In comparison to microtubule based transport, far less is known about the mechanisms regulating actin based transport of mitochondria. The actin motor myosin 19 has been localised to mitochondria, and increases mitochondrial transport in neuronal cell lines (Quintero et al., 2009). However, how this contributes to, or differs from, microtubule based transport remains to be determined.

### 1.3.2 Static Anchors

Roughly 30% of neuronal mitochondria are motile. Thus the non-motile proportion could be uncoupled from molecular motors or there could be an active sequestration

process involving specific regulators. This would confer a greater capacity for spatio-temporal regulation, allowing docking of mitochondria at sites of high energy demand such as the growth cone and at the base of dendritic spines. These sites are depleted of microtubules, but actin rich. Thus, the actin cytoskeleton could be a key anchor for mitochondria (Pathak et al., 2010). Mitochondria accumulate at sites of high nerve growth factor (NGF) concentration, such as the growth cone. Interruption of the actin cytoskeleton impairs this process (Chada and Hollenbeck, 2004). Myosin V has been shown to oppose microtubule based transport in Drosophila axons. Knockdown of this actin based motor results in an increase in motile mitochondria and a decrease in time spent stationary (Pathak et al., 2010). In dendrites, mitochondria are anchored proximal to and within dendritic protrusions, or spines, in an activity dependent manner (Li et al., 2004; Macaskill et al., 2009b). This has been shown to be dependent on actin binding protein, WAVE1 (Sung et al., 2008).

The microtubule cytoskeleton is also known to be involved in mitochondrial docking in axons, dependent upon syntaphilin (SNPH). SNPH is specifically localised to stationary mitochondria, and knockout of this protein results in a drastic increase in the motile fraction of these organelles (Chen and Sheng, 2013; Kang et al., 2008). This docking is dependent upon its microtubule binding ability, and is upregulated upon neuronal activity. SNPH interacts with KIF5 via TRAK2 to inhibit the ATPase activity of the motor, arresting mitochondrial motility (Chen and Sheng, 2013), giving rise to the 'engine switch and brake' model for regulating mitochondrial transport. Please refer to Figure 1-4 part C. In addition to TRAK2 and KIF5, a dynein complex component, LC8, is also involved in SNPH-microtubule association. However, it is unknown if this involves regulation of the dynein motor itself, or a secondary signalling role of LC8 (Chen et al., 2009).

### 1.3.3 Miro

Miro proteins are evolutionarily conserved, found in multiple Eukaryotes including yeast, Drosophila, nematodes and mammals. There are two isoforms in mammals – Miro1 and Miro2 – which share 60% identity and are around 70kDa in mass. This is in contrast to Drosophila, where one isoform is present – dMiro. These proteins were originally identified as atypical Rho GTPases due to their unusual domain structure.

Miro proteins contain an N-terminal Rho GTPase domain followed by two calcium binding EF hand motifs in tandem (Fransson et al., 2003). The significance of the calcium binding domains will be discussed in 1.4.1. There is a second GTPase domain towards the C-terminus of the protein which is diverged from a Rho-like GTPase. This has been recently reported to be comparable to Ras GTPase domains according to crystal structure (Klosowiak et al., 2013). The multi domain structure marked Miro proteins as hubs for intracellular signalling. The presence of Rho GTPase domains suggested a role in regulation of the actin cytoskeleton. However, upon overexpression in cell lines, these proteins adopted an obvious mitochondrial distribution as demonstrated by colocalisation with Mitotracker and cytochrome C staining. Thus the proteins were named Mitochondrial Rho GTPases. Further, this localisation was proposed to be dependent upon the C-terminus of the protein, as expression of only the N-terminal GTPase domain led to a cytosolic distribution (Fransson et al., 2003). A follow up study demonstrated a conserved C-terminal transmembrane domain, essential for anchoring the protein in the outer mitochondrial membrane (Fransson et al., 2006).

Further work has defined a role for Miro proteins as mediators of mitochondrial trafficking. Studies in Drosophila show knockout of dMiro causes an accumulation of mitochondria in the cell bodies and a depletion from axons. By electron microscopy, a loss of mitochondria from presynaptic terminals was evident. Moreover, the authors suggested a role for Miro in the anterograde transport of mitochondria due to this observation (Guo et al., 2005). Thus, correct mitochondrial distribution is dependent upon dMiro. A yeast two hybrid screen proposed an interaction between dMiro and Milton, a kinesin adaptor, raising the possibility that Miro may be involved in kinesin based transport of mitochondria (Giot et al., 2003). Subsequently, an interaction between Miro and kinesin adaptors, TRAK proteins (see 1.3.4) was confirmed biochemically, marking Miro as a candidate anchor for kinesin based transport of mitochondria (Fransson et al., 2006).

These studies have been followed up with numerous live imaging experiments confirming the necessity of Miro in mitochondrial dynamics. Saotome and colleagues showed an increase in mitochondrial movement events upon overexpression of Miro1 and Miro2 in cell lines (Saotome et al., 2008). This result was built upon in a study by

Russo and colleagues in Drosophila axons. Here, motile mitochondria were reduced to 4% of control values upon dMiro knockout and mitochondria spent more time paused. These experiments in axons also revealed the necessity of Miro for bidirectional trafficking – trafficking events in both anterograde and retrograde directions were decreased upon loss of Miro (Russo et al., 2009). These effects of Miro were recapitulated in rodent neurons in culture. Overexpression of Miro1 caused an increase in mitochondrial motility, whereas knockdown decreased percentages of moving mitochondria (Macaskill et al., 2009b). This contrasts with a recent study describing mitochondrial motility in Miro1 knockout mice. In this model, comparable numbers of mitochondria are moving in knockout and wild-type cultures. However, there is a decrease in the time spent in motion in the knockout neurons, suggesting a role for Miro1 in processivity of mitochondrial trafficking (Nguyen et al., 2014).

Miro has been confirmed to be a mitochondrial kinesin adaptor (Macaskill et al., 2009b). Overexpression of Miro recruited kinesin to the mitochondrial fraction. A biochemical interaction was confirmed in rat brain, and so Miro can bind kinesin motors in situ. *In vitro* translated kinesin-1 was shown to interact with Glutathione sepharose transferase (GST) tagged Miro. This interaction not only confirms Miro as a mitochondrial kinesin anchor to regulate transport, but also demonstrates that these two proteins interact directly – other adaptor proteins such as TRAKs are absent in this *in vitro* system (Macaskill et al., 2009b). These findings are in slight contrast to another study which proposes the necessity for TRAK in Miro-kinesin binding. *In vitro* immunoprecipitation experiments using exogenous proteins show an interaction between Miro and kinesin only in the presence of TRAK1 (Wang and Schwarz, 2009). A role for Miro as a mitochondrial adaptor for retrograde transport by the dynein motor complex has been proposed (Morlino et al., 2014; Nguyen et al., 2014). Interactions between Miro and dynein/dynactin complex components p50, dynein heavy chain and p150 have been demonstrated in HEK cells and lymphocytes (Morlino et al., 2014). However, the calcium dependence of this interaction has not been investigated and it is unknown if these interactions are direct or via other motor/adaptor complexes such as TRAK/kinesin. Further, knockout of Miro1 has been shown to cause a specific defect in retrograde motility (Nguyen et al., 2014).

### 1.3.4 TRAKs

TRAK1 and 2, also known as OIP106 [OGT (O-GlcNAc transferase)-interacting protein 106 kDa] and OIP98/Grif-1 respectively, are kinesin adaptor proteins and the mammalian orthologues of Drosophila Milton. They are coiled-coil proteins with an N-terminus similar to that of kinesin adaptor HAP1 (huntingtin associated protein 1) (Birsa et al., 2013). Whilst Milton appears to regulate mitochondrial transport specifically, TRAKs have been linked to trafficking of other cargoes including the $\beta_2$ subunit of $GABA_A$ receptors, endosomes and potassium channels in addition to mitochondria (Beck et al., 2002; Grishin et al., 2006; Kirk et al., 2006). As well as acting as adaptor proteins to couple cargo to motors, TRAKs may also act to increase motor ATPase activity (Chen and Sheng, 2013).

The first evidence for Milton/TRAKs as mediators of mitochondrial trafficking lies in electron microscopy studies in Drosophila (Stowers et al., 2002). In this study, Milton mutant flies were found to lack mitochondria in presynaptic terminals of photoreceptor cells – suggestive of a role in neuronal mitochondrial transport. Consistent with this, Milton was found to localise to mitochondria both by subcellular fractionation and by immunocytochemistry. Coimmunoprecipitation experiments revealed an interaction with kinesin heavy chain (Stowers et al., 2002). Thus, Milton was an exciting candidate molecule to promote mitochondrial trafficking and there has been much interest in characterising its interaction with molecular motors. To this end, experiments investigating the composition of the mitochondrial trafficking complex revealed Milton to act independently of kinesin light chain (Glater et al., 2006). Upon expression of kinesin light chain, the Milton-kinesin heavy chain interaction was abolished. Further, mitochondria were present in axons of kinesin light chain null Drosophila (Glater et al., 2006). Studies in mammalian systems confirm these findings. TRAK2 has been shown to interact with kinesin in rodent brain and precise studies of this association reveal the interaction to be with the kinesin cargo binding domain. Further, this interaction is known to be direct due to evidence from yeast two hybrid and FRET experiments. In contrast to Drosophila systems, TRAK forms a ternary complex with kinesin light and heavy chains (Smith et al., 2006).

TRAKs are known to mediate mitochondrial trafficking in mammalian cells (Brickley and Stephenson, 2011; van Spronsen et al., 2013). A biochemical interaction between TRAK1 and TRAK2 and Miro has been demonstrated in a heterologous expression system and TRAK2 and Miro are known to interact *in vivo* (Fransson et al., 2006; MacAskill et al., 2009a). Live imaging has revealed TRAKs to be positive regulators of mitochondrial transport in neurons. In axons, knockdown of TRAK1 or expression of a dominant negative construct blocking association with kinesin decreases motile mitochondria in both anterograde and retrograde directions. In contrast, knockdown of TRAK2 yielded no effect on mitochondrial motility in axons (Brickley and Stephenson, 2011). This has been recently proposed to be due to differential targeting of TRAK1 and TRAK2. TRAK1 has been shown to localise to axonal mitochondria, while TRAK2 is expressed in proximal dendrites of hippocampal neurons (Loss and Stephenson, 2015; van Spronsen et al., 2013). This distribution is reportedly due to a preferential interaction between TRAK2 and dynein/dynactin components in the dendrites. This preferential interaction is achieved via a 'head-to-tail' conformation of TRAK2, whereby its N- and C-terminal can interact with each other. This conformation was determined by FRET and a rapalog inducible folding of TRAK2, which preferentially bound to the p150 dynactin subunit over KIF5B. Overexpression of kinesin motor KIF5B appears to decrease the FRET signal, unfolding TRAK2. In contrast, TRAK1 was proposed to be more dynamic in this respect, and so able to interact with both kinesin and dynein motor components (van Spronsen et al., 2013). The conformation dependent interaction of TRAKs with opposing motor complexes raises the important question of which factors may bring about conformational change endogenously, and if this is a critical mechanism for a change in direction of organellar transport.

### 1.3.5 Other mitochondrial trafficking proteins

Other adaptor proteins for kinesin based mitochondrial transport in neurons include Syntabulin, FEZ1 and Alex3 (Birsa et al., 2013; MacAskill and Kittler, 2010) (Figure 1-3). Syntabulin interacts directly with kinesin-1 motors and was first identified in transport of presynaptic vesicles, but also partially localises to mitochondria and promotes anterograde transport (Cai et al., 2005). FEZ1 is known to interact with KIF5 and tubulin and promote anterograde transport of mitochondria in differentiating PC12

cells and hippocampal neurons (Fujita et al., 2007a; Ikuta et al., 2007). Whether these proteins act in concert with or independently of Miro mediated mitochondrial trafficking has yet to be determined. Alex3 – a member of a protein family specific to higher vertebrates - is also a positive regulator of mitochondrial trafficking and interacts with Miro proteins, KIF5C and TRAK2. This regulation is abolished upon increased calcium concentrations, highlighting a potential role for Alex3 in calcium dependent mitochondrial trafficking (Lopez-Domenech et al., 2012). In addition to mitochondrial fusion, Mfn2 has also been shown to regulate mitochondrial trafficking. Knock-out of Mfn2, or expression of CMT2A (Charcot Marie Tooth 2A) mutants causes a decrease in mitochondrial velocity and an increase in time spent immotile. Interestingly, this is proposed to be independent of fusion activity, as knockdown of inner membrane fusion protein OPA1 has no effect on trafficking. Further, expression of Mfn2 CMT2A mutants which maintain their fusion activity are unable to compensate for loss of Mfn2 in trafficking assays. Mfn1 has not been investigated in this manner. Both Mfn1 and Mfn2 were shown to interact with Miro and TRAK proteins in a heterologous system, thus it is likely, these proteins are acting in a similar pathway as Miro and TRAK to regulate trafficking, but this remains to be determined (Misko et al., 2010)

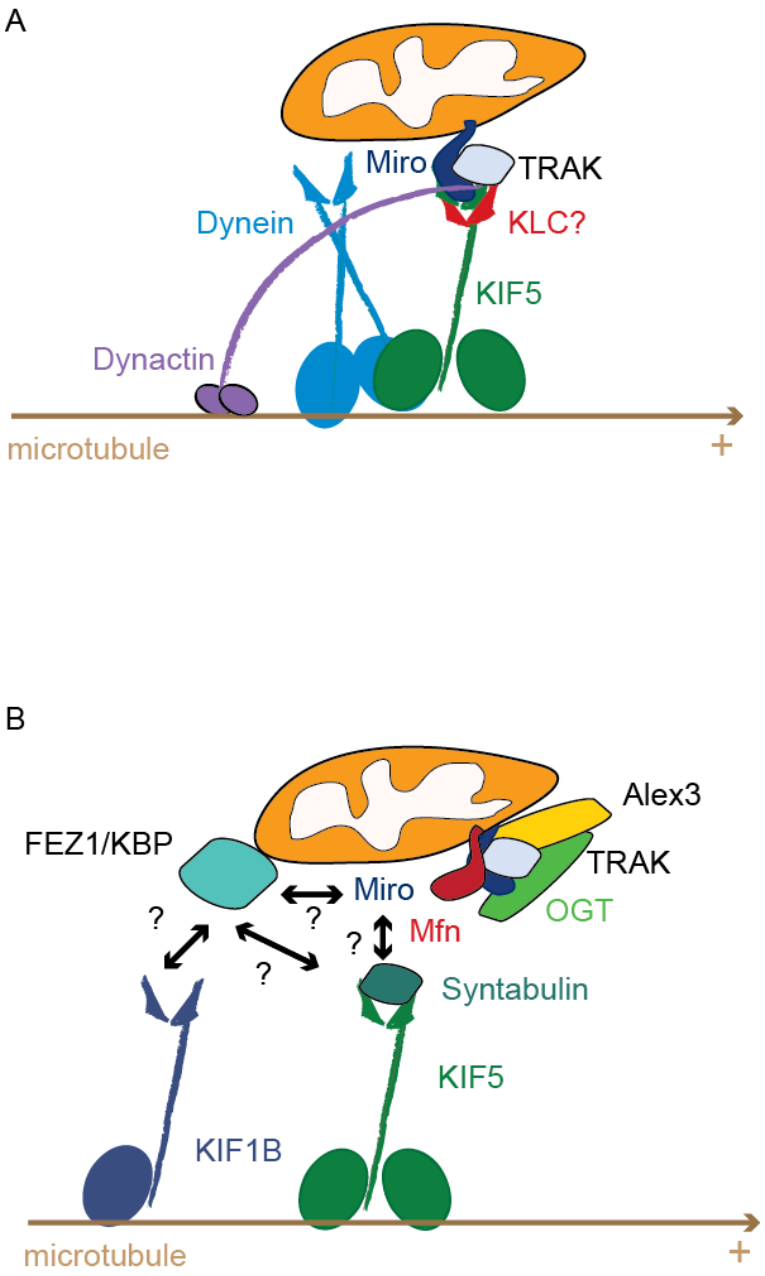



**Figure 1-3 The mitochondrial trafficking complex**

(A) Schematic representing kinesin-1 and dynein mediated mitochondrial transport. (B) Proposed components of the mitochondrial trafficking machinery. Known interactions with Miro and TRAK are shown. These include alternative adaptor proteins, which may act in concert with, or independently of Miro.

## 1.4 Mechanisms regulating mitochondrial trafficking

### 1.4.1 Neuronal activity and calcium signalling

As discussed in 1.3, correct spatio-temporal regulation of mitochondrial dynamics is essential to neuronal function. Mitochondrial dynamics are negatively regulated by calcium levels. Increasing calcium levels leads to a decrease in mitochondrial motility and mitochondrial length (Rintoul et al., 2003; Yi et al., 2004). This is of particular significance in neurons as glutamate receptor activation during neuronal firing leads to influx of calcium, and so mitochondria are stopped specifically at these energetically demanding sites, as shown by an increase in mitochondria opposing presynaptic zones (Macaskill et al., 2009b). This effect can be inhibited by addition of D-APV ((2R)-amino-5-phosphonovaleric acid, a competitive inhibitor of NMDA receptors) prior to glutamate treatment, highlighting the physiological importance of NMDA (N-methyl-D-aspartate) receptor signalling in this mechanism (Macaskill et al., 2009b). This mechanism of calcium-dependent stopping has been well studied and is known to be dependent on Miro, more specifically, its calcium binding EF hand domains. Mutation of these domains to render them calcium insensitive abolishes the calcium dependent stopping effect (Saotome et al., 2008; Wang and Schwarz, 2009) and decreases proximity to synaptic zones (Macaskill et al., 2009b). Calcium binding to these domains is thought to trigger a conformational change, causing an uncoupling of mitochondria from the microtubules. Two major mechanisms have been proposed for this. In one model, increased calcium decreases the interaction between Miro and KIF5, whereas the EF hand mutant remained bound to kinesin at higher calcium concentrations, as demonstrated by GST pull down experiments and coimmunoprecipitation assays from rat brain lysate. The interaction between TRAK and Miro was unaffected. Consistent with this, increased intracellular calcium led to a decrease in kinesin in the mitochondrial fraction (Macaskill et al., 2009b). Alternatively, in axons, it has been shown that kinesin heavy chain remains associated with mitochondria upon calcimycin treatment to increase intracellular calcium (Wang and Schwarz, 2009). In this model, kinesin heavy chain is released from the microtubules upon increased intracellular calcium, and instead co-precipitates with Miro. However, the concentrations of free calcium required to see this interaction are far greater than those in Macaskill et al. (2mM, compared with 5μM) perhaps

accounting for the discrepancy (Macaskill et al., 2009b; Wang and Schwarz, 2009). A schematic representing these mechanisms is shown in Figure 1-4 parts A and B. In contrast to knockdown experiments (Macaskill et al., 2009b), data from a novel mouse model lacking Miro1 shows no difference in calcium-dependent stopping of mitochondria (Nguyen et al., 2014) and *in vivo* imaging experiments in stimulated saphenous nerves show an increase in mitochondrial motility (Sajic et al., 2013). However, this could reflect an increase in mitochondrial fission prior to trafficking that allows even distribution of mitochondria along the axon, or differential mechanisms in axons versus dendrites (Sajic et al., 2013).

In addition to cytosolic calcium levels, intra mitochondrial calcium levels can influence trafficking. Studies in neurons transfected with a mitochondrial matrix calcium reporter indicated a negative correlation between matrix calcium levels and transport velocity. Consistent with this, stationary mitochondria had a higher matrix calcium level than did motile mitochondria. Calcium uptake into the mitochondrion is dependent on the mitochondrial calcium uniporter at the inner membrane, and pharmacological block of this uniporter inhibits the calcium dependent arrest in transport. Interestingly, this mechanism is also dependent on the calcium sensing capabilities of Miro1. Mutation of the EF hand domains to render them calcium insensitive impairs calcium uptake into the mitochondrion, and so the mitochondria do not stop (Chang et al., 2011). This may reflect a mechanism to allow continued trafficking of mitochondria with low matrix calcium, and so high calcium buffering capabilities. Thus, Miro can act as a calcium sensor to regulate mitochondrial trafficking dependent upon cytosolic and matrix calcium levels.

### 1.4.2 Glucose

In contrast to other cargo, such as presynaptic vesicles, mitochondrial motility is negatively regulated by high glucose concentrations. This is dependent upon post translational modification of Milton/TRAK by the O-linked-β-N-acetylglucosamine (O-GlcNAc) transferase enzyme (OGT) that adds N-acetyl glucosamine moieties (Iyer and Hart, 2003; Pekkurnaz et al., 2014). The activity of this enzyme is dependent upon the hexosamine biosynthetic pathway, of which glucose is a major substrate. GlcNAcylation of TRAK decreases mitochondrial motility, anchoring mitochondria at

sites of high glucose, conferring potential for high ATP production. It remains to be determined how modification of TRAK in this way leads to uncoupling of mitochondria from microtubule based transport.

### 1.4.3 Mitochondrial membrane potential

The correlation between mitochondrial membrane potential – indicative of ATP production – and trafficking is controversial. It is of particular importance to study this in the context of spatial regulation of mitophagy – selective clearance of damaged mitochondria. It has been shown that mitochondria with a lower membrane potential are preferentially trafficked in the retrograde direction. Those with a higher membrane potential were trafficked out into the axon, purportedly to replace old or damaged mitochondria (Miller and Sheetz, 2004). This is supported by observations of mitophagy by live imaging (Cai et al., 2012). In this study, cortical neurons overexpressed E3 ubiquitin ligase Parkin (crucial for initiating mitophagy) and CCCP treatment to damage mitochondria decreased anterograde, and increased retrograde mitochondrial trafficking. This is consistent with antimycin treatment, which depolarises mitochondria, upregulating retrograde transport. However, CCCP treatment in the absence of Parkin, decreased transport in both directions (Miller and Sheetz, 2004). Thus, further study will be necessary to determine the motors involved in mitophagy. It is of particular interest to investigate the role of Miro in this context. Miro is a known substrate of the E3 ligase, Parkin (Birsa et al., 2014; Liu et al., 2012). Therefore, ubiquitination of this protein could render it unable to couple to kinesin motors, arresting damaged mitochondria (Rintoul et al., 2003; Wang et al., 2011c).

### 1.4.4 GSK3 beta activity

GSK3 beta (glycogen synthase kinase) has a myriad of substrates. One of these substrates is kinesin light chain (KLC). Phosphorylated kinesin light chain is uncoupled from its cargo, as shown by a decrease in KLC in vesicle fractions in an ATP-dependent manner. This corresponds with an electrophoretic shift, indicative of a phosphorylation, and it is reversed by treatment with alkaline phosphatase. This was confirmed by incubation of squid axoplasm with GSK3 beta. Membrane fractions from these samples were depleted of kinesin. Thus, GSK3 beta is a negative regulator of fast axonal transport, and this occurs in the anterograde direction specifically (Morfini

et al., 2002). Studies in cultured neurons have extended these findings to show inhibition of GSK3 beta leads to an increase in anterograde mitochondrial transport (Chen et al., 2007b) and that overexpression of GSK3 beta, or a constitutively active version thereof, decreased anterograde mitochondrial trafficking. Velocity was unaffected in this study, implying an uncoupling of mitochondria from motor complexes (Morel et al., 2010).

GSK3 beta is inhibited by the kinase, Akt, which itself lies downstream of metabotropic receptors such as dopamine and 5HT (5-hydroxytryptamine, or serotonin) receptors. The contributions of each of these signalling pathways to mitochondrial motility have been investigated. Treatment of neuronal cultures with 5HT or a 5HT receptor agonist increased the fraction of motile mitochondria in proximal axons. Treatment with 5HT led to an increase in phosphorylated, active Akt, and so a decrease in active GSK3 beta, providing mechanistic insight into the effects of serotonergic signalling on mitochondrial motility (Chen et al., 2007b). Conversely, treating hippocampal cultures with dopamine resulted in an increase in stationary mitochondria. Dopamine was shown to decrease phospho-Akt levels, increasing GSK3 beta activity, potentially increasing KLC phosphorylation and uncoupling from cargo (Chen et al., 2008). Both serotonergic and dopaminergic signalling are dysregulated in psychiatric disease (Beaulieu, 2011). Therefore it could be of interest to investigate further alterations in these signalling pathways and their contribution to mitochondrial trafficking in the onset of mental illness.

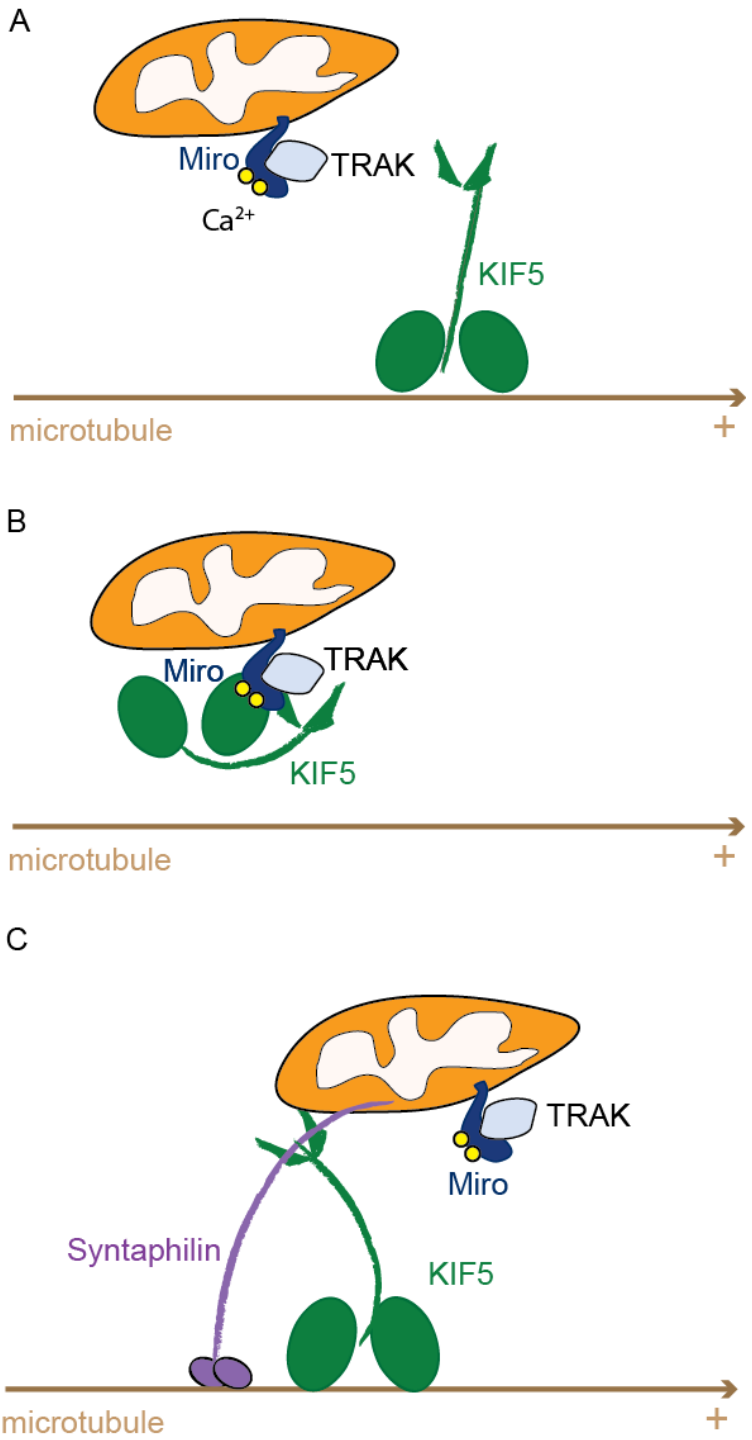
### 1.4.5 Miro GTPase domains

In comparison to the functions of the EF hand domains, contributions of the Miro GTPase domains remain poorly understood. Studies in HEK cells have shown the mitochondrial network to be altered upon expression of a dominant negative (S18N) or constitutively active (P13V) – point mutations which preclude guanine nucleotide binding or mimic a GTP bounds state respectively. Each of these mutations can induce mitochondrial clustering in the perinuclear region and the V13 mutant can also cause mitochondrial elongation (Fransson et al., 2006). Further investigation revealed these mutations differed in their ability to recruit TRAK2 to the mitochondria by immunofluorescence (MacAskill et al., 2009a). The N18 (proposed dominant

negative) construct recruited TRAK to the mitochondria to a similar extent to that of wild-type Miro1. However, the V13 mutant (proposed dominant negative) was unable to recruit TRAK compared to overexpression of wild-type Miro1. Colocalisation of TRAK and mitochondria was comparable to that in cells not expressing Miro1. This corresponds to an increase in the number of mitochondria in neurites upon overexpression of wild-type or N18 Miro, while Miro1 V13 had no effect. This might suggest an increase in mitochondrial trafficking dependent on the Miro GTPase state and mitochondrial TRAK levels (MacAskill et al., 2009a). Interestingly, coimmunoprecipitation assays do not show a difference in the interaction between Miro and TRAK in a GTPase-dependent manner. Instead, the levels of TRAK in cell lysates are decreased when Miro1 is co-expressed. Further, the levels of Miro1 V13 were reduced upon coexpression with TRAK (Fransson et al., 2006).

Recently, a role for the Miro GTPase domains in regulating directionality of mitochondrial trafficking has been proposed (Babic et al., 2015). Studies in Drosophila motor neurons which expressed Miro T25N (orthologue of S18N) revealed no mitochondria were trafficked into axons, and in fact, this mutation was lethal before adulthood. Those expressing A20V (orthologue of P13V) showed a normal mitochondrial distribution. This is consistent with the T25N mutation preventing kinesin based motility. Moreover, a dominant negative mutation in the C-terminal GTPase domain impaired dynein based motility without affecting kinesin based motility– these processes were devoid of mitochondria. This can be clearly addressed in dendrites of Drosophila sensory neurons, where the minus ends are oriented outwards, overcoming the need for normal anterograde transport to investigate retrograde transport (Babic et al., 2015). Therefore, Miro may control the balance between anterograde and retrograde transport, dependent upon its GTPase state. This mechanism may involve TRAK recruitment or conformation. Thus, it would be of great interest to identify enzymes regulating Miro GTPase activity.

**Figure 1-4 Mechanisms of Calcium dependent mitochondrial stopping**




(A) Miro1 calcium binding induces a conformational change that uncouples it from kinesin motors. From (Macaskill et al., 2009b). (B) Miro1 calcium binding induces a conformational change in kinesin that uncouples the motor from the microtubules. From (Wang and Schwarz, 2009). (C) The engine switch and brake model where syntaphilin acts

an anchor to arrest mitochondrial motility. From (Chen and Sheng, 2013).

## 1.5 Contribution of mitochondria to dendrite outgrowth

That dendritic morphology is linked to the connectivity and function of neuronal circuits is well documented (Spruston, 2008). A useful method for quantifying dendritic complexity is Sholl analysis. First demonstrated in 1953, this involves quantification of intersections and branch points of dendrites within concentric circles of evenly increasing distances from the soma (Sholl, 1953). Dendritic morphology is controlled by concerted action of cell extrinsic and cell intrinsic factors throughout the life of a neuron. Initially these cues act to establish polarity, and then promote dendrite outgrowth, followed, at later time points, by fine tuning of the dendritic arbour. These cues can be positive regulators – acting to increase dendritic length or branching, or negative regulators – decreasing or preventing neurite outgrowth or branching. Extrinsic cues fall into three major classes; synaptic activity, secreted cues and contact mediated factors (Valnegri et al., 2015). Intrinsic cues include regulation of protein levels by transcriptional control, regulation of local translation in dendrites and ubiquitin-proteasome mediated protein turnover. Another crucial factor contributing to dendrite growth and maintenance is the cytoskeleton, organised in dendrites by Golgi outposts acting to nucleate microtubules (Ori-McKenney et al., 2012). Actin and microtubule networks act to structure dendrites. Cargo transported along these tracks by motor proteins is essential for neurite maintenance (Puram and Bonni, 2013).

The importance of cytoskeletal-based transport to dendritic morphology is exemplified by loss of dendritic complexity upon knock-out of motor protein subunits. It has been shown in Drosophila that loss of dynein light chain leads to downsizing of the dendritic arbour and a shift towards increased branching in the proximal region compared to distal. Loss of kinesin heavy chain had a comparable effect (Satoh et al., 2008). It is not just motor proteins themselves which contribute to neurite outgrowth, but, ostensibly, their cargo. The distribution of Rab5 positive endosomes is correlated with dendritic branching (Satoh et al., 2008). Furthermore, microtubule plus-end directed transport mediated by kinesins has been shown to contribute to dendrite outgrowth. This is demonstrated by the necessity for KIF5 and adaptor, GRIP1, (Glutamate

receptor interacting protein-1) in the transport of Glutamate and EphB (Ephrin B) receptors for correct dendritic development (Hoogenraad et al., 2005). Notably, this study provides evidence for cooperation between extrinsic and intrinsic cues; EphB receptors respond to secreted ephrins and glutamate receptors to presynaptically released glutamate.

Another crucial cargo for neurite outgrowth is mitochondria themselves. The distribution of these organelles has been characterised as both a positive and negative regulator of dendritic development. Depletion of TRAK2 by RNAi leads to a decrease in moving mitochondria in dendrites and also a decrease in dendritic length in cultured rodent hippocampal neurons (van Spronsen et al., 2013). Mitochondrial fission and fusion proteins have also been linked to dendritic development. Loss of Mitofusin 2 leads to accumulation of mitochondria in the soma of cerebellar Purkinje neurons. These neurons have shorter, thinner dendritic processes than wild-type neurons which contribute to cerebellar degeneration and motor deficits in Mitofusin2 knockout mice (Chen et al., 2007a). These findings would be consistent with mitochondria as positive regulators of dendritic development – namely increasing dendritic length and branching in the neuron. This is consistent with reports of other organelles such as Golgi and early endosomes as positive regulators of dendrite morphogenesis (Horton et al., 2005; Satoh et al., 2008). Alternatively, Mitofusin has also been shown to act as a negative regulator of dendrite outgrowth (Kimura and Murakami, 2014). In this *in vivo* study, Mitofusin1 overexpression by *in utero* electroporation led to depletion of mitochondria from processes. In turn, an increase in total dendritic length and dendritic branching per cell was reported. However, it should be noted that these neurons appeared to have increased numbers of neurites, each shorter than controls. This would not only account for the reported effect, but would concur with mitochondria being positive regulators of dendritic branching, as there is increased branching proximal to the soma – the site of mitochondrial accumulation.

In addition to distribution, mitochondrial function has also been linked to dendrite outgrowth (Fukumitsu et al., 2015). In this study, depletion of dendritic mitochondria led to a decrease in dendritic complexity, once more supporting the hypothesis that mitochondria are positive regulators of neurite outgrowth. Further, this could be rescued by supplementation with creatine – a component of an ATP buffering system.

This implies the importance of mitochondrial ATP production for dendritic outgrowth. Moreover, this local depletion of ATP was shown to impair actin turnover, as demonstrated by decreased phospho-cofilin – an actin polymeriser (Fukumitsu et al., 2015). Thus in this model, mitochondria contribute to dendrite outgrowth by regulating the cytoskeleton.

In addition to mitochondria and endosomes, ER (endoplasmic reticulum) distribution has also been shown to regulate dendritic complexity by stabilising branchpoints. This has been shown to be due to confinement of cargo such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunits (Cui-Wang et al., 2012). Recently, the contribution of ER-endosome contact sites to neurite outgrowth has been studied. The Stenmark group showed contact sites between the ER and late endosomes served to load these organelles onto kinesin-1 motors. This facilitates transport of late endosomes to the periphery and promotes neurite outgrowth in PC12 cells. This is proposed to occur by providing lipids to the plasma membrane via endosome fusion (Raiborg et al., 2015). However, the involvement of contact sites between ER and mitochondria in dendritic branching and outgrowth has yet to be investigated.

## 1.6 Mitochondrial dysfunction as a potential pathophysiological mechanism in psychiatric disease

Psychiatric diseases including bipolar disorder, depression and schizophrenia are associated with abnormal neuronal development and plasticity. These diseases do not present with classical mitochondrial dysfunction, as do multiple neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis (Mattson et al., 2008). However, mitochondria are essential to power and regulate neuronal development and function and so, there has been an effort to investigate the contribution of these organelles to onset of major mental illness, as reviewed in (Manji et al., 2012). These studies have identified decreased respiratory chain activity in rat models of both mania and depression (Rezin et al., 2008; Valvassori et al., 2010). Post mortem tissue from patients with bipolar disorder display decreased expression of mitochondrial encoded genes and in particular, a decrease in creatine kinase levels, essential for buffering ATP. In terms of schizophrenia, animal

models have been generated harbouring a 22q11 deletion, detected in schizophrenic cohorts. In these mice, an up regulation of components of the electron transport chain was noted (Jurata et al., 2006). These observations are all supportive of altered mitochondrial function in psychiatric disease. In post mortem schizophrenia samples distribution of mitochondria was investigated. A reduction in the number and density of mitochondria was detected in the prefrontal cortex, striatum and substantia nigra (Kolomeets and Uranova, 2010; Kung and Roberts, 1999; Manji et al., 2012). This evidence for altered mitochondrial function and distribution in psychiatric disease is suggestive of association between defects in mitochondrial trafficking and mental illness. Thus, it is of great importance to investigate mechanisms underlying these potential abnormalities in mitochondrial transport.

## 1.7 Disrupted in schizophrenia 1 (DISC1)

DISC1 was first identified in a Scottish pedigree with a major chromosomal translocation between chromosomes 1 and 11 (t1:11). This family had a high incidence of major mental illness (specifically, schizophrenia, schizoaffective disorder and major recurrent depression), which segregated with this chromosomal rearrangement. Of 77 screened family members, 34 carried the translocation. Of these 34, 16 had psychiatric diagnoses compared with 5 of the unaffected 43 members (St Clair et al., 1990). Please see Figure 1-6 for a representation of the pedigree genotypes and phenotypes as published in 2001 (Blackwood et al., 2001). There is a dearth of genes in this breakpoint region of chromosome 11, highlighting the role of a gene on chromosome 1 as a major candidate susceptibility factor for psychiatric disease. Analysis of this region revealed two genes at the breakpoint – DISC1 and DISC2 – for Disrupted in schizophrenia 1 and 2. By northern blot, it was shown that DISC1 was enriched in adult brain and heart tissue, and within the brain, to be ubiquitous. DISC1 was predicted to have an original protein structure containing a globular N-terminus and a coiled-coil rich C-terminus (see Figure 1-5). DISC2 on the other hand is found on the complementary strand to DISC1 and has been suggested to be a regulatory RNA sequence to influence DISC1 expression (Millar et al., 2000). The outcome of this translocation at the protein level remains under debate (see page 103 in Chapter 3). The translocation may cause a haploinsufficiency, a DISC1 truncation or expression of a chimaeric fusion protein (Brandon and Sawa, 2011).

In addition to the Scottish pedigree, multiple other linkage analyses and sequence analyses have proposed DISC1 as a risk factor in psychiatric illness. For example, polymorphisms in DISC1 such as a 4 base pair deletion, or point mutations giving rise to amino acid substitutions R37W, S704C and L607F segregate with schizophrenia and other major mental illness (Callicott et al., 2005; Hodgkinson et al., 2004; Sachs et al., 2005; Thomson et al., 2014). Polymorphisms such as these have a range of incidence and association with disease in the population. It should be noted that, likely because of this heterogeneity, DISC1 has not been labelled a major risk factor in genome wide association studies (Brandon and Sawa, 2011; Chubb et al., 2008). Much work into the effects of these mutations remains to be carried out, but, at the biochemical level, DISC1 is linked to sporadic schizophrenia. It has been shown to form insoluble aggregates in post mortem schizophrenia tissue, perhaps induced by cellular stress (Leliveld et al., 2008).

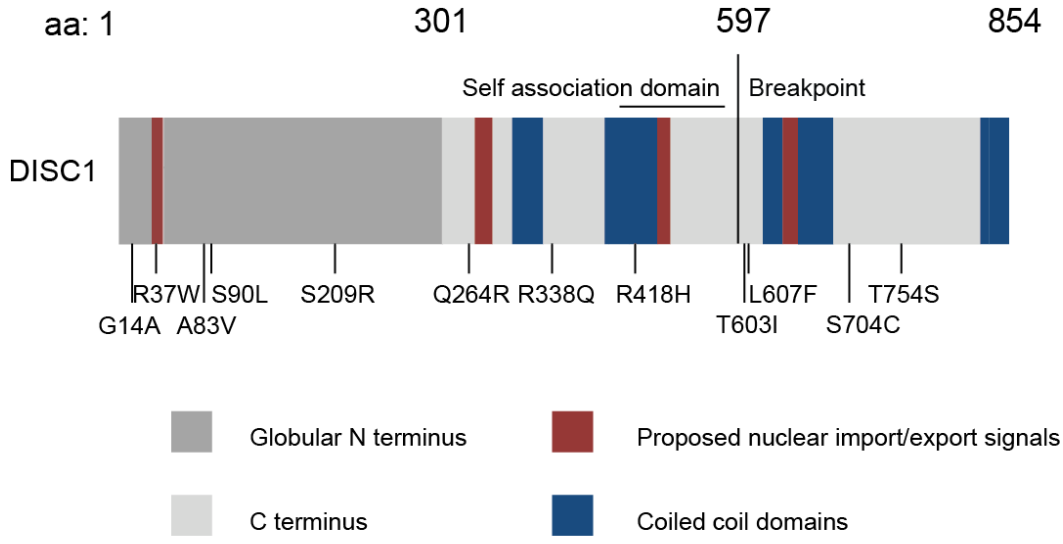



**Figure 1-5 Schematic of DISC1 domains and psychiatric disease risk variants**

Schematic of the DISC1 protein showing coiled coil domains and nuclear import/export signals. The breakpoint refers to the site of the balanced chromosomal translocation in the Scottish pedigree. Amino acid substitutions conferring increased risk of psychiatric disease are indicated below. Adapted from (Brandon and Sawa, 2011).

As described in Table 1-1, further evidence for DISC1 as a candidate susceptibility factor in major mental illness is supported by behavioural phenotypes of mouse models. A variety of models exist, either aiming to replicate the t1:11 translocation or impair DISC1 expression by knockout strategies. These mice show impairments in working memory (Koike et al., 2006; Kvajo et al., 2008), and decreases in pre pulse inhibition (Clapcote et al., 2007; Hikida et al., 2007; Kuroda et al., 2011) – a lower intensity stimulus (often acoustic) precedes a higher one, and should decrease the startle response. Increased startle is a typical phenotype for schizophrenia suggesting the inability to filter out unnecessary information. Developmental abnormalities such as increased ventricle size have also been noted in models of the DISC1 truncation (Pletnikov et al., 2008; Shen et al., 2008a).

These genetic and behavioural data mark DISC1 as a major candidate susceptibility factor for psychiatric disease and so, its function within the neuron is undergoing intense study. DISC1 adopts varying distributions within the cell pointing to multiple roles in the correct function of the neuron. Therefore, studies of DISC1 protein complexes have been of great use in identifying not only the cellular functions of DISC1, but also the pathways affected in major mental illness. Of particular importance in this context is the DISC1 interactome – a large yeast two hybrid screen published by Camargo and colleagues (Camargo et al., 2007). Protein-protein interaction networks point to a role for DISC1 as a regulator of multiple facets of signalling and transport and so, to be a key player in neurodevelopment, to be discussed in the following sections.

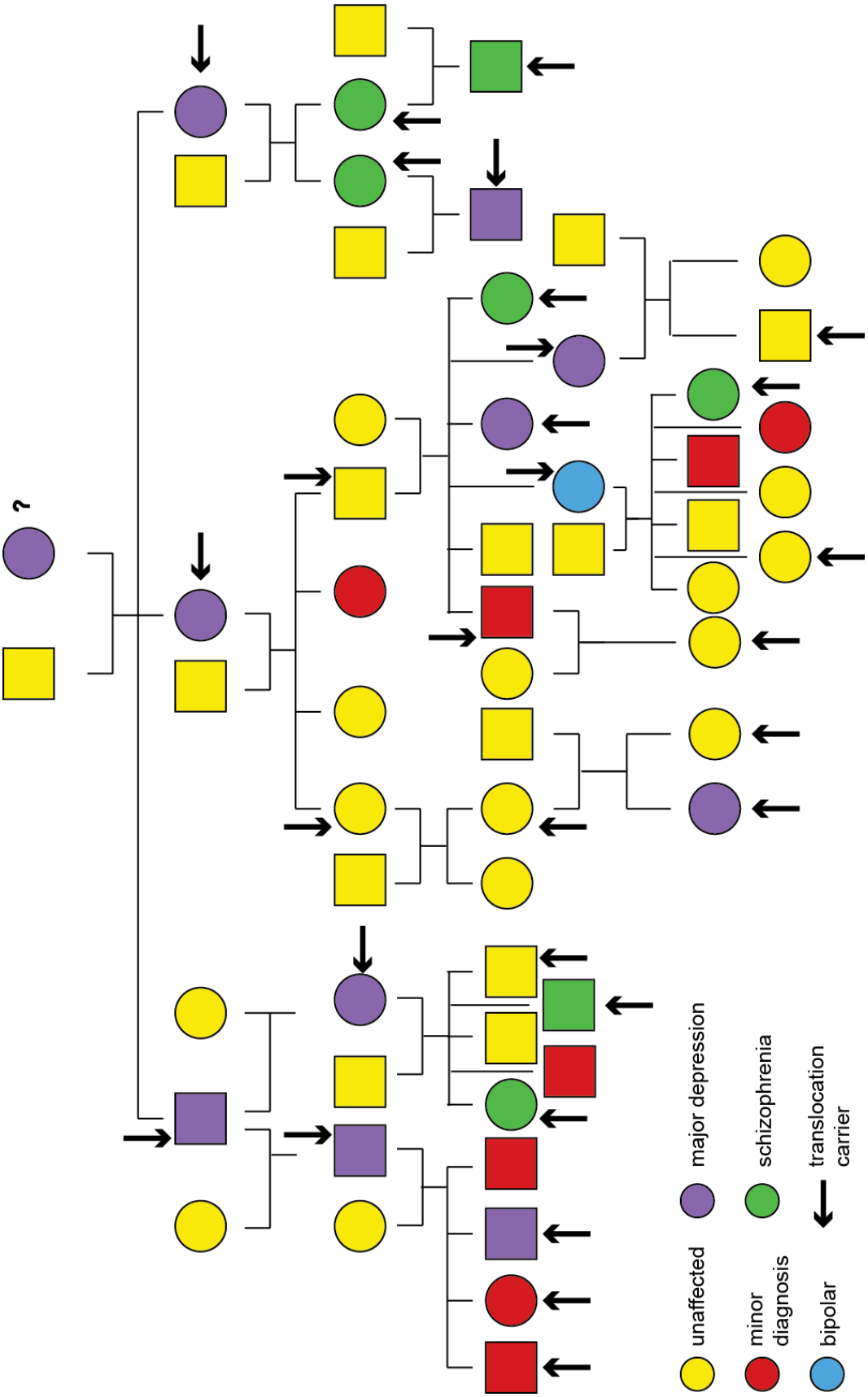



**Figure 1-6 A balanced chromosomal translocation segregates with major mental illness** (adapted from (Brandon and Sawa, 2011))

| Model | Reference | Description | Behavioural and developmental phenotypes |
|---|---|---|---|
| 129S6/SvEv DISC1 introduced onto C57/BL6 background | (Koike et al., 2006) | 129S6/SvEv 25bp deletion in exon 6 of DISC1 purportedly leads to no full length DISC1 expression. | Decrease in working memory by T maze test |
| DISC1$^{TM1Kara}$ | (Kvajo et al., 2008) | DISC1 truncation by introduction of 25bp deletion of DISC1 to C57/BL6 background. Gives stop codon in exon 7. Stop codon and polyadenylation signal in exon 8. No FL DISC1 expression in homozygous mice. Approx 60kDa species present. | Impaired working memory. Decreased novel object recognition. Impaired fear conditioning. |
| DN DISC1 | (Hikida et al., 2007) | Truncated DISC1 (aas 1-597) introduced under CAMKII promoter for forebrain expression. | Hyperactivity. Decreased mobility in forced swim test. Impaired prepulse inhibition. |
| L100P | (Clapcote et al., 2007) | ENU mutagenesis on C57/BL6 mice | Impaired prepulse inhibition. Reduced brain volume. |

| | | | |
|---|---|---|---|
| Inducible human DISC1 expression | (Pletnikov et al., 2008) | Tetracycline inducible human DISC1 expression on C57/BL6 background. Murine DISC1 unaltered. | Hyperactivity. Increased Lateral ventricles. |
| DISC1tr | (Shen et al., 2008a) | Introduction of bacterial artificial chromosome carrying DISC1 exons 1-8 fused to GFP. | Enlarged ventricles, reduced cortices. Decreased mobility in tail suspension test and forced swim test. |
| DISC1 Δ2-3 | (Kuroda et al., 2011) | Homologous recombination interrupts DISC1 between exons 2 and 3. No DISC1 expression in homozygotes. | No gross structural abnormalities. Methamphetamine induced increase in locomotion in females. Overall decrease in pre pulse inhibition. Increased cliff jumping events. |
| DISC1-Boymaw mouse | (Ji et al., 2014) | Model Scottish mutation by introduction of DISC1-Boymaw and Boymaw-DISC1 into DISC1 mouse locus. 129S6/SvEv background. | Decreased mobility in tail suspension test. Hyperactive upon ketamine treatment. |

**Table 1-1 Behavioural and Developmental phenotypes of DISC1 mouse models**

## 1.8 DISC1 as a regulator of neurodevelopment

### 1.8.1 GSK3 beta signalling and neuronal migration

DISC1 has thoroughly studied roles in regulating neural development and this is best characterised in terms of GSK3 beta signalling – itself a renowned regulator of neuronal maturation (Hur and Zhou, 2010). Retroviral mediated knockdown of DISC1 or *in utero* electroporation in E13 mouse embryos resulted in a decrease in proliferating neural progenitors and an increase in migration and differentiation of these cells into mature neurons (Duan et al., 2007a; Mao et al., 2009). This was shown by increased GFP positive cell numbers in the cortical plate, and a concomitant decrease in the proliferative population of the sub ventricular zone. This DISC1-dependent switch from proliferation to migration is known to be dependent upon the ability of DISC1 to inhibit GSK3 beta. Under these conditions, the cell cycle progressor, beta catenin, is not phosphorylated by GSK3 beta, and thus not degraded. Upon DISC1 knockdown, this inhibition is lost, causing neural progenitors to exit the cell cycle. This is supported by decreased protein levels of beta catenin targeted transcripts, e.g. Cyclin D1 and axin. This can be rescued by phospho-null, degradation resistant beta catenin. The role for DISC1 in inhibiting GSK3 beta in this pathway was confirmed by western blot, specifically for GSK3 beta pY216 (an autophosphorylation site indicative of kinase activity). Knockdown of DISC1 increased these levels compared to control.

The influence of schizophrenia associated DISC1 mutations on GSK3 beta signaling and neurodevelopment was investigated in a follow up study from the Tsai lab (Singh et al., 2011). Once more, *in utero* electroporation studies were carried out to knockdown DISC1, in parallel with coexpression of DISC1 common variants R264Q, S704C, L607F – each potentially linked to aberrant brain structure and psychiatric phenotypes – and the rare variant A83V; located in the DISC1-GSK3 beta binding domain. As previously, there was a decrease in proliferating cells upon DISC1 knockdown. Concurrently, there was an increase in neuronal Tuj1 staining. Expression of wild-type DISC1 was able to rescue this effect, but expression of DISC1 A83V, R264Q or L607F showed no significant rescue. Notably, S704C behaved comparably to wild-type DISC1. Each of the mutants that failed to rescue the proliferation

phenotype showed a decreased interaction with GSK3 beta (Singh et al., 2011). Similar results were obtained by the Sawa group upon *in utero* electroporation of truncated DISC1 (Kamiya et al., 2005). Each of these results is consistent with a subset DISC1 variants being unable to maintain the neural progenitor pool via loss of GSK3 beta inhibiton, leading to an increase in progenitor migration and differentiation into mature neurons.

This role for DISC1 in regulating GSK3 beta activity in cortical development was further explored in a study by Ishizuka et al. 2011. Mass spectrometry showed DISC1 to be phosphorylated at serine 713 (or serine 710 of murine DISC1), suggesting levels of phosphorylated DISC1, as well as total protein levels to be important. *In vitro* kinase assays revealed DISC1 to be a substrate of PKA and CDK5, although another study has reported DISC1 phosphorylation to be unaffected by PKA inhibition (Murdoch et al., 2007). Phosphorylation at this site confers the ability of DISC1 to switch between pro-proliferative and pro-migratory roles. In its unphosphorylated form, DISC1 activates beta catenin signalling, via a preferential, inhibitory, interaction with GSK3 beta compared to the phospho-mimic. The phospho-mimic was found to have an enhanced interaction with centrosomal Bardet-Biedl syndrome proteins (BBS), promoting neuronal migration and cortical development. Also, immunoprecipitation assays from E14 and E18 brains mimicked *in vitro* assays and showed DISC1 to interact preferentially with GSK3 beta at E14 and BBS1 at E18. The role for DISC1 phosphorylation as a switch in cortical development is strongly supported by increased levels of phospho-DISC1 in mouse E18 brains (a major stage of neuronal migration) compared to E14 brains (when the neural progenitor pool is proliferating). Additionally, phosphorylated DISC1 was shown to be predominantly in the cortical plate and absent from the subventricular zone by immunohistochemistry. This study confirms the role for DISC1 phosphorylation in proliferation versus migration by *in utero* electroporation of DISC1 knockdown constructs alongside phospho null and phospho mimic DISC1 constructs. Phospho-null DISC1 behaves in a similar fashion to DISC1 knockdown, increasing the number of cells in the subventricular zone, whereas phospho-mimic DISC1 rescued the migration defect induced by DISC1 knockdown. Electroporation of a CDK5 dominant negative construct phenocopied DISC1 knockdown in this assay, marking it as a DISC1 kinase (Ishizuka et al., 2011).

Therefore, phosphorylated DISC1 is permissive of GSK3 beta activity and a promoter of neuronal maturation.

### 1.8.2 Dendrite outgrowth

In addition to mitochondrial dysfunction, there are also multiple reports of altered dendritic structure in neurologicalal disease, including dementia, autistic spectrum disorders and schizophrenia. These diseases often, but not exclusively, present with decreased dendritic complexity (reviewed in (Kulkarni and Firestein, 2012)). In schizophrenia, post mortem imaging studies have shown a decreased cortical volume, with no change in cell number or axon number. This is consistent with decreased dendritic complexity, and supports the neurodevelopmental theory of schizophrenia, whereby the appearance of symptoms in early adulthood can be partially attributed to earlier developmental events (Kulkarni and Firestein, 2012). Observations such as these prompted study into the contribution of DISC1 to dendritic development.

DISC1 is often reported as a positive regulator of dendritic branching. This has been carried out *in vitro* and *in vivo*. Early studies in differentiating PC12 cells showed an upregulation of neurite outgrowth to be dependent on interaction of DISC1 and FEZ1 (Miyoshi et al., 2003). This result was built upon by studies of DISC1 overexpression and knockdown in PC12 cells. DISC1 overexpression led to an increase in process length, whilst knockdown decreased this metric (Hattori et al., 2010). Other interactors of DISC1 have been reported to contribute to its effect on dendrite outgrowth, such as Ndel1 and dysbindin – also psychiatric susceptibility factors. DISC1 has been shown to stabilise dysbindin protein level. Furthermore, overexpression of dysbindin upon DISC1 knockdown rescued a defect in neurite outgrowth in differentiating CAD cells (Cath.-a-differentiated cells, a murine neuroblastoma cell line). In primary culture, a mutant of DISC1 unable to interact with, and thus stabilise, dysbindin causes decreased neurite outgrowth (Lee et al., 2015).

The effect of DISC1 on dendrite development has also been studied in mouse models. Dissociated cultures of neurons from P0 mice expressing truncated DISC1 (Table 1-1) demonstrate fewer and shorter neurites compared to controls (Shen et al., 2008b). *In vitro* and *In vivo* preparations from another mouse model carrying a truncating lesion

in DISC1 concur with these findings (Kvajo et al., 2011; Lepagnol-Bestel et al., 2013). *In vitro* preparations of hippocampal neurons from both heterozygous and homozygous mice display decreased dendritic length and branching at DIV4, with cortical cultures displaying a similar, although less severe, phenotype. In each case this effect was rescued by overexpression of wild-type DISC1 (Lepagnol-Bestel et al., 2013). *In vivo*, decreased dendritic complexity was noted in developing neurons of the dentate gyrus at post natal day 11 and in adult born neurons. This effect appeared to be caused by cell extrinsic factors (see 1.5 on page 42) - expression levels of semaphorin3A were downregulated (Kvajo et al., 2011).

In contrast, evidence for DISC1 as a negative regulator of dendritic development has also been demonstrated. Knockdown of DISC1 by stereotaxic injection of RNAi into proliferating neural progenitors of the hippocampus revealed adult born neurons to have more elaborate dendritic arbours by Sholl analysis (Duan et al., 2007b). This is consistent with data presented by Kang and colleagues (Kang et al., 2011) showing DISC1 knockdown in adult born neurons to confer increased complexity in collaboration with FEZ1. It remains to be confirmed whether DISC1 has opposing effects on regulating dendritic development during embryonic development compared to adult born neurons. Alternatively, these contrasting observations could be specific to cell sub-type, or, as recently proposed, an off target effect of the shRNA (Tsuboi et al., 2015).

## 1.9 DISC1 as a regulator of intracellular signalling

### 1.9.1 Cyclic AMP

Cyclic AMP (or cyclic Adenosine monophosphate - cAMP) is a secondary messenger with multiple roles within the neuron including neurite outgrowth, glucose metabolism and transcriptional control. cAMP is generated by adenylyl cyclase and degraded by phosphodiesterase family enzymes (Averaimo and Nicol, 2014). It is at this level that DISC1 is able to spatially and temporally regulate cAMP signaling cascades by direct interaction with phosphodiesterase 4 enzymes, subsequently altering their activity (Murdoch et al., 2007). PDE4B itself is a candidate susceptibility factor for mental illness as demonstrated by a t(1:16) chromosomal translocation in two related patients

with a history of psychotic episodes. DISC1 and PDE4B were shown to interact directly by *in vitro* translation binding assays and the sites required deduced to be the UCR2 domain of PDE4B – common to all splice variants – and the N-terminus of DISC1 between amino acids 219-283 (Millar et al., 2005b). This interaction was confirmed in neurons and noted to be enriched in the mitochondrial fraction, highlighting the potential for this pathway to be involved in mitochondrial trafficking. Furthermore, this interaction is dependent on intracellular cAMP levels. Pharmacologically increasing cAMP by addition of PDE inhibitor, IBMX, and adenylate cyclase activator forskolin causes the dissociation of the PDE4-DISC1 complex. This dissociation is associated with an increase in PDE4 activity, denoted by an increase in PKA mediated-PDE4 phosphorylation and cAMP hydrolysing activity (Millar et al., 2005b). In a contrasting publication, further investigation into PDE4 isoform selectivity was carried out, revealing that PDE4D was susceptible to cAMP mediated release from DISC1 whereas PDE4B and DISC1 maintain a stable interaction (Murdoch et al., 2007).

As well as regulation by direct interaction, DISC1 has been recently shown to regulate PDE4 activity by transcriptional regulation in concert with Activating transcription 4 (ATF4). Chromatin immunoprecipitation revealed DISC1 to bind a PDE4 locus specific to PDE4D9, and repress transcription at that locus. This transcriptional repression is likely dependent on cAMP levels as shown by PKA dependent phosphorylation of DISC1 on S58 causing it to exit the nucleus and release ATF4 (Soda et al., 2013). This pathway may be augmented upon DISC1 mutation. Two point mutations associated with schizophrenia (R37W and L607F) lose the ability to repress ATF dependent transcription. In the case of L607F, it appears the interaction with ATF is attenuated (Malavasi et al., 2012). Thus, PDE4D transcription, among other genes, may be increased.

### 1.9.2 Synaptic activity

DISC1 has been implicated in regulating activity at multiple types of synapse. It has been reported to localise to the excitatory post synapse by fractionation and immunogold labelling (Wang et al., 2011b). Further, DISC1 has been shown to regulate synaptic transmission via modulation of post-synaptic composition. Traf and Nck interacting kinase (TNIK) has been demonstrated to be a key player in this process. TNIK is proposed to be a postsynaptic density protein according to mass spectrometry screens of post synaptic density fractions (Wang et al., 2011b) and DISC1 interactor according to the large scale yeast two hybrid screen (Camargo et al., 2007). The DISC1-TNIK interaction appears to decrease TNIK kinase activity. Knockdown of TNIK, or inclusion of a DISC1 peptide which inhibits TNIK kinase activity, leads to a decrease in surface levels of GluR1 (AMPA receptor subunit). Likewise, total protein levels of post-synaptic density (PSD) components GluR1, PSD95 (post-synaptic density protein 95) and stargazin were decreased in a proteasome/lysosome dependent manner. This is supportive of a decrease in glutamatergic synapses. Consistent with this, TNIK knockdown or treatment of cells with the TNIK inhibitory peptide caused a decrease in amplitude and frequency of mini excitatory post synaptic currents (Wang et al., 2011b). Additional mechanisms by which DISC1 can regulate glutamatergic synapses have been proposed by the Sawa group (Hayashi-Takagi et al., 2010). DISC1 has been shown to form a 'signalasome' with PSD95, anchoring and inhibiting Kalirin-7 at the post synaptic density. This protein is a guanine nucleotide exchange factor able to activate Rac1 – a key molecule in effecting changes in dendritic spines. DISC1 knockdown leads to free Kalirin-7, able to activate Rac1. Prolonged activation of Rac1 in a DISC-dependent manner leads to a decrease in spine number and volume in organotypic slices (Hayashi-Takagi et al., 2010). Further, inhibitors of PAK (downstream of Rac1 in regulating spine morphology) can rescue this phenotype induced by DISC1 knockdown (Hayashi-Takagi et al., 2014). Taken together, these data support multiple roles for DISC1 in regulating synapse composition and thus synaptic strength, deficits in which are linked to psychiatric disease.

DISC1 has a recently reported role in regulating dopaminergic signalling via interaction with receptor subunit D2R. This interaction is increased in post mortem

brain tissue of schizophrenia patients. As discussed in 1.8.1, DISC1 is able to inhibit GSK3 beta activity, a downstream effector of dopaminergic signalling. The DISC1-D2R interaction caused a decrease in receptor internalisation, as shown by biotinylation experiments in HEK293 cells and rat striatal culture (Su et al., 2014). Thus, enhanced GSK3 beta activity, downstream of impaired dopaminergic signalling, could represent a pathophysiological mechanism in psychiatric illness.

## 1.10 DISC1 as a regulator of intracellular trafficking

DISC1 is implicated in intracellular trafficking of multiple cargoes via interactions with molecular motors and microtubule associated proteins such as MIPT3 and MAP1a (Camargo et al., 2007; Kamiya et al., 2005; Morris et al., 2003; Taya et al., 2007). It can act to regulate the microtubule network via centrosome association and can regulate trafficking of specific cargo via association with molecular motors. This centrosomal localisation of DISC1 is also crucial to its role in neuronal development. Here it can interact with Ndel1 – a nuclear distribution factor known to be crucial for neuronal differentiation and migration. Mitochondria are a crucial cargo of DISC1 and DISC1-dependent trafficking of these organelles will be discussed in 1.11.3. Other known cargoes of DISC1 include mRNA, amyloid precursor protein (APP) and synaptic vesicles (Flores et al., 2011; Shahani et al., 2015; Tsuboi et al., 2015). Depletion of DISC1 by knockdown or knockout decreases surface levels of APP and increases amyloid beta (please refer to section 5.2.5 on page 167) (Shahani et al., 2015). Further study into the implications of this coordination between DISC1 and APP remain to be carried out. DISC1 knockdown or truncation leads to a decrease in motility of presynaptic vesicles (Flores et al., 2011). This can be rescued by mood stabiliser and GSK3 beta inhibitor, lithium. However, in this study, it seemed that lithium was affecting assembly of a FEZ1/DISC1/synaptotagmin trafficking complex to affect presynaptic vesicle transport, rather than affecting kinesin-based motility via GSK3 beta inhibition (Flores et al., 2011; Morfini et al., 2002).

Recently, DISC1 has been shown to be essential in trafficking of certain mRNAs, in particular, the 3'UTR of the Inositol-trisphosphate receptor ($IP_3R$) transcript. In DISC1 knockout neurons, this transcript is not efficiently transported into dendrites, decreasing its availability for local translation. This is the first report linking DISC1 to

local protein synthesis, essential for synaptic plasticity in neurons. Loss of this transcript in dendrites confers a decrease in the maintenance of long term potentiation (LTP), according to experiments carried out with a cell-permeable DISC1 peptide that can act as a dominant negative to uncouple the interaction between DISC1 and the $IP_3R$ transcript (Tsuboi et al., 2015).

Dynein complex components Ndel1 and Lis1 have been shown to be trafficked to the distal axon by kinesin-1 in a DISC1 dependent manner. This is shown to be essential for axon elongation and identifies DISC1 as a potential molecular link between kinesin and dynein motors (Taya et al., 2007). In addition to interaction with kinesin-1 and kinesin-3 motors, DISC1 is known to interact with components of the dynein complex Lis1, p150 and Ndel1. Further, it has been shown to be necessary in localising these proteins to the centrosome and expression of mutant (truncated) DISC1 interferes with the ability of wild-type DISC1 to recruit these proteins. Moreover, mutant DISC1 appears to interfere with the microtubule cytoskeleton, as shown by failure of microtubules to reorganise upon expression of truncated DISC1 in conjunction with nocodazole treatment. This highlights a role for DISC1 as an organiser of the microtubule network, and so able to regulate trafficking independently from motor complexes (Kamiya et al., 2005). This role for DISC1 is supported by its localisation at the centrosome (the major microtubule organising centre) and interaction with centrosome associated proteins such as kendrin and BBS4 (Biedet-Bardl syndrome 4 protein) (Kamiya et al., 2008; Miyoshi et al., 2004). Correct localisation of centrosomal associated proteins is essential for normal neuronal migration, and thus cortical development. This is further evidence for the role of DISC1 in regulation of neuronal development (Wang and Brandon, 2011).

## 1.11 DISC1 and mitochondria

### 1.11.1 Localisation of DISC1 at the mitochondrion

DISC1 has been reported to localise to mitochondria in multiple studies (James et al., 2004; Millar et al., 2005a; Millar et al., 2005b; Ogawa et al., 2014). This has been shown both by cellular fractionation approaches and immunocytochemistry. It would seem this targeting is dependent upon the N-terminus of DISC1. The first 358 amino

acids of DISC1, corresponding to a globular head domain, appear to be targeted to mitochondria in a similar way to full length DISC1, suggesting the C-terminus is not necessary for mitochondrial localisation (Millar et al., 2005a). Five DISC1 isoforms have been described at the protein level. Transcripts Long and Long variant (of which they are two splice variants) are around 98kDa comprising exons 1-13. The Short transcript of 70kDa comprises exons 1-9 and the Extremely Short transcript of 40kDa comprises exons 1-3 of the 13 exon gene. Of these DISC1 isoforms, only that using the alternative exon 1 (exon 1b, present exclusively in the Extremely Short variant) contain a predicted mitochondrial targeting sequence. However, there is no conclusive evidence for this transcript at the protein level. Therefore, other factors must allow this mitochondrial targeting, such as interaction with mitochondrial proteins. Those that have been studied in this way include trafficking proteins, Miro1 and TRAK1 and the cristae regulator, Mitofilin (Ogawa et al., 2014; Park et al., 2010a). Immunocytochemistry shows a predominantly mitochondrial localisation in two neuroblastoma cell lines (U373 MG and SH-SY5Y), yet puncta are detected both in the periphery and the nucleus (James et al., 2004). Further studies in cortical neurons are consistent with this heterogeneous distribution of DISC1 (Brandon et al., 2005; Park et al., 2010a). Two studies show a redistribution of DISC1 upon destabilisation of microtubules with taxol or nocodazole. Under these conditions, DISC1 is seen to take on a less mitochondrial distribution. Fractionation assays suggest a shift into the cytosolic fraction (Brandon et al., 2005; James et al., 2004). These observations represent early indications of a role for DISC1 in microtubule based transport of mitochondria. Little is known about the effect of schizophrenia-associated DISC1 mutations on mitochondrial distribution. One mutation that has been studied in this regard is the R37W mutation. This mutation lies within an arginine rich sequence shown to be important for the mitochondrial localisation of DISC1 – deletion of this region or mutation of arginine residues to alanine results in DISC1 taking on a diffuse staining in contrast to a mitochondrial localisation in COS7 cells. The R37W mutation itself appears to lead to a more uniform distribution of DISC1 on the mitochondria, and induce mitochondrial clustering. In neurons, this mutation seems to increase colocalisation of DISC1 with the mitochondria (Ogawa et al., 2014).

Further experiments attempted to identify the sub-mitochondrial localisation of DISC1. Ultrastructural analysis has demonstrated the presence of both exogenous and

endogenous DISC1 inside mitochondria in HEK cells and mouse brain sections respectively (Park et al., 2010a). Trypsin digests of mitochondrial fractions have shown DISC1 to be protected, supportive of localisation at internal mitochondrial compartments (Park et al., 2010a). Further investigation of localisation of multiple isoforms has been performed by trypsin digests of mitochondria enriched fractions. James and colleagues state the longer, 100kDa isoforms are degraded by this treatment, consistent with an outer mitochondrial membrane distribution. However, the shorter, 71 and 75kDa isoforms were protected from this treatment. This is supportive of presence of these isoforms within the mitochondria, perhaps in the intermembrane space, inner membrane or matrix. The authors suggest the 75kDa isoform (or perhaps isoforms) may be precursors to the 71kDa form (James et al., 2004). However, a protease involved in this processing has yet to be investigated. Moreover, difference in function between the 100 and 71-75kDa isoforms have yet to be confirmed.

### 1.11.2 DISC1 and mitochondrial function

The previous section has discussed the prospect of DISC1 being present both outside the mitochondrion and within internal compartments. This raises the possibility that DISC1 could act within the mitochondrion to regulate function of these organelles. Further, two independent yeast two hybrid screens have identified the inner mitochondrial membrane protein Mitofilin as a DISC1 interactor (Camargo et al., 2007; Park et al., 2010a). Mitofilin (also known as IMMT for inner membrane protein, mitochondrial) has been shown to regulate cristae formation. Mitofilin knockdown studies in HeLa cells showed less organised cristae associated with an increase in reactive oxygen species (John et al., 2005). Thus, Mitofilin regulates mitochondrial function. The interaction between DISC1 and Mitofilin has been localised to the mitochondrial fraction of cell lysates. Multiple experiments were carried out to investigate crosstalk of these two proteins in regulating mitochondrial function. Knockdown of DISC1 both decreased NADH dehydrogenase activity and ATP production. These effects mimicked those obtained from Mitofilin knockdown cells. Further, expression of truncated DISC1 (amino acids 1-597, resulting from the Scottish mutation) also caused a decrease in these measures (Park et al., 2010a). This phenocopy is likely due to the necessity of DISC1 in stabilising the Mitofilin protein. Knockdown or expression of truncated DISC1 (see Chapter 3 and section 1.7) causes

an increase in Mitofilin ubiquitination and subsequent proteasomal turnover. Thus DISC1 acts as a regulator of mitochondrial function via Mitofilin levels.

In addition to DISC1, another of its interaction partners, CHCHD6, has been shown to regulate Mitofilin levels. This protein is localised to the inner mitochondrial membrane according to fractionation experiments and knockdown of this protein has similar adverse effects on cristae organisation as does Mitofilin. As with Mitofilin, knockdown of this protein leads to a decrease in mitochondrial ATP production (An et al., 2012). This raises the possibility that DISC1, Mitofilin and CHCHD6 exist together in a large complex involved in mitochondrial function by maintaining cristae integrity. In addition to ATP production, Park and colleagues also investigated the ability of mitochondria to buffer calcium upon knockdown of DISC1 or Mitofilin (Park et al., 2010a). This was investigated in differentiating CAD cells with a fluorescent, genetically encoded calcium reporter (GCAMP), targeted to the inner mitochondrial membrane. Upon DISC1 or Mitofilin knockdown, mitochondria were shown to buffer calcium more slowly than control cells. This was exemplified by an increase in the time taken for intra mitochondrial calcium levels to return to baseline after ionomycin treatment (Park et al., 2010a). Therefore DISC1, in conjunction with Mitofilin, can regulate both the bioenergetic and calcium buffering capabilities of mitochondria.

In addition to DISC1 truncation, alternative outcomes of the Scottish mutation have been investigated with a focus on mitochondrial dysfunction (please refer to Figure 3-9 on page 105) (Eykelenboom et al., 2012). The balanced chromosomal translocation has been proposed to give rise to abnormal transcripts encoding DISC1 1-597 plus 60 or 69 novel amino acids derived from the gene on chromosome 11. This gene has been named both Boymaw and DISC1FP1 for DISC1 fusion partner 1. The authors denote the novel DISC1-fusion transcripts CP60 or CP69, for Chimaeric Protein 60 or 69. The abnormal transcripts containing either 60 or 69 novel amino acids have both been shown to adopt a more alpha helical structure than DISC1 1-597. Thus the novel amino acids cause a change in structure, which could contribute to a gain of function effect. To investigate this possibility, the distribution of these transcripts was investigated in COS7 cells and primary neurons. In both cases, the novel transcripts adopted a predominantly mitochondrial localisation, in contrast to DISC1 1-597, which appeared diffuse throughout the cytosol. Thus it appears the novel

amino acids confer a gain of function in terms of distribution also. It was notable that mitochondria of cells expressing CP60 or CP69 showed decreased mitotracker staining in comparison to cytochrome C labelling. This observation might suggest that the novel transcripts decrease mitochondrial membrane potential, as mitotracker is selectively taken up into functional mitochondria (Eykelenboom et al., 2012). Thus it would appear that CP60 and CP69 have deleterious effects on mitochondrial function, independent of wild-type DISC1, although endogenous DISC1 levels were not investigated in cells heterologously expressing the chimaeric proteins. Therefore, indirect effects via loss of DISC1 expression cannot be ruled out.

Consistent with these observations, a recent study of the DISC1-Boymaw fusion protein (CP60 by the alternative nomenclature), was shown to localise to mitochondria. Additionally, a decrease in NADH oxidoreductase acitivity was detected in total lysates and mitochondrial fractions of cells expressing the DISC1-Boymaw fusion protein. This may contribute to a decrease in ATP production via mitochondrial damage (Ji et al., 2014). The Boymaw gene itself has been recently suggested to encode a small protein (Ji et al., 2015). This is in contrast with earlier reports which suggested no open readings frames were present in this gene (Eykelenboom et al., 2012; Zhou et al., 2010; Zhou et al., 2008). When overexpressed in HEK293 cells, this small Boymaw protein localises to mitochondria and inhibits NADH oxidoreductase activity (Ji et al., 2015). Taken together, these data are supportive of mitochondrial damage, caused by the DISC1-Boymaw fusion protein, being a pathological feature of schizophrenia.

### 1.11.3 DISC1 as a mitochondrial trafficking protein

There are many lines of evidence supporting DISC1 as a mitochondrial trafficking regulator. As in 1.11.1 it is known to localise to the mitochondria and cytoskeleton. Further, it can interact with components of molecular motor complexes such as kinesin-1 family members and the adaptor TRAK1, dynein complex proteins Nde1 and Ndel1. As described in 1.3, mitochondrial distribution is essential to neuronal function and development. Thus, a potential role for DISC1 in mitochondrial transport was an enticing prospect to investigate. Confirmation of this role for DISC1 was first demonstrated by Atkin and colleagues (Atkin et al., 2011). These experiments

employed overexpression and knockdown of DISC1 and analysis of percentages of motile mitochondria in axons of hippocampal neurons. DISC1 knockdown revealed a decrease in moving mitochondria, whilst neurons overexpressing DISC1 showed a significant increase in mitochondrial trafficking. Both of these observations are consistent with DISC1 acting to positively regulate mitochondrial trafficking. Analysis of the velocity of moving mitochondria revealed no change upon DISC1 knockdown or overexpression. This is consistent with DISC1 acting to couple mitochondria into the transport pathway, rather than a direct regulation of motor activity, or indeed, a modification to microtubules (Atkin et al., 2011). The exact mechanisms by which DISC1 can regulate trafficking remain to be determined. DISC1 knockdown is known to impair mitochondrial function (Park et al., 2010a), thus impaired bioenergetics could be responsible for the associated decrease in trafficking. Alternatively, DISC1 could be acting as a scaffold via interactions with molecular motors and adaptors at the outer mitochondrial membrane.

Of further interest, the roles of schizophrenia associated DISC1 mutations were investigated in this assay. Upon knockdown of DISC1, expression of S704C was able to restore percentages of motile mitochondria to their control levels. However, another variant, L607F, was unable to rescue this effect. Thus, schizophrenia associated DISC1 mutations have differing abilities to regulate mitochondrial trafficking. This not only serves to highlight the role of DISC1 in mitochondrial trafficking, but shows the potential for this regulation to be lost in certain cases of schizophrenia (Atkin et al., 2011). Thus, decreased mitochondrial motility, and aberrant distribution, may be associated with the onset of schizophrenia like symptoms. The ability of DISC1 to impair mitochondrial trafficking in schizophrenia models has been reported in other studies. For example, the effect of the R37W mutation has been shown to confer a subtle defect on mitochondrial trafficking (Ogawa et al., 2014). By mitochondrial isolation, it has been shown that this mutation further increases the presence of kinesin-1 in this fraction compared to wild-type DISC1. However, when mitochondrial motility was investigated, a decrease in anterograde (kinesin-mediated) mitochondrial trafficking was noted in comparison to overexpression of wild-type DISC1. In this case, overexpression of DISC1 served only to bias trafficking to the anterograde direction, rather than cause an overall increase as in (Atkin et al., 2011).

In addition to point mutations, DISC1 aggregation has been linked to mitochondrial trafficking defects. The ability of DISC1 to aggregate has been demonstrated by its presence in sarkosyl insoluble or resistant fractions of cell lysates and is increased in schizophrenic post mortem brain tissue (Leliveld et al., 2008). Further, these aggregates have been identified as aggresomes – inclusions of misfolded protein to be degraded by proteasomal and autophagic pathways. This was shown by colocalisation of GFP DISC1 with aggresome markers such as ubiquitin and HSP70. GFP tagged DISC1 has a higher propensity to aggregate and has been shown by fluorescence recovery after photobleaching (FRAP) to be stable in these aggresomal structures. Crucially, cytosolic DISC1 can be recruited to these aggregates by misfolded DISC1. This corresponds with a decrease in mitochondrial trafficking, likely by decreasing the availability of DISC1 as in the previously described knockdown experiments, and so contributing to the onset of psychiatric disease (Atkin et al., 2012).

## 1.12 Thesis aims:

Correct mitochondrial transport is essential for neuronal development and synaptic function. DISC1 plays a role in mitochondrial transport in the neuron. DISC1 has also been shown to interact with trafficking proteins such as kinesin-1 and dynein complex components – the motor proteins responsible for mitochondrial trafficking. The exact mechanisms by which DISC1 can regulate trafficking remain to be determined. DISC1 knockdown is known to impair mitochondrial function, thus impaired bioenergetics could be responsible for the associated decrease in trafficking. Alternatively, DISC1 could be acting as a scaffold via interactions with molecular motors and adaptors at the outer mitochondrial membrane. Here it may have structural roles in complex assembly, or influence signalling pathways e.g. via cAMP levels or GSK3 beta activity. Further, how these mitochondrial dynamics contribute to aberrant neuronal function in psychiatric disease remains to be explored.

Therefore, the aims of this thesis are to investigate the contribution of DISC1 to mitochondrial dynamics, and subsequently, neuronal development. More specifically;

- To investigate the protein protein interactions of DISC1, which may regulate mitochondrial dynamics and transport.
- To address how these dynamics may be altered by a disease associated variant of DISC1.
- To investigate the impact of these aberrant dynamics on neuronal development.
- To extend studies on mitochondrial trafficking in neurological disease states into differentiated human neurons, and thus, compare and contrast mitochondrial trafficking in these cells to rodent neurons.

# Chapter 2

# Materials and Methods

## 2.1 Antibodies

For a complete list of primary antibodies used for western blotting (WB), immunofluorescence (IF) or immunoprepcipitation (IP) please refer to Table 2-1. For a complete list of secondary antibodies please see Table 2-2.

## 2.2 Molecular Biology

### 2.2.1 Constructs

Untagged human DISC1 in a pRK5 expression vector was a kind gift from C. Korth (Uni Dusseldorf) (Leliveld et al., 2009). Mitochondrially targeted monomeric DsRed fluorescent protein (MtDsRed2), Synaptophysin$^{GFP}$, $^{GFP}$Miro1 and $^{GFP}$Miro2 and $^{Myc}$Miro1 have been previously described (Birsa et al., 2014; Fransson et al., 2003; Fransson et al., 2006; Macaskill et al., 2009b). Endoplasmic reticulum targeted DsRed fluorescent protein (ERDsRed) was from Clontech. $^{GFP}$TRAK1 and $^{GFP}$TRAK2 were cloned by insertion of the mouse TRAK sequences into the EGFP-C1 vector from Clontech. HA tagged DISC1 deletion constructs were previously described and were a kind gift from M. Houslay (Wang et al., 2011b). $^{HA}$Boymaw was a kind gift from M. Geyer (University of California, San Diego) and subcloned into the pRK5 expression vector (Zhou et al., 2010) as detailed in section 2.2.3 to 2.2.7. The following constructs were from Addgene: $^{myc}$Mitofusin1 (plasmid 23212) and 2 (23213), su9-EGFP (23214) (Chen et al., 2003), $^{myc}$Mitofusin2 K109A (26051) mito PAGFP (23348) (Karbowski et al., 2004).

### 2.2.2 Reagents

Chemicals are from Melfords unless stated otherwise. Bacteria used throughout were XL10 Gold. Bacteria were cultured in Luria Bertani (LB) medium or LB-agar. See Table 2-3 Bacterial Culture Media for media composition. Selection of bacteria expressing relevant plasmids was achieved with ampicillin (100µg/ml) or kanamycin (30µg/ml). Enzymes were from NEB (New England Biolabs).

| Primary Antibody | Species | Company | catalogue number | Western Blotting | IF | IP |
|---|---|---|---|---|---|---|
| DISC1 14F2 supernatant | mouse | gift from C. Korth | | 1:200 | | |
| DISC1 14F2 affinity purified | mouse | gift from C. Korth | | | 1:200 | 2ug |
| Myc 9E10 | mouse | prepared in house | | 1:100 | 1:100 | |
| Myc A-14 | rabbit | Santa Cruz | sC 789 | | 1:100 | |
| GFP FL | rabbit | Santa Cruz | sC 8334 | 1:200 | | |
| HA 12CA5 | mouse | prepared in house | | 1:100 | 1:100 | |
| MAP2 | guinea pig | Synaptic Systems | 188004 | | 1:1000 | |
| GFP | rat | Nacalai Tesque | GF090R | | 1:2000 | |
| RFP | rabbit | Abcam | ab62341 | | 1:500 | |
| Miro1 | mouse | Atlas | AMAb90854 | | | 2ug |
| Miro1 | rabbit | Atlas | HPA010687 | 1:1000 | | |
| TRAK1 | rabbit | Atlas | HPA005853 | 1:1000 | | 2ug |
| TRAK2 | rabbit | Atlas | HPA015827 | 1:1000 | | 2ug |
| Mfn1 | mouse | Abcam | ab57602 | 1:500 | | 2ug |
| TOM20 | rabbit | Santa Cruz | FL-145 | 1:1000 | | 2ug |
| TAU | mouse | Dako | A0024 | | 1:500 | |
| MAP2 | rabbit | Cell Signalling Technologies | 4542 | | 1:1000 | |
| BIII tubulin | rabbit | Cell Signalling Technologies | 5568 | | 1:1000 | |
| vGlut2 | mouse | Abcam | ab79157 | | 1:100 | |

**Table 2-1 Conditions for Primary Antibodies**

| Secondary Antibody | Species | Company | Catalogue number | WB | IF |
|---|---|---|---|---|---|
| anti-Mouse horse radish peroxidase | Goat | Biorad | 170-6516 | 1:10,000 | |
| anti-Rabbit horse radish peroxidase | Goat | Rockland | 611-103-122 | 1:10,000 | |
| anti-mouse 488 | donkey | Invitrogen | A-21202 | | 1:1000 |
| anti-rabbit 647 | donkey | Invitrogen | A-31573 | | 1:1000 |
| anti-guinea pig 405 | donkey | Jackson | 706-475-148 | | 1:1000 |
| anti-mouse 647 | goat | Invitrogen | A-21236 | | 1:1000 |
| anti-rabbit 568 | donkey | Invitrogen | A-10042 | | 1:1000 |
| anti-rat 488 | donkey | Invitrogen | A-21208 | | 1:1000 |

**Table 2-2 Conditions for Secondary Antibodies**

| | |
|---|---|
| LB | 10g NaCl<br>10g tryptone<br>5g yeast extract<br>$H_2O$ to 1litre |
| LB-agar | 2.5g NaCl<br>2.5g tryptone<br>1.25g yeast extract<br>2.5g agar<br>$H_2O$ to 250ml |

**Table 2-3 Bacterial Culture Media**

### 2.2.3 Polymerase chain reaction

In order to express the DISC1-Boymaw fusion protein in mammalian cells, it was subcloned into pRK5. PCR was carried out using the Phusion polymerase (Finnzymes) according to the manufacturer's instructions. The reaction was assembled as described in Table 2-4 PCR Components. For details of the PCR cycle please refer to Table 2-5. Steps in bold were repeated 25 times. Sequences for primers were as follows;

FWD: 5' CATCAT*GAATTC*CCACCATGCCAGGCGGGGGTC 3'

REV: 5' CATCAT*GTCGAC*CTAAGCGTAGTCTGGAACGTCGTATGG 3'

Underlined italics indicate the restriction enzyme sites EcoRI in the forward primer and SalI in the reverse.

| | **PCR reaction** | **Negative Control** |
|---|---|---|
| Forward Primer (100pmol/µl) | 0.5 µl | 0.5 µl |
| Reverse Primer (100pmol/µl) | 0.5 µl | 0.5 µl |
| Template DNA | 0.05µg | - |
| 5x HF buffer | 10 µl | 10 µl |
| 10mM dNTP mix | 1 µl | 1 µl |
| Phusion enzyme | 0.5µl | 0.5µl |
| Water | 37µl | 38µl |

**Table 2-4 PCR Components**

| | **Temperature/ ºC** | **Time** |
|---|---|---|
| Melting | 95 | 5 min |
| **Melting** | 95 | 30 sec |
| **Annealing** | 65 | 30 sec |
| **Extension** | 72 | 2 min |
| Extension | 72 | 5 min |
| Hold | 4 | ∞ |

**Table 2-5 PCR cycle for cloning of DISC1-Boymaw into pRK5**

5µl of the resultant PCR product was resolved on an agarose gel to check for DNA quality and purity of amplification. If the product consisted of a single band at the correct molecular weight, PCR clean up was carried out with a PCR clean up kit (Qiagen) according to the manufacturer's instructions. If the product also contained non-specific bands, the whole PCR was resolved on an agarose gel and the band at the correct molecular weight was excised and prepared with a gel extraction kit (Qiagen) according to the manufacturer's instructions.

### 2.2.4 Agarose gel electrophoresis

0.6% agarose gels were used to resolve DNA bands and determine length in kilobases in comparison to standards (NEB). Gels were prepared by dissolving appropriate mass of agarose (Melfords) into 1x TBE (tris-borate EDTA - 89mM Tris base, 89mM boric acid, 2mM $Na_2EDTA$), National Diagnostics). 1µl of ethidium bromide was added per 50ml of gel prior to running to allow visualisation of DNA bands upon exposure to ultraviolet light. Samples were prepared for loading by addition of 6X loading buffer (0.25% bromophenol blue and 40% sucrose in $H_2O$). Gels were subjected to electrophoresis for 45 minutes at 90V in 1x TBE.

### 2.2.5 Restriction enzyme digestion

Restriction enzyme mediated digestion of PCR products and pRK5 vector was prepared as in Table 2-6 and incubated for 2 h at 37ºC.

| | |
|---|---|
| DNA | 1µg vector/ 10µl PCR product |
| EcoRI | 1µl |
| SalI | 1µl |
| 10x Buffer | 2µl |
| 10x BSA | 2µl |
| Water | To 20µl |

**Table 2-6 Restriction Digest Components**

### 2.2.6 Ligation

Extracted insert and vector DNA were incubated at a ratio of 10:1 with 1µl of T4 DNA ligase and 2µl 10x ligase buffer in a total volume of 20µl. The ligation reaction was carried out at room temperature for 15 min and then overnight at 4ºC.

### 2.2.7 Transformation

Competent bacteria were kindly prepared by Nathalie Higgs. Cells were thawed on ice and incubated together with DNA in thin walled falcon tubes for 30 min. Cells were subjected to heat shock at 42ºC for 45 sec and allowed 2 min recovery on ice. 250µl

LB + 4% glucose was added and cells were allowed one h shaking at 37ºC for expression of selection markers prior to plating on ampicillin or kanamycin LB-Agar plates.

### 2.2.8 Miniprep and Maxiprep of plasmid DNA

Maxiprep kits were obtained from Promega. Miniprep kits were obtained from Sigma. For preparation of DNA, 5ml (miniprep) or 250ml (maxiprep) of bacterial culture transformed to contain the plasmid of interest was grown overnight by shaking in LB including the appropriate antibiotic at 37ºC. Bacteria were pelleted, resuspended and subjected to alkaline lysis and neutralisation. Centrifugation was carried out to remove cellular debris and genomic DNA. Plasmid DNA was bound to miniprep or maxiprep columns, washed and eluted in molecular biology grade water.

## 2.3 Cell Culture

### 2.3.1 Cell line culture

**Materials**

Media, sera and supplements were from GIBCO (Invitrogen) unless otherwise stated. Small molecules for neural progenitor culture and differentiation were from Tocris unless otherwise stated.

**COS7 and SHSY-5Y cell culture**

COS7 cells or SHSY-5Y cells were maintained in 10cm dishes in 10ml DMEM (Dulbecco's Modified Eagle Medium) as described in Table 2-7 Cell Line Culture Solutions. Cells were passaged at 80-90% confluency or transfected at 50-60% confluency. Removal from plates was carried out by removal of media and washing plates with phosphate buffered saline (PBS), then adding 1ml prewarmed trypsin followed by quenching with DMEM and pelleting by centrifugation at 1000rpm for 2 minutes. Cells were then plated at appropriate density in fresh cell culture media.

**Cell line transfection**

For transfection, cell pellets were resuspended in electroporation buffer (see Table 2-7 Cell Line Culture Solutions) at a volume of 100µl per transfection. 1-5µg of DNA was used per transfection. Cells were electroporated using an Amaxa nucleofector and immediately plated in DMEM. Cells were left 24-48 h to allow plasmid expression.

**Hybridoma Culture**

9E10 and 12CA5 monoclonal antibodies were prepared from hybridoma culture in a CELLine bioreactor (Integra). Media is described in Table 2-7 Cell Line Culture Solutions. Cells were maintained in suspension, fed every two days and split every 4 days. At these points, supernatant was collected and filtered for use as antibody.

| | |
|---|---|
| PBS | 137mM NaCl |
| | 2.7mM KCl |
| | 10mM $Na_2HPO_4$ |
| | 2mM $KH_2PO_4$ |
| DMEM | 10% FBS |
| | 50U/ml Penicillin |
| | 50ug/ml Streptomycin |
| | DMEM |
| Hybridoma media | 20% FBS |
| | 50U/ml Penicillin |
| | 50ug/ml Streptomycin |

| | |
|---|---|
| | DMEM |
| Electroporation buffer | 15mM $NaH_2PO_4$ |
| | 35mM $Na_2HPO_4$ |
| | 5mM KCl |
| | 10mM $MgCl_2$ |
| | 11mM Glucose |
| | 100mM NaCl |
| | 20mM HEPES |

**Table 2-7 Cell Line Culture Solutions**

### 2.3.2 Primary Neuronal culture

For preparation of rat primary neuronal cultures E18 pups were removed from the dam under sterile conditions. Brains were removed from the skulls and hippocampal dissection was carried out in HBSS (See Table 2-8 Primary Neuronal Culture Media) at 4°C prior to incubation in 0.125% Trypsin EDTA solution for 15 min at 37°C. Hippocampi were washed three times with 10 ml of HBSS (Hank's balanced salt solution) and triturated 10 times using a fire polished Pasteur pipette in pre-warmed attachment media (See Table 2-8 Primary Neuronal Culture Media). Cells were counted using erythrosine B dye as an indicator of viability with an improved Neubauer haemocytometer. Cells were plated at 350,000 cells in 5 ml of pre-warmed attachment media in 6 cm dishes containing washed glass coverslips pre-coated overnight in 500 μg/ml poly-L-lysine. After 5 hours media was removed and replaced with pre-warmed maintenance media (See Table 2-8 Primary Neuronal Culture Media).

| | |
|---|---|
| HBSS | 1x HBSS |
| | 10mM HEPES |
| Attachment media | MEM |
| | 10% Horse serum |
| | 1mM Sodium pyruvate |
| | 33mM Glucose |
| Maintenance media | Neurobasal |
| | 2% B-27 supplement |
| | 1% Glutamax |
| | 6% Glucose |
| | Penicillin-Streptomycin |

**Table 2-8 Primary Neuronal Culture Media**

### Transfection of primary neuronal cultures

Hippocampal cultures were transfected at DIV 7-8 (days *in vitro*). Transfection was carried out using Lipofectamine 2000 (Invitrogen) in unsupplemented Neurobasal with 6% glucose. 0.5µg DNA and 0.5µl Lipofectamine were used per coverslip. In the case of multiple DNAs, constructs were transfected at a ratio of 1:3, where 1 is the fluorescently tagged gene, and 3 is the not fluorescently tagged. The reagents were mixed and incubated for 30 minutes at room temperature to allow for complexation. The complex was added to coverslips for 2 hours 30 minutes at 37$^{o}$C, 5% $CO_2$ at which point original, conditioned culture media was replaced. The neurons were allowed 2 – 3 days for plasmid expression.

### 2.3.3 induced Pluripotent Stem Cell derived neural progenitor cell culture

Neural progenitor cells (NPCs) were prepared from induced pluripotent stem cells (iPSCs) by J. Kasuboski and L. Wilson according to a protocol by Reinhardt et al (Reinhardt et al., 2013). Neural progenitors were maintained in NPC media (Table 2-9 Neural Progenitor Cell Culture Media) in 6 well plates pre-coated with matrigel (BD

Biosciences), prepared according to the manufacturer's instructions. Cells were plated at a density of 0.8 to 0.9 million cells per well and passaged every two days. Accutase (Invitrogen) was used to lift the cells. Cells were pelleted at 1000 rpm and counted using a Countess automated cell counter and Trypan blue staining. Upon plating, thiazovivin (Sigma) was added to the media at a concentration of 10µM to facilitate cell adhesion. The total volume of media was replaced the day after plating, and cells were fed by replacing half the volume of media daily.

| | |
|---|---|
| N2B27 base media | 0.5x DMEM:F12 |
| | 0.5x Neurobasal |
| | 0.5mM Glutamax |
| | 0.5x N2 |
| | 0.5x B27 |
| | Normocin 100µg/ml (Invivogen) |
| NPC media | N2B27 base media |
| | 150µM Ascorbic Acid |
| | 3µM CHIR |
| | 0.5µM Purmorphamine |

**Table 2-9 Neural Progenitor Cell Culture Media**

**Neural progenitor differentiation into glutamatergic neurons**

Two days prior to differentiation, 0.82 million neural progenitors were plated onto matrigel coated plates. 3cm Mattek glass bottom dishes were pre coated with 0.01% Poly-L-ornitihine (Sigma) for one hour at 37$^{o}$C, washed twice with cell culture grade water, and then coated with 3.3µg/ml laminin (Sigma). Plates were incubated overnight at 4$^{o}$C and washed twice with cell culture grade water. 0.86 million neural progenitors were plated per 3cm dish in glutamatergic differentiation media. Media was replaced every two days (see Table 2-10 Glutamatergic Differentiation Media and Figure 2-1 Schematic showing differentiation of Neural Progenitors). At day 8, 25µl of cell lights mito RFP (Invitrogen) was added to each dish for labelling of mitochondria for live imaging at day 11.

| | |
|---|---|
| Glutamatergic Differentiation base media | N2B27 base media<br>10µM SB431542<br>0.2µM LDN 193189 (StemGent)<br>1µM Cyclopamine<br>10ng/mL FGF2 (Invitrogen) |
| Day 0-2 | Glutamatergic Differentiation base media<br>1µM DAPT (Gamma secretase inhibitor)<br>10µM Thiazovivin |
| Day 2-6 | Glutamatergic Differentiation base media<br>30µM 5-Fluoro-2-Deoxyuridine (Mitotic inhibitor) |
| Day 6-8 | Glutamatergic Differentiation base media |
| Day 8-11 | Glutamatergic Differentiation base media<br>10ng/mL BDNF<br>10ng/mL GDNF |

**Table 2-10 Glutamatergic Differentiation Media**

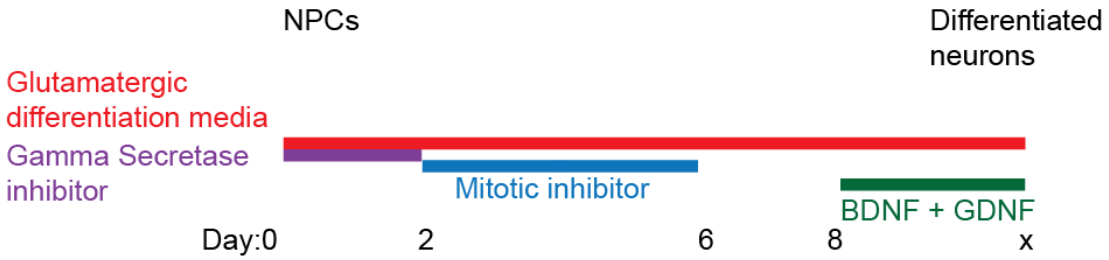



**Figure 2-1 Schematic showing differentiation of Neural Progenitors**

## 2.4 Biochemistry

### 2.4.1 Coimmunoprecipitation

#### Coimmunoprecipitation from COS7 cells

Cells were washed twice in PBS to remove media and scraped in 500µl ice cold lysis buffer for detachment (See Table 2-11 Biochemistry Buffers). For lysis, samples were incubated at 4°C with end over end rotation for one hour. Cellular debris was pelleted by centrifugation at 20,000 x g at 4°C for 10 minutes. Subsequently, input samples were taken and the remainder of the lysate was incubated with GFP-trap beads (Chromotek) or myc beads (Sigma) to allow specific pull down of tagged proteins and associated complexes. Samples were incubated at 4°C with end over end rotation for one hour. To remove non-specifically bound protein complexes, beads were pelleted at 100 x g for one minute at 4°C, the supernatant was removed and 1ml of lysis buffer was added. This was repeated 3-5 times. After the final wash 15µl of 3X sample buffer was added and the samples were boiled at 98°C for 5 minutes for denaturation.

#### Coimmunoprecipitation from rat brain lysates

To identify native protein complexes, coimmunoprecipation experiments were carried out from rat brain lysate. This was prepared by removal of the brain of an adult rat and homogenisation in high Triton lysis buffer (see Table 2-11 Biochemistry Buffers). Lysis was carried out by end over end rotation at 4°C for 2 hours. Lysate was then subjected to centrifugation at 44,000 x g for 40 minutes at 4°C. Protein content was quantified using a Bradford assay. For immunoprecipitation, 5mg of protein was

incubated with 2µg antibody in immunoprecipitation buffer (See Table 2-11 Biochemistry Buffers) with end over end rotation at 4ºC overnight. Subsequently, 15µl protein A agarose slurry (sigma) was added. The protein A agarose beads were prepared by incubation with PBS 0.05% Triton and 1% BSA. After 2 hour incubation, the IPs were washed thrice in immunoprecipitation buffer and once in lysis buffer. Input samples were diluted 1:3 with lysis buffer without Triton. Samples were boiled at 98ºC for 5 minutes in 3x Sample buffer.

### 2.4.2 Mitochondrial fractionation

Mitochondrial fractionation was carried out from cultured cells as previously described (Frezza et al., 2007). Briefly, cells were washed in PBS and lysed in mitochondrial isolation buffer (Table 2-11 Biochemistry Buffers) by homogenisation. Lysates were subjected to centrifugation at 600 x g for 10 minutes. Input samples were taken after this step. Supernatants were subjected to centrifugation at 7000 x g for 10 minutes. Pellets, comprising a mitochondrial enriched fraction, were washed with 1ml mitochondrial isolation buffer and the centrifugation step was repeated. Pellets were resuspended in 3x Sample buffer and boiled for 5 minutes at 98ºC.

| | |
|---|---|
| Lysis Buffer | 50mM Tris pH7.5<br>150mM NaCl<br>0.5% Triton X-100<br>1mM EDTA<br>1mM PMSF<br>1µg/mL Antipain, leupeptin, pepstatin |
| High Triton Lysis Buffer | 50mM Tris pH7.5<br>150mM NaCl<br>1.5% triton X-100<br>1mM EDTA<br>1mM PMSF<br>1µg/mL Antipain, leupeptin, pepstatin |

| | |
|---|---|
| Immunoprecipitation Buffer | 50mM Tris pH7.5 |
| | 150mM NaCl |
| | 1.5% triton X-100 |
| | 1mM EDTA |
| | 1mM phenylmethanesulfonyl fluoride (PMSF) |
| | 1µg/mL Antipain, leupeptin, pepstatin |
| | 1% BSA |
| Mitochondrial isolation buffer | 200mM Sucrose |
| | 10mM Tris-MOPS pH 7.4 |
| | 1mM Tris-EGTA pH 7.4 |
| 3X Sample Buffer | 150mM Tris pH 8 |
| | 6% sodium dodecyl sulphate (SDS) |
| | 0.3M dothiothreitol (DTT) |
| | 0.5% Bromophenol Blue |
| | 30% Glycerol |

**Table 2-11 Biochemistry Buffers**

### 2.4.3 Polyacrylamide Electrophoresis and Western Blotting

**SDS PAGE**

Polyacrylamide gels were prepared using 10% running gels and 5% stacking gels in Novex 1.5mm (see Table 2-12 Protein Electrophoresis and Western Blotting Buffers) Cassettes and run using the Novex XCell SureLock Mini-Cell system. Rat brain lysate IPs were resolved on gels made up half of 15% acrylamide for lower molecular weights and half of 8% acrylamide for higher molecular weights. Gels were submerged in Running Buffer (see Table 2-12 Protein Electrophoresis and Western Blotting Buffers). Samples were loaded into individual lanes and at least one lane was loaded with Page Ruler plus prestained molecular weight marker (BioRad). Gels were

subjected to electrophoresis at 150V for 90 minutes or until the gel front reached the bottom of the cassette. Gels were then transferred onto Hybond-C nitrocellulose membranes (GE Healthcare). Gels and membranes, pre-soaked in transfer buffer (Table 2-12 Protein Electrophoresis and Western Blotting Buffers), were sandwiched between 3mm Whatman filter papers. Novex supplied transfer sponges were placed either side. The system was submerged in transfer buffer and transfer was carried out at 30V for 2 hours or overnight at 4ºC at 20V for rat brain lysate coimmunoprecipitation experiments.

**Western Blotting**

After transfer, proteins were visualised by ponceau staining. Membranes were sectioned according to molecular weight using a scalpel and blocked in 4% milk for one hour to prevent non-specific antibody binding (see Table 2-12 Protein Electrophoresis and Western Blotting Buffers). Membranes were incubated with relevant primary antibodies diluted in 4% milk at 4ºC overnight with shaking. Unbound primary antibody was removed by washing thrice in 4% milk for 5 minutes each. Secondary HRP conjugated antibodies were diluted in 4% milk and incubated with membranes for one hour at room temperature with shaking. Unbound secondary antibody was removed by 3x 10 minute washes in 4% milk with shaking at room temperature and one wash in PBS 0.05% Triton. Bands were visualised using Crescendo Chemiluminescent substrate (Millipore) using an ImageQuant LAS 4000 CCD camera system (GE Healthcare).

**Stripping a previously exposed membrane**

For detection of proteins at similar molecular weights, sections of nitrocellulose were stripped to remove previously bound primary and secondary antibodies. Membranes were incubated in stripping buffer (see Table 2-12 Protein Electrophoresis and Western Blotting Buffers) pre-warmed to 37ºC for 25 minutes with shaking. Subsequently, membranes were washed twice with PBS 0.05% Tween 20 and blocked again in 4% milk. Membranes were then treated as described in Western Blotting.

| | |
|---|---|
| 8 or 10% Resolving Gel | 8 or 10% Protogel (acrylamide solution)<br>375mM Tris pH 8.8<br>1% SDS<br>1% Ammonium persulphate<br>0.04% TEMED |
| 15% Resolving Gel | 15% Protogel (acrylamide solution)<br>375mM Tris pH 8.8<br>1% SDS<br>1% Ammonium persulphate (APS)<br>0.06% TEMED |
| 5% Stacking gel | 5% Protogel<br>125mM Tris pH 6.8<br>1% SDS<br>1% APS<br>0.1% TEMED |
| Running Buffer | 25mM Tris<br>192mM Glycine<br>0.1% SDS |
| Transfer Buffer | 25mM Tris<br>192mM glycine<br>2% Methanol<br>0.035% SDS |
| Ponceau | 5% acetic acid<br>0.1% Ponceau s |

| | |
|---|---|
| 4% Milk | 4% Non-fat milk<br>0.05% Tween<br>PBS |
| Stripping buffer | 6.25mM Tris pH 6.8<br>2% SDS<br>0.7% β mercapto ethanol<br>PBS |

**Table 2-12 Protein Electrophoresis and Western Blotting Buffers**

## 2.5 Immunocytochemistry

Fixed cell imaging was carried out on cells plated on coverslips by fixation with 4% paraformaldehyde (PFA - see Table 2-13 Fixed Imaging Solutions) for 10 minutes at room temperature followed by blocking for 10 minutes in blocking solution (see Table 2-13 Fixed Imaging Solutions). Coverslips were incubated in relevant primary antibodies diluted in blocking solution for 1 hour, washed 5x in PBS and incubated in secondary antibodies diluted in blocking solution. Coverslips were washed 5x in PBS and mounted onto slides using Prolong® Gold antifade reagent (Invitrogen) and later sealed with nail varnish. Cell fusion assays were carried out as follows; the cells were transfected with either MtDsRed or Su9GFP and plated together. After 24 hours, the media was replaced with 50% polyethylene glycol 1500 in unsupplemented DMEM for 45 seconds to allow plasma membrane fusion and washed thrice every 10 minutes, 3 times. Normal media was replaced, supplemented with 30µg/ml cycloheximide to prevent de novo synthesis of fluorophores. Cells were fixed 3 hours later.

| | |
|---|---|
| Paraformaldehyde | 4% Paraformaldehyde<br>4% Sucrose<br>PBS |
| Blocking Solution | 10% Horse serum<br>0.2% Triton X-100<br>0.5% BSA<br>PBS |

**Table 2-13 Fixed Imaging Solutions**

## 2.6 Imaging

### 2.6.1 Fixed Imaging

Samples were imaged on a Zeiss LSM 700 upright microscope using an Apochromat 63x oil immersion objective, NA 1.4 with appropriate excitation of fluorophores. For Sholl analysis, an Apochromat 40x oil immersion objective, NA 1.3 was used. Experiments to characterise differentiations of neural progenitors into neurons were carried out on a Zeiss LSM 700 using a 63x oil immersion objective, NA 1.4. Images were digitally captured using ZEN 2010 or 2012 software.

### 2.6.2 Live Imaging

For neuronal imaging of mitochondria, embryonic day 18 (E18) primary hippocampal neurons were transfected at 7-8 Days *in vitro* (DIV) and imaged at 9–10 DIV under perfusion with imaging media (see Table 2-14 Live Imaging Media) warmed to 37°C and flowed at a rate of 1-2 ml/minute throughout the duration of each experiment (Atkin et al., 2012). For acquisition, fluorescence was captured using an Olympus microscope (BX60M) with a water immersion 60× Olympus NA 1.0 objective coupled to an EM-CCD camera (Ixon; Andor). Excitation was provided by a mercury arc lamp (Cairn) with the appropriate filters (Muir et al., 2010). Images were acquired at 1 frame per second for two minutes throughout. Axonal regions were acquired at a distance of 100 to 200 µm from the cell body and dendritic imaging was acquired at a distance of

50 µm from the cell body due to their reduced length. The length of process assayed was ≈ 150 µm. Photoactivation assays were carried out on a Zeiss LSM 700 upright confocal microscope with an apochromat 60x water-immersion lens with NA 1.0. Photoactivation was carried out at 405nm after 5 frames. Acquisition was carried out over 100 frames at 1 frame per 6 seconds.

| | |
|---|---|
| Live Imaging Media | 125mM NaCl |
| | 10mM HEPES |
| | 10mM Glucose |
| | 5mM KCl |
| | 2mM CaCl2 |
| | 1mM MgCl2 |
| | pH7.4 |

**Table 2-14 Live Imaging Media**

## 2.7 Image processing

To create kymographs image sequences were opened within ImageJ. Curved processes were straightened using the 'straighten' macro and kymographs created by the 'multiple kymograph' macro. Resultant kymographs show the process along the x-axis and time across the y axis. Thus, moving organelles appear as diagnonal lines and stationary organelles as straight lines. Mobility was assessed by counting the percentage of objects moving during an imaging period. Mitochondria and synaptophysin$^{GFP}$ positive vesicles were classed as moving if they moved more than 2µm between the initial and final frame of acquisition. For photoactivation assays, spread of GFP signal was measured in ImageJ.

Morphological analysis of neurons was carried out in Neuron Studio. Dendrites were manually traced. From these traces, the total dendritic length and total number of branch points per cell is measured. The Sholl plugin was used to generate numbers of branch points per 10µm concentric circle. Measure of the number of intersections with each circle was carried out in ImageJ using the 'simple neurite tracer' plugin.

'Percentage of colocalisation' analysis was carried out on thresholded images in ImageJ using the 'image calculator' function. This generates an image containing just pixels positive in both channels and colocalised signal over total can be analysed. Pearson and Manders coefficients were calculated using the JACoP plugin within image J.

## 2.8 Statistical analysis

All data were obtained using cells from three different preparations unless otherwise stated. Data are presented as mean ± standard error of the mean (SEM). Individual differences were assessed using individual student's unpaired t-tests at a 95% significance level. Statistical significance across groups was analyzed using one-way analysis of variance and Tukey's post hoc test to compare all data groups. For Sholl analysis, two-way repeated measure ANOVA was used with post hoc Bonferroni test for comparison of dendritic crossing and branch points. Data are shown as mean ± SEM. NS = not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

# Chapter 3
# DISC1 regulates mitochondrial trafficking via interaction with Miro and TRAKs

## 3.1 Introduction

The roles of the Miro and TRAK proteins in regulation of mitochondrial trafficking have been well studied (Glater et al., 2006; Macaskill et al., 2009b; van Spronsen et al., 2013; Wang and Schwarz, 2009) and consistently demonstrated to be positive regulators of mitochondrial trafficking. The DISC1 protein has been previously reported to be involved in mitochondrial trafficking (Atkin et al., 2012; Atkin et al., 2011; Ogawa et al., 2014). RNAi mediated knockdown of DISC1 has been shown to decrease percentages of motile mitochondria in rat hippocampal cultures, also indicative of positive regulation (Atkin et al., 2011). Further, DISC1 has been shown to promote a bias towards anterograde mitochondrial trafficking (away from the soma in axons where microtubule plus ends are uniformly directed outwards) (Ogawa et al., 2014). This would be consistent with DISC1 acting to positively regulate kinesin based motility (plus end directed), or potentially, negatively regulate dynein based motility (minus end directed). Consistent with this role for DISC1, it is also partially mitochondrially localised (Brandon et al., 2005; James et al., 2004; Millar et al., 2005a; Park et al., 2010b) and known to interact with components of the motor complexes involved in microtubule based trafficking; namely dynein and dynactin (Kamiya et al., 2005) and kinesin-1 family member KIF5A (Taya et al., 2007). However, the mechanisms by which DISC1 can regulate mitochondrial trafficking remain unclear.

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It is possible that DISC1 exerts its effects via distal regulation of signalling proteins such as GSK3β and phosphodiesterases, known DISC1 interactors (Ishizuka et al., 2011; Mao et al., 2009; Murdoch et al., 2007). Regulating the activity of these proteins could cause a global change in mitochondrial motility, via second messenger cyclic AMP or via phosphorylation mediated effects. However, the large scale, iterative, yeast two hybrid screen conducted to identify DISC1 binding partners proposed an indirect interaction with TRAK1 – the kinesin adaptor protein and Miro interactor – via microtubule associated protein MIPT3. The direct DISC1 interactor MIPT3 was selected as bait for a secondary screen due to its microtubule association and likely involvement in intracellular trafficking (Camargo et al., 2007). This raises the possibility that DISC1 can interact with components of the mitochondrial trafficking complex in concert with the signalling proteins mentioned above. This interaction may allow DISC1 to mediate mitochondrial trafficking specifically, rather than via an interaction with the motors which themselves have multiple cargoes. Therefore, DISC1 could act as a local regulator of mitochondrial trafficking, rather than upregulating all kinesin or dynein based transport via distal signalling or non- selective interaction with molecular motors.

In addition to regulating mitochondrial trafficking under wild-type conditions, schizophrenia associated mutations in DISC1 (namely amino acid substitutions R37W and L607F) have been shown to impair these functions in overexpression or knockdown-rescue experiments respectively (Atkin et al., 2011; Ogawa et al., 2014). Expression of the R37W mutation decreases a bias for anterograde transport, without affecting overall mitochondrial transport – net displacement and total motile mitochondria were unchanged (Ogawa et al., 2014). In contrast, the L607F mutation was investigated in a knockdown-rescue experiment also in axons. In this case it failed to rescue total percentages of moving mitochondria, whereas wild-type DISC1 was able to do so. Furthermore, aggregation of DISC1 and incorporation into aggresomes inhibits mitochondrial trafficking (Atkin et al., 2012). These aggresomes have been shown to recruit soluble DISC1, therefore decreasing its availability to regulate trafficking of mitochondria. DISC1 aggregation is associated with psychiatric illness as demonstrated by the presence of DISC1 aggregates in post mortem tissue from schizophrenic patients (Leliveld et al., 2008). DISC1 aggregation can be induced by cellular stress (Atkin et al., 2012) and interrupt normal interactions such as that with

Ndel 1 (Leliveld et al., 2008). Notably, these observations show that dysfunction wild-type DISC1 could contribute to the onset of sporadic schizophrenia. A crucial mutation in DISC1 that has yet to be considered in terms of mitochondrial transport is that resulting from a balanced chromosomal translocation described in the Scottish pedigree reported by Millar and colleagues (Millar et al., 2000) and introduced in section 3.2.5 on page 103. This mutation has been proposed to result in expression of a chimaeric protein; the DISC1-Boymaw fusion protein (Zhou et al., 2008), containing DISC1 amino acids 1-597, but lacking the C-terminus. Instead, novel amino acids derived from the Boymaw gene are fused to the C-terminus.

To allow investigation of the potential for DISC1 to locally regulate mitochondrial trafficking, interactions with known components of the trafficking complex were studied in both native and heterologous systems. This serves to confirm interactions proposed by the large scale yeast two hybrid - the DISC1 interactome - at the protein level (Camargo et al., 2007). Additionally, these experiments serve to confirm previous reports of DISC1 interaction with mitochondrial transport proteins (Ogawa et al., 2014) and extend these findings to show a native complex in brain tissue. In order to explore the necessity of these interactions for correct mitochondrial trafficking, the region of DISC1 responsible for these interactions was determined by coimmunoprecipitation assays between Miro or TRAK and constructs encoding regions of DISC1 corresponding to previously described domains. The effect of expression of the DISC1-Miro/TRAK interacting region on mitochondrial trafficking was assessed by live imaging in rodent hippocampal neurons. Finally, the effect of the schizophrenia associated DISC1-Boymaw fusion protein on mitochondrial trafficking was explored by similar live imaging experiments. These experiments aim to provide a means by which the balanced chromosomal translocation may contribute to the onset of psychiatric disease.

## 3.2 Results

### 3.2.1 DISC1 interacts with mitochondrial trafficking complex proteins

DISC1 has been previously linked to mitochondrial transport, but the investigation into mechanisms involved remains limited. However, the involvement of Miro1 and the TRAKs in this process has been well documented. Therefore, coimmunoprecipitation experiments were carried out to explore potential interactions between these proteins. COS7 cells were transiently transfected with untagged human DISC1 and GFP-tagged Miro or TRAK constructs. Transfection of DISC1 with GFP alone was carried out as a negative control. GFP trap coimmunoprecipitation, followed by western blotting, confirmed a biochemical interaction between DISC1 and mitochondrial trafficking complex proteins. This result can be confirmed as a specific interaction due to the greatly increased DISC1 signal in the Miro or TRAK GFP conditions compared to the GFP control condition. This result builds upon data published by Camargo and colleagues (Camargo et al., 2007) which suggested an indirect interaction between DISC1 and TRAK by yeast two hybrid screen. Furthermore, the interaction between DISC1 and both TRAK1 and TRAK2 proposes a role for DISC1 in both axonal and dendritic trafficking of mitochondria, due to differential compartmentalisation of the TRAK proteins (Loss and Stephenson, 2015; van Spronsen et al., 2013) with TRAK1 shown to be axonal and TRAK2, dendritic. See Figure 3-1.

In order to confirm the interaction between DISC1 and mitochondrial trafficking complex proteins in neuronal tissue, coimmunoprecipitation assays were carried out from rat brain lysate prepared from transgenic rats expressing human DISC1. This provided the advantage of detecting DISC1 with the anti- human DISC1 antibody used previously in COS7 cell experiments. Miro1 and both TRAK1 and TRAK2 were immunopurified using antibodies specific for these proteins followed by western blotting for DISC. Example western blots are shown in Figure 3-2 on page 93. In each condition, DISC1 is seen to coimmunoprecipitate, whilst the control conditions containing rabbit or mouse IgGs do not result in specific DISC1 pull down. Therefore, the presence of DISC1 in a native, neuronal mitochondrial trafficking complex can be confirmed.

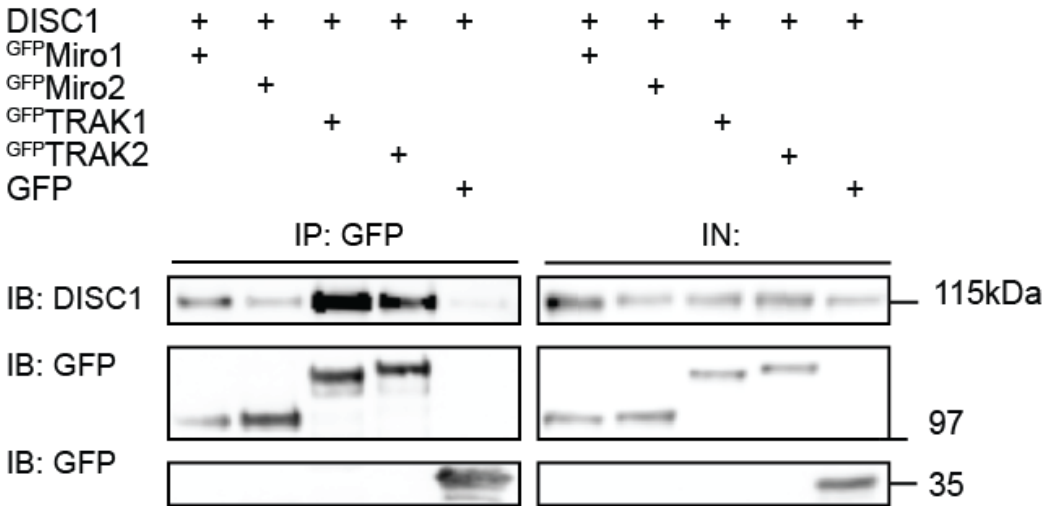


**Figure 3-1 DISC1 interacts with GFP tagged Miro and TRAK proteins in COS7 cells**

Untagged human DISC1 and GFP tagged mitochondrial trafficking complex proteins (Miro1,2 and TRAK1,2) were transiently expressed in COS7 cells. DISC1 transfected with GFP alone formed the negative control condition. Cells were lysed in lysis buffer, lysates were centrifuged and supernatants incubated with GFP trap beads to immunoprecipate GFP tagged proteins. Complexes were resolved by SDS page and detected by western blotting. As demonstrated by the presence of a DISC1 band in the Miro1,2 and TRAK1 and 2 conditions, and the absence thereof in the GFP control, DISC1 interacts with these proteins in a heterologous expression system. n=3.

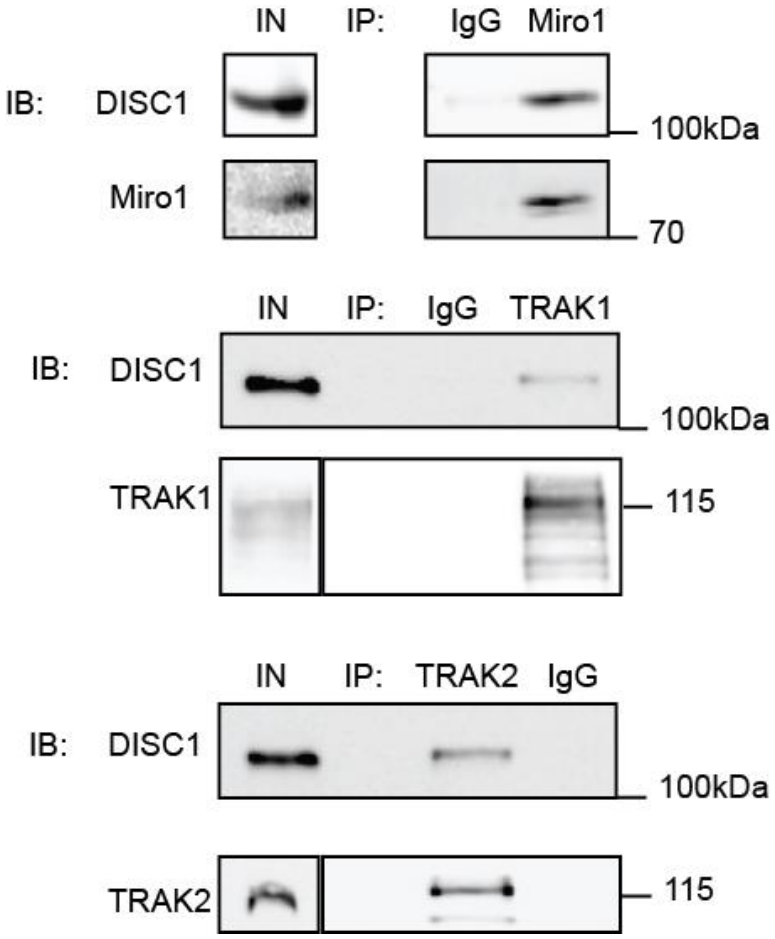


**Figure 3-2 DISC1 forms a native complex with Miro and TRAK proteins in brain tissue.**

Coimmunoprecipitation assays were carried out from brain lysates prepared from transgenic animals expressing human DISC1. Miro1, TRAK1 and TRAK2 were immunopurified using specific antibodies and protein A coupled to Sepharose beads. Non-specifically bound protein complexes were removed by washing and specific components of the protein complex were resolved by electrophoresis. Non-immune immunoglobulins (IgG) provide a negative control. n=3.

### 3.2.2 DISC1 is recruited to mitochondria by overexpression of Miro

The subcellular distribution of DISC1, including its mitochondrial localisation, has been well studied. DISC1 has been shown to be targeted to many subcellular compartments including the ER, post-synapse, nucleus and mitochondria (Brandon et al., 2005; Hayashi-Takagi et al., 2010; Malavasi et al., 2012; Park et al., 2015). In order to investigate any Miro-mediated effect on DISC1 localisation, confocal microscopy and colocalisation analysis was carried out in COS7 cells transfected with MtDsRed2 to visualise the mitochondrial network and human DISC1, with or without overexpression of myc tagged Miro1. In Figure 3-3 DISC1 is recruited to mitochondria by Miro1 overexpression. Example cells are shown in (A) and quantification of DISC1 signal on mitochondria is shown in (B). Colocalisation analysis in imageJ by calculation of DISC1 area overlapping with mitochondrial area revealed 20% of DISC1 to be present on mitochondria in control conditions, where cells express DISC1 and MtDsRed only. This was significantly increased (around 2.5 fold) upon overexpression of Miro, where 48% of the DISC1 signal colocalised with mitochondria ($p=2.3 \times 10^{-5}$). This is consistent with an interaction between these two proteins (see Figure 3-1). It also highlights the role of Miro1 – anchored by its C-terminus in the outer mitochondrial membrane (see 1.3.3 in the Introduction) - as a mitochondrial acceptor of DISC1. This is comparable to the effect of Miro1 overexpression on subcellular distribution of the TRAKs, relating to mitochondrial trafficking, and the E3 ubiquitin ligase Parkin relating to mitochondrial quality control (Birsa et al., 2014; MacAskill et al., 2009a). This result is dependent on the mitochondrial area being unchanged between the two conditions – an increase in mitochondrial area could account for an increased colocalisation of DISC1 with MtDsRed2 signal. Therefore, mitochondrial area in imaged cells was calculated with and without Miro1 overexpression (see part (C) of Figure 3-3). This measure was unchanged; 5.5 AU in control cells compared to 6.0 in Miro overexpressing cells, p= 0.49, not significant. Therefore the increase in DISC1 signal on mitochondria is specific to Miro overexpression.

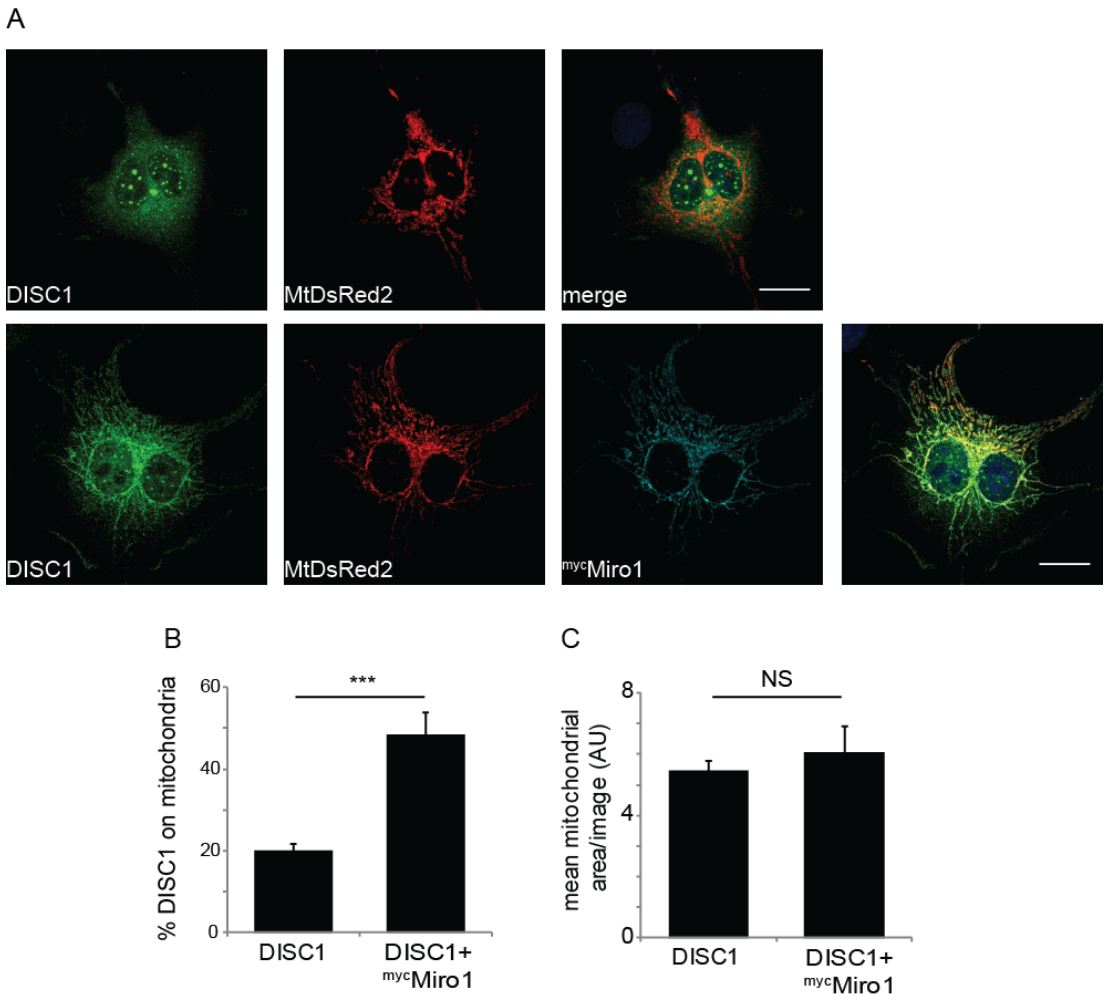


**Figure 3-3 DISC1 is recruited to mitochondria by Miro1 overexpression**

(A) Immunocytochemistry in COS7 cells showing localisation of exogenous DISC1 with and without $^{myc}$Miro1 overexpression. Mitochondria are labeled with MtDsRed2. Scale bar = 20µm. (B) Percentage of DISC1 on mitochondria in (A) (n = 13-15 cells from 3 individual experiments, p = $2.29 \times 10^{-5}$ unpaired T-test). (C) Area of mitochondrial signal per image in control compared to $^{myc}$Miro1expressing cells. p=0.49, NS = not significant (n=13-15 cells from 3 individual experiments).

### 3.2.3 The DISC1 N-terminus is necessary for interaction with Miro and TRAK

The DISC1 protein is comprised of an N-terminal globular domain and a C-terminal region containing multiple coiled-coil regions (Chubb et al., 2008). The N-terminus is shown in black in Figure 3-4 and the coiled-coil regions in blue. These coiled-coil regions have been shown to mediate DISC1 self-association (Leliveld et al., 2009). In order to investigate the region of DISC1 responsible for the interaction with the trafficking complex proteins, coimmunoprecipitations from COS7 cells were carried out between GFP-tagged TRAK1 or myc tagged Miro1, and HA-tagged deletion constructs of DISC1 encoding just the regions indicated in Figure 3-4, including the full length DISC1 protein as a positive control. Myc Miro and GFP-TRAK1 were pulled down with anti-myc beads or GFP trap beads respectively as detailed in the methods section.

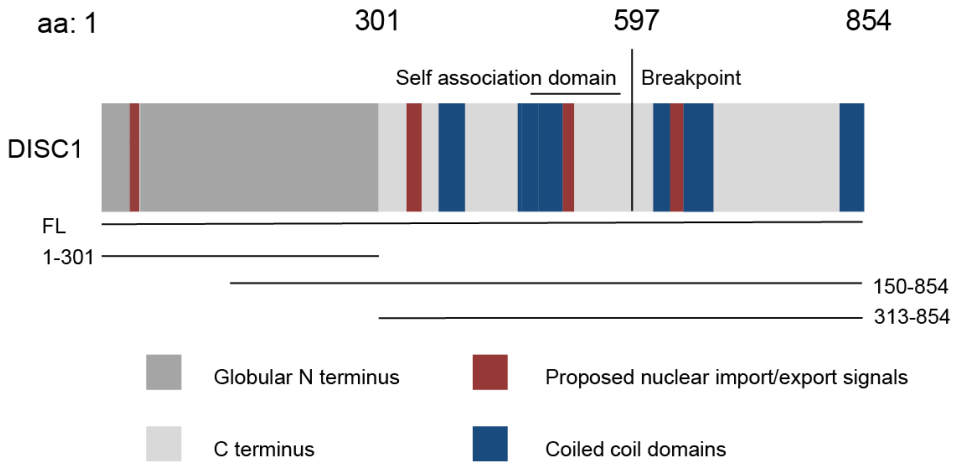


**Figure 3-4 Schematic showing DISC1 deletion constructs used for interaction mapping experiments**

The full length wildtype DISC1 sequence was used along with the N terminal globular domain (1-301), which lacks any coiled coil regions and the DISC1 self association domain. The intermediate 150-854 fragment contains part of the globular N terminal domain as well as the self association domain and coiled coil regions. The C terminal 313-854 fragment lacks the globular N terminus, but contains the coiled coil regions and self association domain.

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Coimmunoprecipitation experiments in Figure 3-5 show that the DISC1 N-terminal region (amino acids 1-301) and the longer sequence, amino acids 150-854 are pulled down with myc Miro. However, the construct encoding the C-terminal region of DISC1, (amino acids 313-854), including the coiled-coil regions and self-association domain (amino acids 403-504) is not precipitated with myc Miro. Thus, the interaction between DISC1 and myc Miro occurs at the DISC1 N-terminus and the C-terminal coiled-coil regions are not necessary for this interaction. Furthermore, it is likely the region of DISC1 responsible for the interaction lies between amino acids 150-301, the overlapping region between the two constructs which are immunoprecipitated with myc Miro.

These experiments were repeated with the same regions of DISC1, but with GFP-TRAK1 expression in place of Miro, and are shown in Figure 3-6. Once more, the full length DISC1 construct is coimmunoprecipitated with GFP-TRAK1. The N-terminal region (amino acids 1-301) is pulled down with TRAK, whereas the C-terminal region (amino acids 313-854) is not. Similarly, the intermediate region (amino acids 150-854) is pulled down with GFP-TRAK1. These results mirror those seen with myc Miro and raise the possibility that these three proteins form a ternary complex.

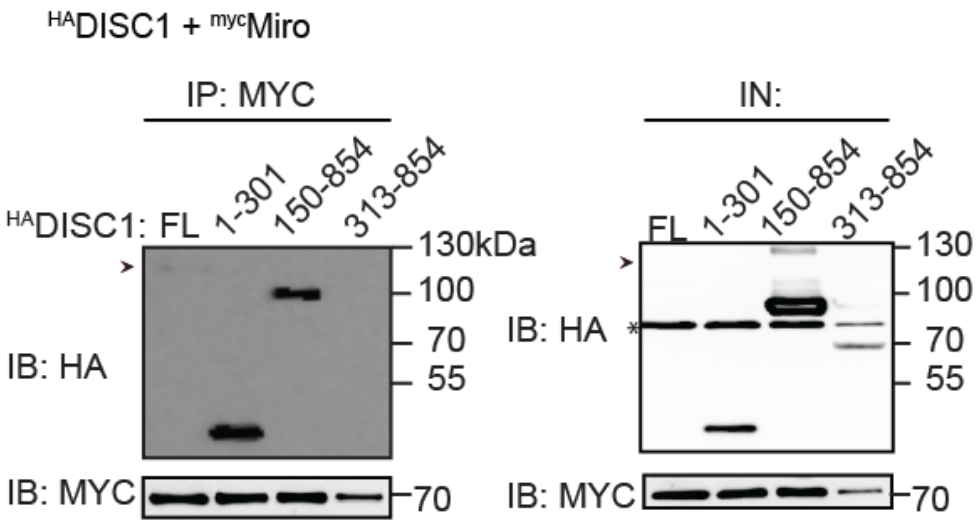


**Figure 3-5 The DISC1 N-terminus interacts with Miro1**

Mapping the region of DISC1 which interacts with mycMiro1 by co expression of full length mycMiro1 and HA DISC1 deletion constructs. Data show the N-terminal 301 amino acids to interact with Miro whilst amino acids 313-854 are not pulled down, arrowhead highlights FL HADISC1 band, * indicates non-specific band.

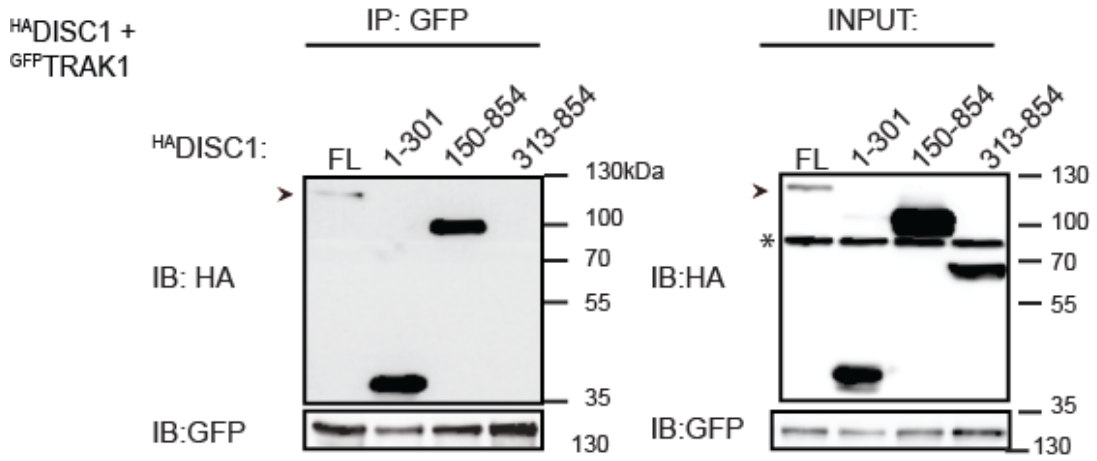


**Figure 3-6 The DISC1 N-terminus interacts with TRAK1**

Coimmunoprecipitation experiments were carried in transiently transfected COS7 cells. Cells were expressing GFP TRAK1 and HA DISC1 deletion constructs detailed in Figure 3-4. Cells were lysed in lysis buffer and after centrifugation incubated with GFP-trap beads as in Figure 3-1. The interaction profile is the same as that shown with Miro1. * indicates a non-specific band.

### 3.2.4 The DISC1-Miro/TRAK interaction is necessary for normal mitochondrial transport.

Overexpression of the DISC1-Miro interacting region (DISC1 residues 1-301) can be used to interrupt the biochemical interaction between DISC1 and the trafficking complex proteins by displacing endogenous DISC1. To investigate the consequences of disrupting the DISC1/Miro interaction, experiments were performed exploring the impact of expressing the DISC1-Miro interacting domain (DISC1 residues 1-301) on mitochondrial transport dynamics by live cell imaging as detailed in the methods section. Hippocampal neurons were transfected with MtDsRed2 (control) or co-transfected with MtDsRed2 and $^{HA}$DISC1 1-301. $^{HA}$DISC1 1-301 expression was confirmed by immunocytochemistry after live imaging. Upon co-expression of the DISC1-Miro interacting domain, a significant decrease in moving mitochondria was detected, (Figure 3-7, Ctrl = 14.7% ± 2.25, 1-301 = 5.07% ± 1.84, n = 23 ctrl and n = 21 1-301 expressing neurons). Kymographs were generated as detailed in the methods section. In these images, a line scan through a process is projected sequentially to give time on the y-axis, so motile organelles appear as diagonal lines and stationary organelles as straight lines. The decrease in diagonal lines in the 1-301 expressing condition compared to control indicates the decrease in moving mitochondria. Thus, the DISC1 1-301 region acts as a dominant negative to impair mitochondrial trafficking.

In addition to the DISC1-Miro interacting region, the effect of DISC1 313-854 (which does not interact with Miro) was investigated. Overexpression of this region showed no significant alteration in mitochondrial trafficking, (Figure 3-8 Control = 14.6% ± 2.42, 313-854 = 12.6% ± 3.18 n = 12 control neurons and 11 313-854 expressing neurons). This can be seen by comparable numbers of diagonal versus straight lines in the kymographs in each condition. Therefore, the impairment in mitochondrial trafficking is reliant on the DISC1-Miro interacting domain and the interaction between DISC1 and the trafficking complex proteins is necessary to regulate normal mitochondrial trafficking.

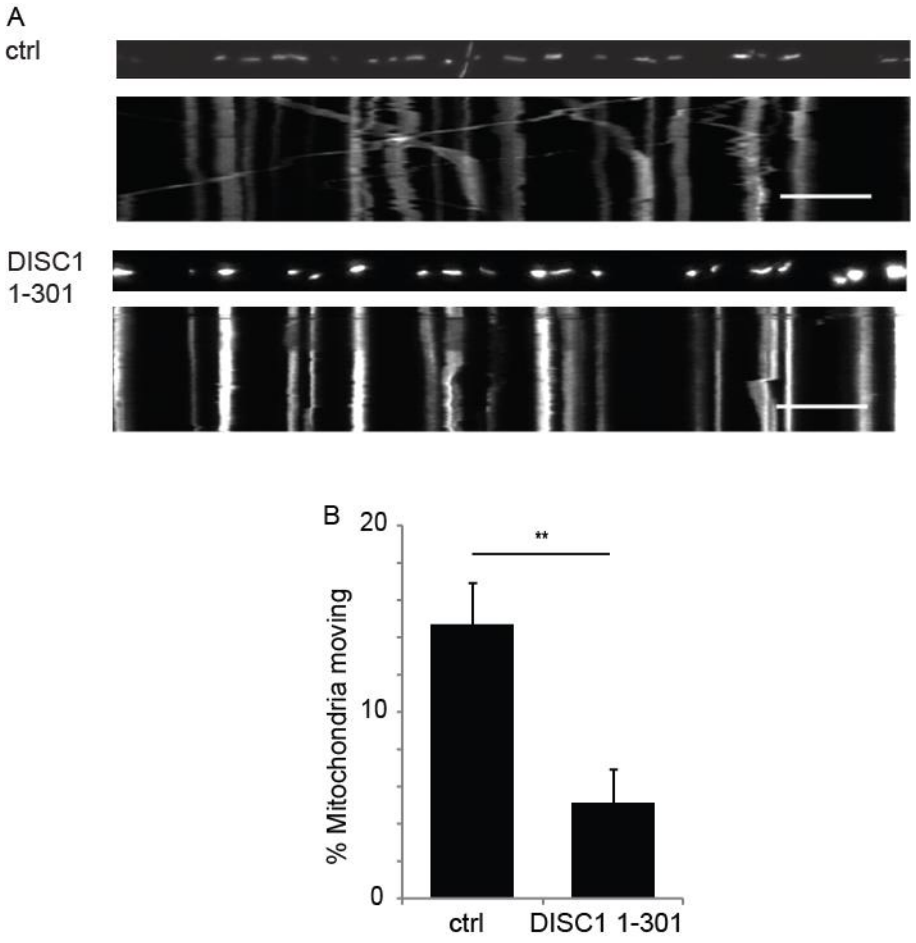


**Figure 3-7 The DISC1-Miro/TRAK interaction is necessary for correct mitochondrial trafficking.**

(A) Kymographs showing effect of overexpressing the DISC1-TRAK binding domain on mitochondrial transport. Scale bar = 10μm. (B) Percentage of moving mitochondria. Data are pooled from recordings in axons and dendrites. Overexpression of the DISC1-TRAK binding domain prevents mitochondrial transport (n = 23 ctrl and n = 21 DISC1 1-301 expressing neurons from 3 preparations, p= 0.002 unpaired T-test).

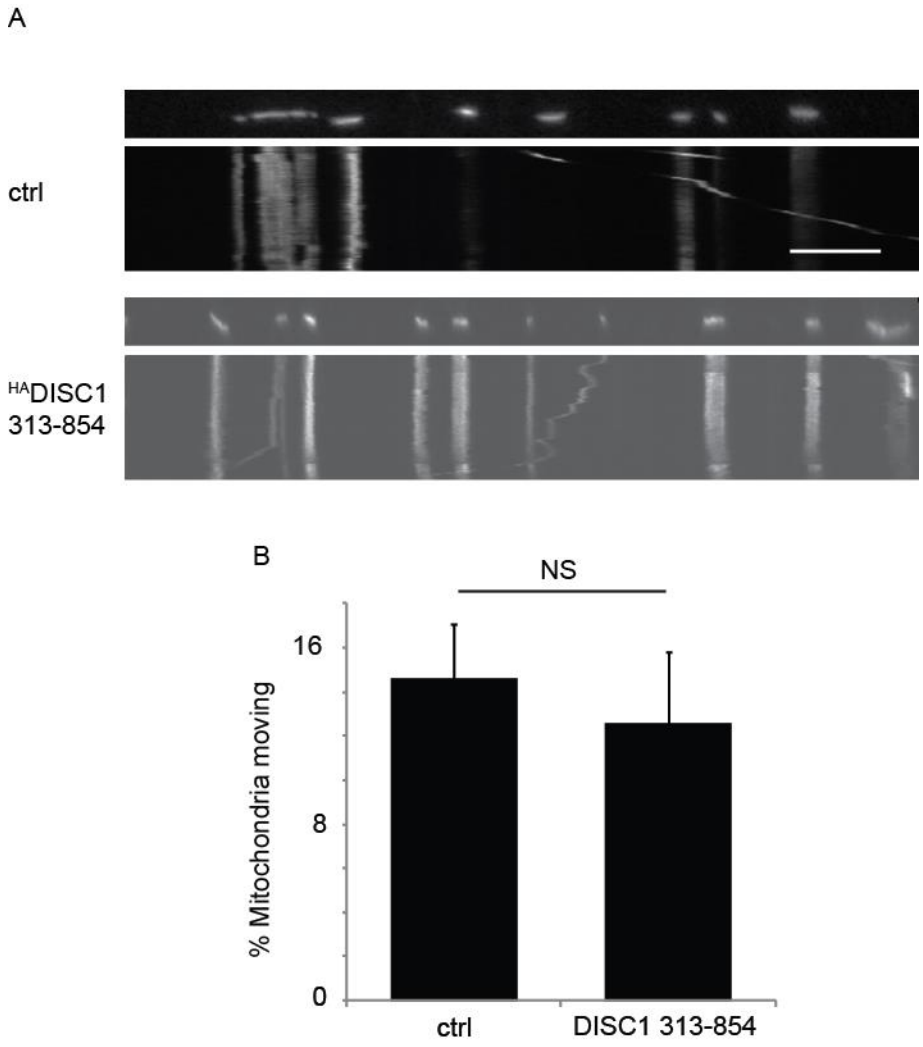


**Figure 3-8 The DISC1 C-terminus does not affect mitochondrial trafficking**

(A) Kymographs showing mitochondrial transport in axons expressing MtDsRed2 and co- expressing $^{HA}$DISC1 313-854. Scale bar = 10μm. (B) Percentage of moving mitochondria in axons and dendrites was quantified with and without expression of $^{HA}$DISC1 313-854. Presence of the DISC1 C-terminus does not alter percentage of mitochondria moving (p=0.6 unpaired T-test) (n = 7 ctrl and n = 10 DISC1 313-854 expressing neurons).

### 3.2.5 The Schizophrenia associated DISC1-Boymaw fusion protein is localised to mitochondria.

Disruptions in normal mitochondrial transport have been linked to neurodegenerative disease and so could also be a causative factor in psychiatric illness. Indeed mitochondrial mislocalisation, consistent with aberrant trafficking, has been demonstrated in post mortem brain samples of schizophrenic patients (Somerville et al., 2011). As has been discussed in 1.7, certain mutations in DISC1 are linked to schizophrenia and other major mental illness (Brandon and Sawa, 2011). Some of these mutations have been shown to confer impairments in mitochondrial dynamics. The R37W point mutation – associated with schizophrenia in one patient – abrogates the ability of DISC1 to bias mitochondrial trafficking in the anterograde direction (Ogawa et al., 2014). L607F is unable to rescue a decrease in moving mitochondria upon knockdown of DISC1 (Atkin et al., 2011)

As described in 1.7, a balanced chromosomal translocation led to the discovery of DISC1. Research has focussed on the DISC1-Boymaw fusion rather than the Boymaw-DISC1 fusion (see Figure 3-9 part A for an illustration of the novel chromosomes). This is because there is little evidence for protein expression of the wild-type Boymaw gene on chromosome 11 – the gene appears to lack significant open reading frames (Eykelenboom et al., 2012; Zhou et al., 2008). Thus, there has been no evidence for this transcript. However, this has been recently refuted, and it is possible this protein could contribute to gain of function effects of DISC1 as a disease mechanism (Ji et al., 2015). In the case of the DISC1-Boymaw fusion, there are three proposed outcomes of the novel chimaeric chromosomes (see Figure 3-9 part B). In each case the expression of wild-type DISC1 from the unaltered chromosome is predicted. The DISC1-Boymaw fusion could lead to a haploinsufficiency if the novel transcript were not to be expressed. Therefore, these patients would be reliant solely on the remaining DISC1 allele as originally proposed (Millar et al., 2005c). The second possible outcome depicted in Figure 3-9 B is the expression of a DISC1 truncation; with expression of the DISC1 protein up to the breakpoint. This could be considered the least likely as there is no evidence for this transcript at the protein or mRNA level. The third potential outcome would be the expression of a fusion protein comprised of DISC1 up to the breakpoint and 69 novel amino acids at the C-terminus, encoded for

by the remaining region of the Boymaw gene. There is evidence for this transcript at the mRNA level (Eykelenboom et al., 2012). However, expression of this transcript at the protein level remains under debate. Antibodies are capable of detecting this construct when heterologously expressed (Eykelenboom et al., 2012). However, via western blot, these antibodies fail to detect the putative fusion protein in cells derived from members of the pedigree carrying the translocation (Millar et al., 2005c). It has since been shown that this protein is insoluble and so could have been absent from the fraction investigated (Zhou et al., 2010). In acknowledgement of this advance, further attempts to detect this protein in a total lysate (compared to soluble fraction alone) have been made. In these experiments too, the fusion protein is absent (Eykelenboom et al., 2012). More recently, it has been shown that the DISC1-Boymaw fusion protein is constitutively degraded (Ji et al., 2014). This high turnover could account for its absence in patient derived samples to date. Further analysis must be carried out to determine if this is the case and if impairing this turnover allows detection of a DISC1-Boymaw fusion protein.

Despite multiple thorough investigations into the impacts of a DISC1 truncation (Atkin et al., 2011; Kamiya et al., 2005; Kvajo et al., 2011; Park et al., 2010b), the cellular outcomes of expression of a DISC1-Boymaw fusion protein remain poorly examined. Thus, the mitochondrial effects of this fusion protein were studied. To do this, the full DISC1-Boymaw fusion protein ($^{HA}$Boymaw) was transfected into hippocampal neurons along with MtDsRed2. Its subcellular localisation was investigated by confocal microscopy. Figure 3-10 on page 106 shows the DISC1-Boymaw fusion protein to colocalise with mitochondria. This is demonstrated in the line scan taken along the process shown in the zoomed image, where DISC1-Boymaw fusion protein signal and MtDsRed2 signal coincide. Moreover, Pearson's correlation coefficient, which measures covariance in intensity of two signals, between DISC1-Boymaw and mitochondria is 0.65 ± 0.08, suggesting a preferential localisation to mitochondria.

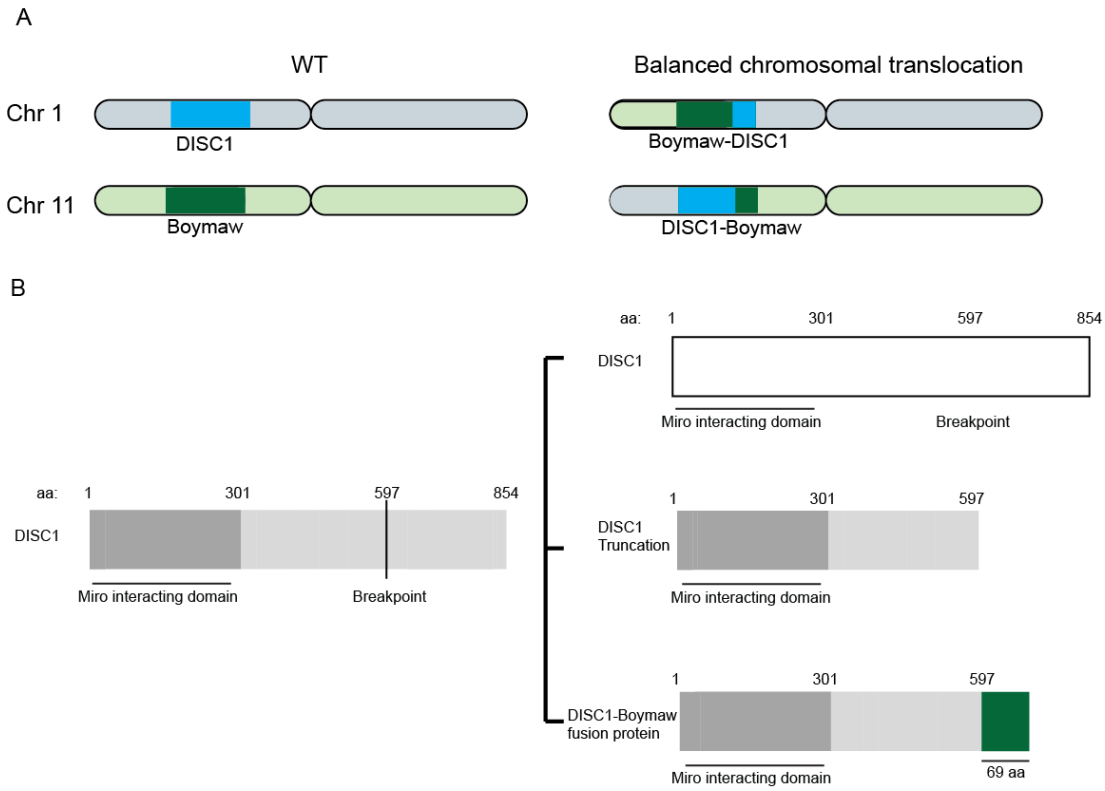


**Figure 3-9 Potential outcomes of a balanced chromosomal translocation**

(A) shows an illustration of structures of chromosomes after a balanced translocation. (B) shows possible outcomes of these novel chromosomes at the protein level. This could be expression of wild-type DISC1 from the unaltered chromosome alone – a haploinsufficiency, expression of wild-type DISC1 and of a DISC1 truncation, or expression of wild-type DISC1 and of a fusion protein of DISC1 and the Boymaw gene – the DISC1-Boymaw fusion protein.

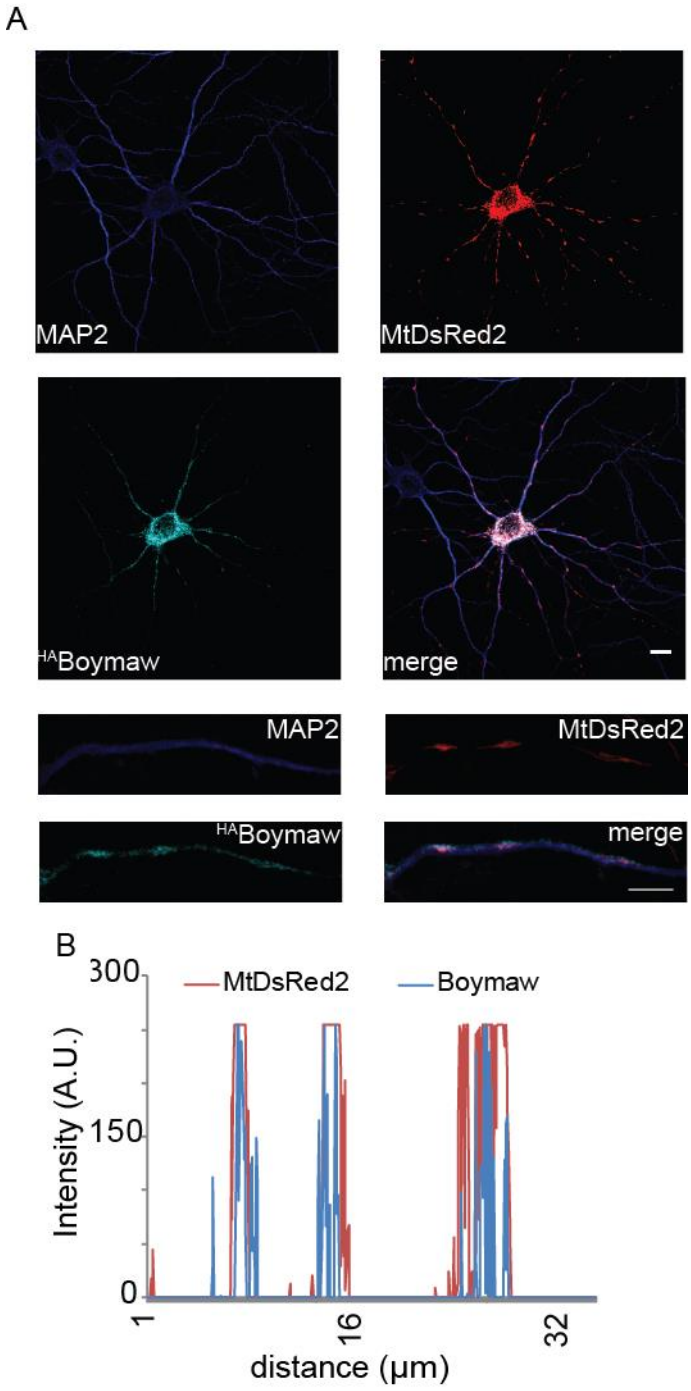


**Figure 3-10 The DISC1-Boymaw fusion protein is localised to mitochondria in processes of hippocampal neurons**

(A) Immunocytochemistry in 10DIV hippocampal neurons showing localization of the Boymaw protein. Scale bar = 20µm, 5 µm on zoomed image. (B) Line scan of zoomed process showing the DISC1-Boymaw fusion protein to localise to mitochondria.

### 3.2.6 The DISC1-Boymaw fusion protein impairs mitochondrial trafficking

In addition to subcellular localisation, the effects of DISC1-Boymaw fusion protein expression on mitochondrial trafficking were investigated. As in the previous section, hippocampal neurons were transfected with MtDsRed2 along with the DISC1-Boymaw fusion protein. MtDsRed2 alone was expressed in control neurons. DISC1-Boymaw fusion protein expression was confirmed by immunocytochemistry after live imaging experiments (see Figure 3-10). Trafficking assays were carried out as detailed in the methods section. Quantitation of percentage of moving mitochondria revealed expression of the DISC1-Boymaw fusion protein to significantly decrease the percentage of moving mitochondria compared to control (Figure 3-11 control = 16.1% ± 2.20, $^{HA}$Boymaw = 6.59% ± 1.40, n = 32 control neurons and n = 26 $^{HA}$Boymaw expressing neurons from 3 preps). This is demonstrated by the decrease in diagonal lines in the kymographs compared to control.

In order to investigate the effect of DISC1-Boymaw fusion protein expression on trafficking of another cargo, synaptophysin GFP was transfected into hippocampal neurons with or without expression of the DISC1-Boymaw fusion protein. In contrast to mitochondria, $^{HA}$Boymaw expression did not significantly impact trafficking of synaptophysin$^{GFP}$ positive vesicles (control = 27.9% ± 2.7, Boymaw = 28.5% ± 4.3, n =17-19 neurons; see Figure 3-12) confirming that the Boymaw fusion protein is not responsible for an overall decrease in microtubule based transport, but specifically disrupts the trafficking of mitochondria.

The impact of DISC1-Boymaw fusion protein expression is consistent with the dominant negative effect of the DISC1-Miro interacting domain on mitochondrial trafficking and suggests a disruption in DISC1-mediated mitochondrial trafficking could be a pathological mechanism in schizophrenia and other psychiatric disease.

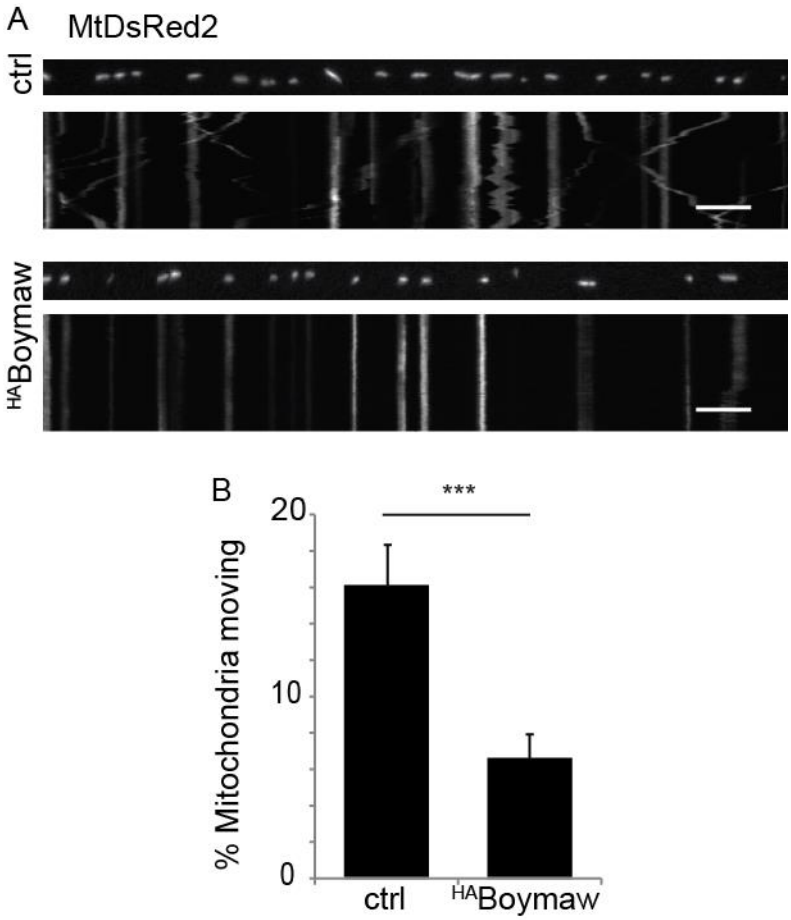


**Figure 3-11 Expression of the DISC1-Boymaw fusion protein impairs mitochondrial transport**

(A) Kymographs showing mitochondrial transport in axons expressing MtDsRed2 and co expressing $^{HA}$Boymaw. Scale bar = 10μm. (B) Percentage of moving mitochondria in axons and dendrites was quantified with and without expression of $^{HA}$Boymaw. Presence of the $^{HA}$Boymaw fusion protein inhibits mitochondrial trafficking ($p = 0.001$, unpaired T-test. n = 32 control neurons and n = 26 $^{HA}$Boymaw expressing neurons from 3 preps)

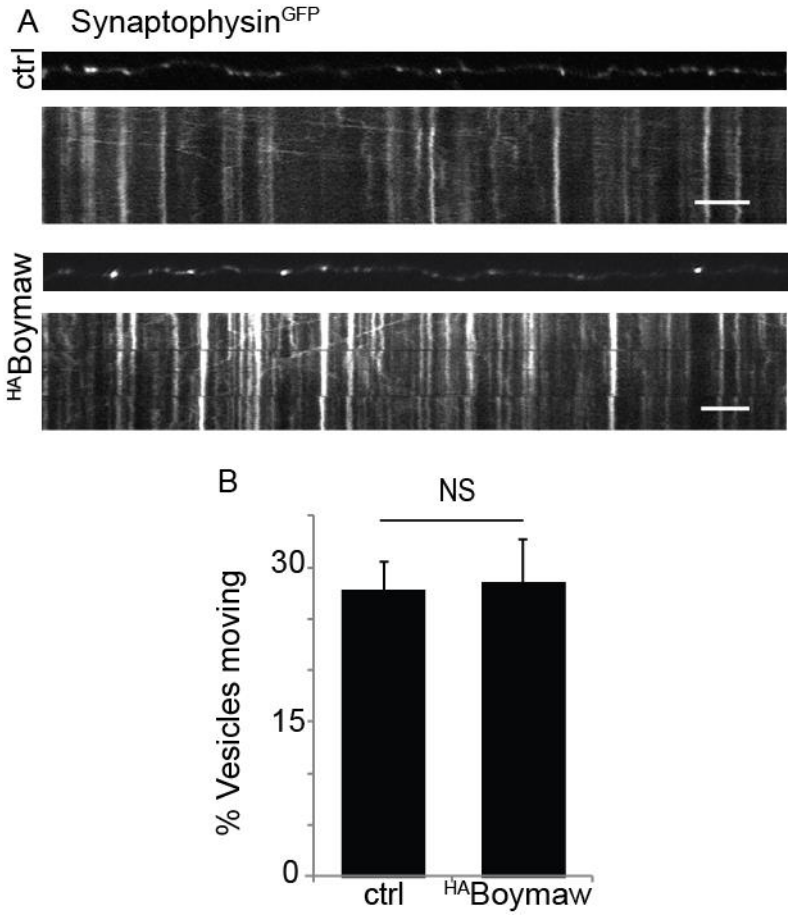


**Figure 3-12 The DISC1-Boymaw fusion protein does not cause a global trafficking impairment**

(A) Kymographs showing the effect of $^{HA}$Boymaw expression on synaptophysin trafficking in axons of rat hippocampal cultures. Synaptophysin GFP was used as a reporter to track presynaptic vesicles. (B) shows quantification. No significant alterations in trafficking of synaptic vesicles was detected. (n = 17 control and n = 19 $^{HA}$Boymaw expressing neurons from 3 preparations, p = 0.9, unpaired T-test).

## 3.3 Discussion

In this chapter, multiple experiments have shown DISC1 to be a component of the mitochondrial trafficking complex. This has been demonstrated by biochemical interaction and subcellular localisation. Using overexpressed protein in COS7 cells, DISC1 has been shown to interact with Miro and TRAKs – canonical mitochondrial trafficking proteins. This finding is supported and extended by demonstration of an interaction between endogenous DISC1 and Miro and TRAKs by coimmunoprecipitation assays from rat brain lysates. This agrees with, and builds upon data reported by Ogawa *et al* where DISC1 was shown to interact with Miro and TRAK1 in cell lines (Ogawa et al., 2014). Future work is necessary to address the direct interactions in this complex. The DISC1 interactome, as determined by yeast two hybrid (Camargo et al., 2007), would suggest an indirect interaction with TRAK via microtubule associated protein MIPT3. This potential indirect interaction is supported by data from the Millar group. An interaction between *in vitro* translated DISC1 and TRAK1 was not detected (Ogawa et al., 2014). This suggests other components are necessary to mediate the interaction. These could be other proteins, such as TRAF3IP or kinesin-1 – known to bind DISC1 directly from *in vitro* experiments (Taya et al., 2007). Alternatively, post-translational modification, e.g. phosphorylation, of DISC1 or TRAK1 may be necessary for the interaction. These modifications would be absent in *in vitro* translated protein and may represent a method for regulating assembly or function of the complex. Further, the interaction between DISC1 and Miro could be dependent on TRAK. Coimmunoprecipitation assays could be carried out from TRAK1/2 knock-out tissue to determine its necessity in this complex.

The interaction with both TRAK1 and TRAK2 suggests DISC1 could be acting as a mitochondrial trafficking protein in both axonal and dendritic compartments. TRAK1 has been localised to axons and TRAK2 to dendrites in cultured hippocampal neurons (Loss and Stephenson, 2015; van Spronsen et al., 2013). This observation is suggestive of separate mechanisms of mitochondrial trafficking in these two neuronal compartments. This is also implied by studies on the mitochondrial anchor, syntaphilin, whose action has been described in axons specifically (Chen and Sheng, 2013). Therefore, via interaction with each of the TRAK proteins, DISC1 could instead

be acting as a common factor in mitochondrial targeting into neuronal compartments and trafficking within these regions.

DISC1 was shown to be recruited to mitochondria by Miro expression, which can thus be described as an adaptor for DISC1 at the outer mitochondrial membrane. This is consistent with effects of Miro on both TRAK2 and Parkin (Birsa et al., 2014; MacAskill et al., 2009a) and previous studies in DISC1. Ogawa and colleagues reported DISC1 to be recruited to mitochondria by overexpression of either TRAK1 or Miro1 (Ogawa et al., 2014). Taken together these data provide evidence for DISC1 to be responsible for local regulation of mitochondrial trafficking, rather than via distal mediation of cellular signalling pathways. Examples may include GSK3β dependent phosphorylation of multiple targets, or cyclic AMP levels via phosphodiesterase 4 activity. These two proteins have been previously demonstrated to interact with, and be held inactive by DISC1 (Murdoch et al., 2007; Singh et al., 2011). This demonstrates the ability of DISC1 to act locally to integrate these signals at the trafficking complex, consistent with its role as a scaffolding protein. In this way, DISC1 may allow specific spatiotemporal control of mitochondrial trafficking, in contrast to global effects on the entire mitochondrial network.

In this chapter, the region of DISC1 necessary for the interaction with Miro and TRAK was determined to lie in the N-terminus of DISC1 – the DISC1 1-301 and 150-854 constructs were able to interact with Miro and TRAK while the C-terminal 313-854 construct was not. These results suggest the interaction is likely dependent upon a region of DISC1 between amino acids 150-300, due to a sustained interaction between DISC1 amino acids 150-854 and Miro or TRAK. This is consistent with experiments from Millar and colleagues which shows a construct encoding the first 358 amino acids to be targeted to the mitochondria (Millar et al., 2005a). However, results reported here differ somewhat with data presented by Ogawa et al. In this case an arginine rich sequence within amino acids 30-50 was deemed to be important for DISC1 recruitment to mitochondria (Ogawa et al., 2014). Coimmunoprecipitation experiments showed a decrease in the interaction between TRAK and DISC1 lacking amino acids 1-47, or a DISC1 construct where the arginine residues in this motif have been mutated to alanine residues, abolishing this region of positive charge. However, some interaction remained, implying this motif is not essential for the DISC1-TRAK interaction. Data

presented in this study from the Millar group also suggest this region of DISC1 is necessary for TRAK-dependent recruitment of DISC1 to the mitochondria – consistent with the decreased interaction (Ogawa et al., 2014). The slight discrepancies could be due to the different methods employed in each of these experiments, or could suggest that multiple factors affect the interaction of DISC1 with the trafficking complex, such as post-translational modifications within the N-terminus.

Via elucidation of the region of DISC1 responsible for interaction with the trafficking complex proteins, the interaction of DISC1 with Miro and TRAK was shown to be necessary for correct mitochondrial trafficking. Overexpression of the interacting region, which could act to uncouple the endogenous DISC1-Miro interaction, significantly depleted motile mitochondria. This is consistent with previous reports of DISC1 as a positive regulator of mitochondrial trafficking (Atkin et al., 2011) and place DISC1 at the mitochondrial trafficking complex to effect transport. This is consistent with co immunoprecipitation assays reported in this chapter. Future work will investigate the function of DISC1 at the trafficking complex, aiming to elucidate any signalling roles it may have in this context such as GSK3 beta or PDE4 inhibition. These potential mechanisms will be further discussed in Chapter 6. Notably, the DISC1 C-terminus did not impair mitochondrial trafficking - despite containing the Lis1/Ndel1 binding site (Brandon et al., 2004). These are dynein complex components, necessary for neurodevelopment but also implicated in mitochondrial trafficking (Shao et al., 2013). Overexpression of this C-terminal region of DISC1 could act as a dominant negative to recruit them away from the trafficking complex. However, this appears not to be the case, perhaps reflecting a specificity of DISC1 for anterograde mitochondrial transport (Ogawa et al., 2014) or a necessity for DISC1 to be localised to the trafficking complex via its N-terminus for regulation of bidirectional trafficking.

This chapter also describes the effects of expression of the schizophrenia associated DISC1-Boymaw fusion protein on mitochondrial trafficking. This fusion protein is shown to constitutively localise to mitochondria, in contrast to wild-type DISC1 which required recruitment by other members of the trafficking complex. This could be caused by loss of the C-terminus of wild-type DISC1 in this transcript or the influence of the novel 69 amino acids at the C-terminus of this chimaeric protein. Yet, this finding is consistent with the DISC1 Miro interacting region remaining unaltered in

this fusion transcript. Thus, it may exert a greater effect on the distribution of this protein than the wild-type DISC1. This protein acted consistently with the dominant negative construct to impair mitochondrial trafficking. This effect could be due to a lack of binding sites available to DISC1 at the trafficking complex, and so a loss of DISC1's ability to act as a local regulator of mitochondrial trafficking. These data show the DISC1-Boymaw fusion protein to be another DISC1 mutation unable to mediate mitochondrial trafficking.

Alternatively, the DISC1-Boymaw fusion protein could impair trafficking by a gain of function activity. For example, the DISC1-Boymaw fusion protein has been shown to be insoluble – found in the pellets of lysates (Zhou et al., 2010). This could represent gain of aggregation tendency associated with psychiatric disease and impaired mitochondrial trafficking (Atkin et al., 2012; Leliveld et al., 2008). However, the aggregates previously described were not localised to the mitochondria as is the DISC1-Boymaw fusion protein. Instead, gain of toxic function may arise from impaired mitochondrial function by the Boymaw protein. The fusion protein is known to decrease NADH oxidoreductase activity in both mitochondrial and ER compartments (Ji et al., 2014). Decreased respiratory capacity and thus mitochondrial membrane potential could account for decreased trafficking (Miller and Sheetz, 2004). Further, the Boymaw protein itself has been proposed to be localised within mitochondria due to colocalisation with cytochrome C (Ji et al., 2015). Therefore, the DISC1-Boymaw fusion protein could be mistargeted from the outer mitochondrial membrane and be localised within the mitochondrion. However, colocalisation experiments which have suggested this distribution were not carried out at high enough resolution to confirm this localisation. Studies on the Boymaw protein itself will be necessary to address a gain of toxic function compared to loss of wild-type DISC1 function.

Whether due to gain or loss of function, these impairments in mitochondrial trafficking could contribute to the onset of schizophrenic symptoms via a lack of correctly positioned mitochondria. This would contribute to a mismatch between supply and demand at sites where high calcium buffering capacity or high energy levels are required. Examples of these locations include the pre and post synapse. Data presented here suggest this trafficking defect to be specific to mitochondria as trafficking of

presynaptic vesicles, labelled with synaptophysin GFP, was unaltered. A role for DISC1 in trafficking of other cargoes, (e.g. synaptic vesicles) has been demonstrated, (Flores et al., 2011). Moreover, the interaction reported here with the TRAK proteins implicates cargo beyond mitochondria – TRAKs have known roles in $GABA_A$ receptor and potassium channel trafficking (Beck et al., 2002; Smith et al., 2006). However, a specificity for mitochondria is not unexpected given the mitochondrial localisation of the DISC1-Boymaw fusion protein. Data presented here provide a disease mechanism for the balanced chromosomal translocation, but the mechanism of action of this fusion protein yet to be thoroughly explored.

# Chapter 4
# DISC1 regulates mitochondrial fusion and dendrite outgrowth

## 4.1 Introduction

As shown in Chapter 3, DISC1 couples to the mitochondrial trafficking proteins Miro and TRAKs to mediate correct mitochondrial transport. This process can be impaired in disease states, such as upon expression of the DISC1-Boymaw fusion protein. Yet, in addition to trafficking, mitochondria also undergo fission and fusion events to maintain their morphology and health (Youle and van der Bliek, 2012). Fusion is mediated at the outer mitochondrial membrane by Mitofusins. These are GTPases which can tether together two mitochondria by interaction *in trans* via heptad repeat domains (hydrophobic helical regions – see 1.2.1 on page 21) (Koshiba et al., 2004). The role of DISC1 in mitochondrial fusion is, to date, unexplored. However, alterations in mitochondrial morphology have been described upon DISC1 overexpression (Millar et al., 2005a). In this study, mitochondria can be seen to form ring or lariat like structures, perhaps indicative of hyperfusion. Upon overexpression of GFP-tagged DISC1 (shown to form deleterious aggresomes (Atkin et al., 2012)) mitochondria can be seen to cluster in the perinuclear region and appear fragmented (Millar et al., 2005a). Therefore, it is of interest to investigate the possibility of any DISC1-dependent regulation of the fusion machinery.

As well as fusion, Mitofusin2 (Mfn2) may act as a tether between mitochondria and the endoplasmic reticulum (ER). Confocal microscopy based analyses revealed area of ER-mitochondria contact was decreased in Mfn2 knock-out MEFs compared to wild-type. It was also shown that, while wild-type Mfn2 expression in the knock-out MEFs could rescue this phenotype, expression of a fusion deficient construct had no

effect (de Brito and Scorrano, 2008). However, this has since been refuted (Cosson et al., 2012; Filadi et al., 2015). Electron microscopy (EM) studies have suggested that Mfn2 knock-out MEFs in fact display an increased ER mitochondria contact area – suggesting Mfn2 to be a negative regulator of these sites. This difference is proposed to be due to volume changes to organelles contributing to effects in confocal but not EM studies, where just the percentage of mitochondrial perimeter in contact with ER can be studied (Filadi et al., 2015).

Mitochondria-ER contacts facilitate communication between these two organelles, including calcium and lipid transfer (Rizzuto et al., 1998). These experiments were carried out by inducing calcium release from the ER via stimulation of $IP_3$ receptors. Mitochondrial calcium concentration was measured with a mitochondrially targeted, fluorescent calcium indicator - aequorin - and area of contact sites is thought to be commensurate with calcium transfer (de Brito and Scorrano, 2008; Filadi et al., 2015; Rizzuto et al., 1998). This function of the contact sites is crucial to the cell as mitochondrial calcium levels regulate ATP production and apoptotic signalling (Kaufman and Malhotra, 2014). These ER-mitochondria contact sites are known points of autophagosome biogenesis. Upon starvation (to induce autophagy), pre-autophagosomal marker ATG14 localises to these sites, recruited by ER SNARE, syntaxin 17 (STX17). Biochemically, it has been shown that STX17 relocalises ATG14 to the mitochondria associated membrane (MAM) fraction (Hamasaki et al., 2013). Additionally, contacts between the ER and mitochondria are proposed to be involved in both fission-fusion and trafficking of mitochondria (Filadi et al., 2015; Rowland and Voeltz, 2012) and interestingly, the yeast homologue of Miro1 – Gem1 – is also known to be localised to these sites (Kornmann et al., 2011). Further, the mitochondrial trafficking regulator VAPB, an ER protein, has also been localised to these sites via interaction with mitochondrial protein PTPIP51 (Morotz et al., 2012; Stoica et al., 2014).

Of further interest in the study of ER-mitochondria associations is their alteration in disease. To date, alterations in these associations have been linked to neurodegenerative disorders including Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (PD, AD and ALS from here on) (Hedskog et al., 2013; Kornmann, 2013; Stoica et al., 2014). Overexpression of alpha synuclein (mutated or

overexpressed in some PD cases) can cause an increase in ER mitochondria contact as determined by confocal microscopy (Cali et al., 2012). TDP-43, known to carry causative mutations in ALS, has been recently demonstrated to be a modulator of ER-mitochondria contact sites. Overexpression of this protein or ALS associated mutations in cell lines perturbed cellular calcium homeostasis – ER levels were increased, whereas mitochondrial levels were decreased. Electron microscopy in motor neurons of transgenic mice demonstrated a decreased ER-mitochondria contact. Concurrently, overexpression of TDP-43 leads to an uncoupling of tethering proteins VAPB at the ER and PTPIP51 at the mitochondrial outer membrane. Coordination of these two proteins has been shown to be essential for normal mitochondria-ER interface by both electron and confocal microscopy. Further, this uncoupling was proposed to be dependent on activity of the kinase, GSK3 beta. This highlights the dynamic nature of these organelle associations (Stoica et al., 2014). In terms of AD, exposure of cells to pathological amyloid beta peptide causes an upregulation in mitochondria associated membrane proteins. This was also confirmed in tissue from an AD mouse model carrying pathogenic mutations in amyloid precursor protein. This led to an increased number of ER-mitochondria associations as quantified by proximity ligation assay between $IP_3R$ (at the ER) and VDAC1 (at the mitochondrial outer membrane) in primary neurons. This confers an increased shuttling of calcium from the ER to the mitochondria and could contribute to neuropathy (Hedskog et al., 2013).

As described in the introduction (see page 42), organellar distribution has been shown to be crucial for dendritic development. There is a wealth of literature supporting mitochondria as essential mediators of this process. They have been suggested to both positively and negatively regulate dendrite outgrowth in cultured neurons (Fukumitsu et al., 2015; Kimura and Murakami, 2014). Additionally DISC1 is a well studied, and similarly controversial, regulator of dendrite outgrowth. *In vivo* DISC1 knockdown studies have reported increased neurite length and number, and so would propose it to be a negative regulator of dendritic development (Duan et al., 2007b; Kang et al., 2011). However, experiments in mouse models of schizophrenia, which carry truncating lesions in DISC1, show impaired neurite outgrowth. Thus DISC1 could be concluded to act as a positive regulator of dendrite development in these systems (Kvajo et al., 2011; Lepagnol-Bestel et al., 2013; Shen et al., 2008b). This is supported

by studies in differentiating cell lines, which show decreased neurite outgrowth upon uncoupling of interactions between DISC1 and FEZ1 or Dysbindin (Lee et al., 2015; Miyoshi et al., 2003).

In this chapter, a potential involvement of DISC1 in mitochondrial fusion will be investigated by coimmunoprecipitation experiments both in overexpression and native systems. Further, the effect of the DISC1-Boymaw fusion protein on this process will be studied by live imaging in hippocampal neurons, and fixed imaging in COS7 cells. Multiple lines of evidence prompted investigation into DISC1 as a component or regulator of ER-mitochondria contacts. As well as potential involvement with mitochondrial fusion via Mfn2, DISC1 has been shown to localise to the endoplasmic reticulum and mitochondria, and to regulate mitochondrial calcium homeostasis (Park et al., 2015; Park et al., 2010b). Alterations in these sites in conditions of neuropsychiatric disease have yet to be studied, and so the effect of the DISC1-Boymaw fusion protein will also be investigated here. Finally, in this chapter, the contribution of DISC1-dependent mitochondrial dynamics to dendrite outgrowth will be explored. These experiments employ both the DISC1 dominant negative described in Chapter 3 and the DISC1-Boymaw fusion protein and aim to highlight a potential disease mechanism for the DISC1-Boymaw fusion protein in the aetiology of schizophrenia.

## 4.2 Results

### 4.2.1 The DISC1 N-terminus and the DISC1-Boymaw fusion protein alter mitochondrial morphology

As discussed in Chapter 3, expression of the DISC1 N-terminus or the DISC1-Boymaw fusion protein acts dominant negatively to decrease the percentages of motile mitochondria. In addition, mitochondrial length in axons appeared decreased upon expression of either of these constructs. This change in morphology may suggest an imbalance between mitochondrial fission and fusion activity. In order to confirm this, the length of mitochondria was measured in neurons co-expressing MtDsRed2 and DISC1 1-301 or the DISC1-Boymaw fusion protein. A significant

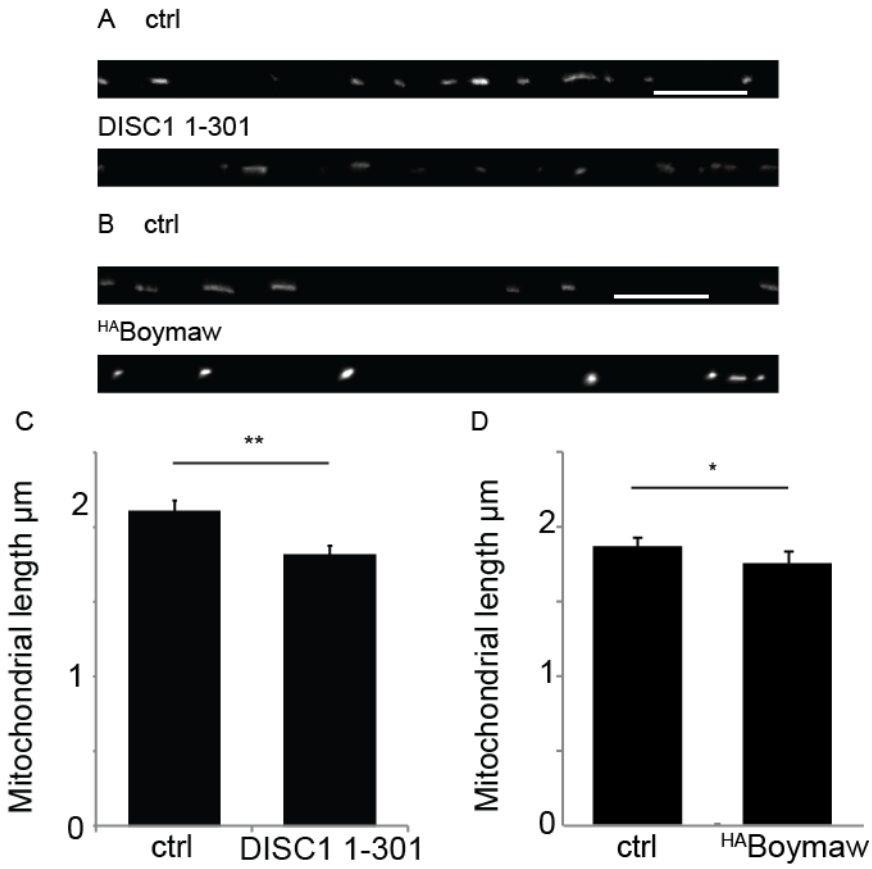


**Figure 4-1 Mitochondrial length is decreased upon expression of DISC1 1-301 or the DISC1-Boymaw fusion protein**

(A) DISC1 1-301 decreases the length of mitochondria quantified in (C) (control = 2.1 µm ± 0.065, 1-301 = 1.8 µm ± 0.063 n = 11 axons, p = 0.004 unpaired T-test). (B) and (D) The DISC1-Boymaw fusion protein decreases the length of mitochondria (control = 1.81µm ± 0.0858, $^{HA}$Boymaw = 1.54µm ± 0.0644, n = 13 axons, p = 0.02, unpaired T-test). Scale bars 5µm.

decrease in the length of axonal mitochondria compared to control was noted in each case (see Figure 4-1). Example images of axonal mitochondria in DISC1 1-301 overexpressing neurons show a shorter, rounder morphology compared to control. (Control = 2.1 µm ± 0.065, 1-301 = 1.8 µm ± 0.063 n = 11 axons). Likewise, DISC1-Boymaw fusion protein expressing neurons show a comparable phenotype, with these mitochondria being less elongated than control. (Control = 1.81µm ± 0.0858, $^{HA}$Boymaw = 1.54µm ± 0.0644.)

### 4.2.2 Mitofusin overexpression can alter DISC1 subcellular localisation

This decrease in mitochondrial length is consistent with an imbalance in mitochondrial fission and fusion (Youle and van der Bliek, 2012), namely, either a decrease in fusion or an increase in fission. This raises the possibility that DISC1 could regulate this machinery. As detailed in the introduction (1.2.1), mitochondrial fusion is regulated by Mitofusins at the outer mitochondrial membrane, whilst fission requires recruitment of the cytosolic GTPase, Drp1. Thus experiments were focussed on Mitofusins as DISC1 has been shown to localise to the mitochondria in section 3.2.2. Moreover, the Mitofusins are known interactors of Miro and TRAK (Misko et al., 2010). The subcellular localisation of a protein is of great importance to its function. Thus the distribution of exogenous DISC1 in COS7 cells was investigated upon overexpression of Mitofusins 1 and 2. This was carried out by immunocytochemistry, as detailed in the methods section, with MtDsRed2 used to label mitochondria. Please see Figure 4-2 where a redistribution of DISC1 to the mitochondria is noted upon expression of either Mitofusin1 or Mitofusin2.

In order to confirm this observation, mitochondrial isolation assays were carried out under similar conditions; overexpression of DISC1 and $^{myc}$Mitofusin1 or 2 in COS7 cells (Figure 4-3). This provides a biochemical readout based on a larger population of cells than the immunocytochemistry experiments. Consistently, an increase in DISC1 in the mitochondrial fraction was observed upon overexpression of either Mitofusin protein. This is in agreement with data presented in the previous chapter regarding DISC1 distribution and Miro1 overexpression. Therefore, the Mitofusins can also act as recruiters of DISC1 at the outer mitochondrial membrane.

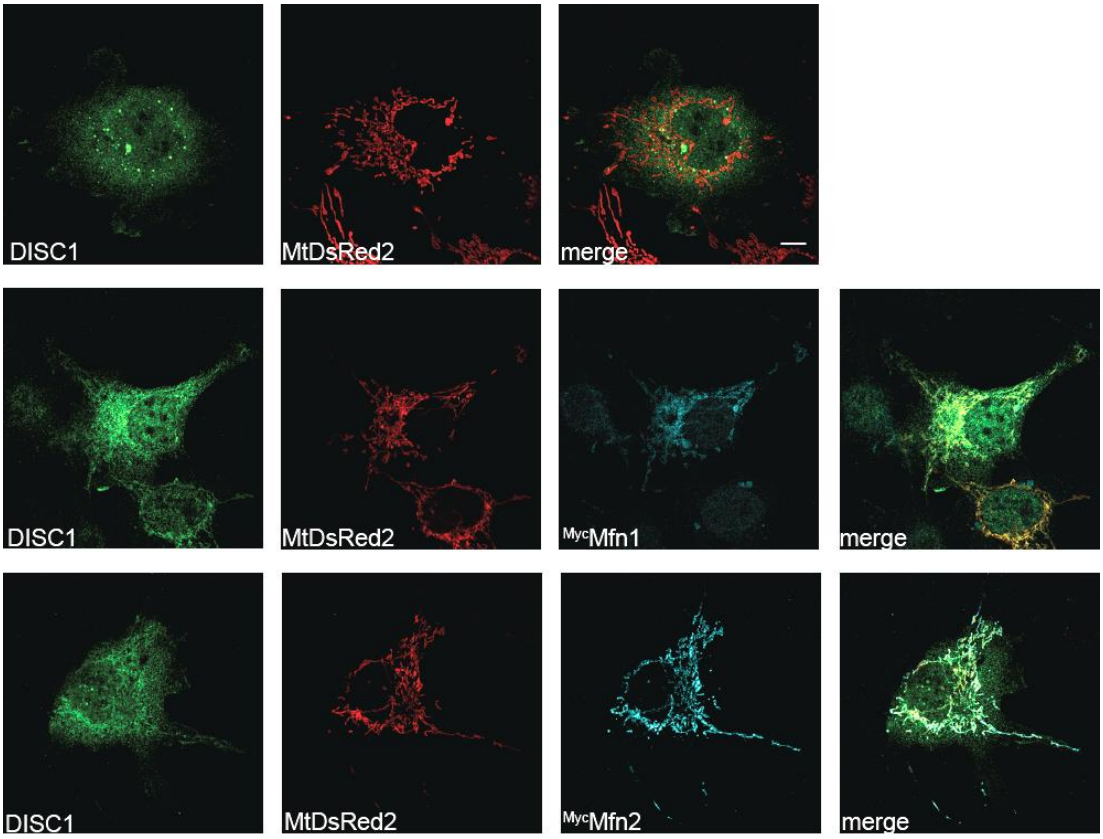


**Figure 4-2 DISC1 is recruited to mitochondria in COS7 cells by overexpression of Mitofusin1 or 2**

Immunocytochemistry in COS7 transiently transfected with untagged human DISC1 and MtDsRed2 to label mitochondria (control condition) or with coexpression of Mitofusin constructs $^{myc}$Mfn1 or $^{myc}$Mfn2. The control condition shows DISC1 to adopt a diffuse staining with occasional puncta, dissimilar to the MtDsRed2 signal. DISC1 distribution is altered upon Mitofusin overexpresion where it can be seen to translocate to the mitochondria. Scale bar = 20μm.

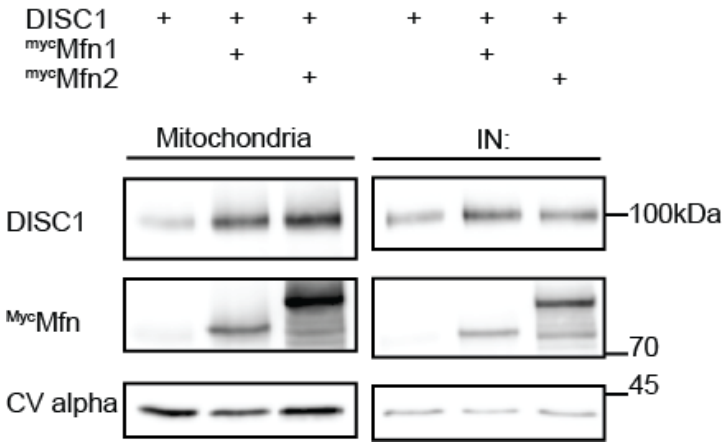


**Figure 4-3 DISC1 is recruited to the mitochondrial fraction by overexpression of Mitofusin1 or 2**

COS7 cells were transfected with untagged human DISC1 and myc tagged Mitofusin1 or Mitofusin2. Cell were subjected to mitochondrial fractionation assays. An increase of DISC1 in the mitochondrial pool compared to total fraction (Input) with overexpression of either Mitofusin1 or 2 is noted.

### 4.2.3 DISC1 interacts with the mitochondrial fusion machinery, Mitofusins 1 and 2.

To extend these findings and to investigate the possibility that DISC1 may interact with the Mitofusins to regulate mitochondrial morphology, coimmunoprecipitation experiments were carried out from COS7 cells. In this experiment, cells were transiently expressing myc tagged Mitofusin constructs (Mfn1 and Mfn2 in figures) and human DISC1 (see Figure 4-4). Experiments were carried out as detailed in the methods section. DISC1 can be seen to be coimmunoprecipitated with either Mitofusin1 or Mitofusin2. This is supportive of a biochemical interaction between these proteins.

In order to confirm this interaction with endogenous Mitofusin1 in neuronal tissue, co immunoprecipitation assays were carried out from rat brain lysate as detailed previously (see 2.4.1 in methods and Figure 3-2 in chapter 3). Results are shown in Figure 4-5. Mitofusin1 was immunopurified with a specific antibody followed by immunoblotting for DISC1. The presence of the DISC1 band in the Mitofusin1 IP indicates the presence of these proteins in a complex under native conditions. In order to investigate the possibility that DISC1 could interact indiscriminately with proteins of the outer mitochondrial membrane, the same experiment was carried out with immunoprecipitation of TOM20 (Translocase of the outer mitochondrial membrane of molecular mass 20kDa). This protein is resident at the outer mitochondrial membrane but has no known role in mitochondrial transport. Instead it acts as a component of the machinery required to import proteins into the inner mitochondrial compartments (Baker et al., 2007). No interaction was detected between DISC1 and TOM20, exemplified by the absence of the DISC1 band in this lane of the blot. So, DISC1 does not interact indiscriminately with outer mitochondrial membrane proteins. Thus DISC1 can interact with, and could regulate the mitochondrial fusion machinery.

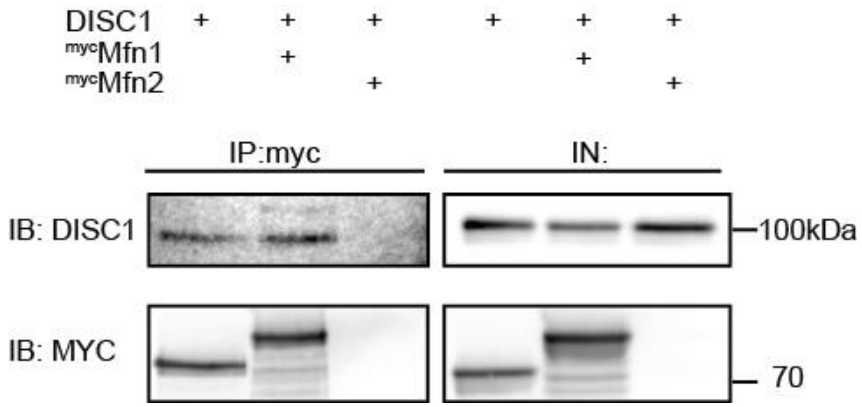


**Figure 4-4 DISC1 interacts with myc tagged Mitofusins in COS7 cells**

*In vitro* coimmunoprecipitation experiments from exogenous proteins expressed in COS7 cells transfected with untagged human DISC1 and myc tagged Mitofusin1 or 2. Experiments were carried out as detailed in the methods section. After lysis, samples were incubated with anti-myc antibody conjugated to agarose beads to immunopurify protein complexes containing myc tagged proteins. Samples were washed to remove non-specifically bound proteins and resolved by SDS-PAGE prior to immunoblotting.

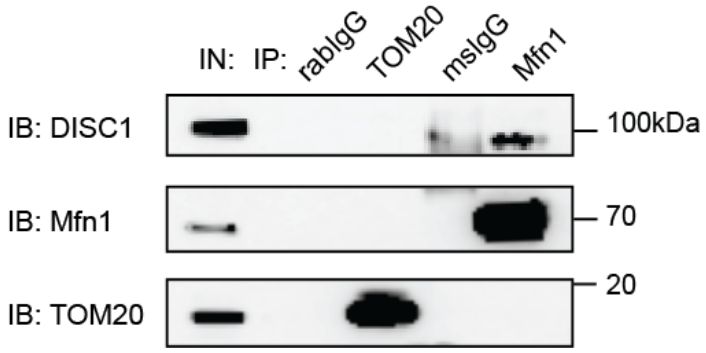


**Figure 4-5 DISC1 forms a native complex with Mitofusin1 in brain tissue**

Coimmunoprecipitation assays were carried out from brain lysates prepared from transgenic animals expressing human DISC1. Mfn1, and TOM20 were immunopurified using specific antibodies and protein A coupled to Sepharose beads. Non-specifically bound protein complexes were removed by washing and specific components of the protein complex were resolved by electrophoresis. Non-immune immunoglobulins (IgG) raised in the relevant species (rabbit for TOM20, mouse for Mitofusin1) provide a negative control.

### 4.2.4 The schizophrenia associated DISC1-Boymaw fusion protein impairs mitochondrial fusion in rodent hippocampal neurons

The interaction of DISC1 with the mitochondrial fusion machinery suggests not only that DISC1 could be important in regulating fusion activity, but also that this activity could be impaired upon DISC1 mutation, such as in schizophrenia. Therefore, the effect of the DISC1-Boymaw fusion protein on mitochondrial fusion was investigated. In order to do this, mitochondrial fusion assays were carried out in rodent hippocampal neurons co-transfected with MtDsRed2 to visualise the mitochondrial network and a photoactivatable GFP construct with a mitochondrial targeting sequence (Karbowski et al., 2004). Photoactivation was carried out in the soma, a site of high mitochondrial density, and thus a region where fusion can occur with a lesser dependence on mitochondrial transport than in neuronal processes (Cagalinec et al., 2013). Measuring the area of GFP signal over time provides a readout for mitochondrial fusion. Please refer to Figure 4-6 for a schematic of the experimental setup.

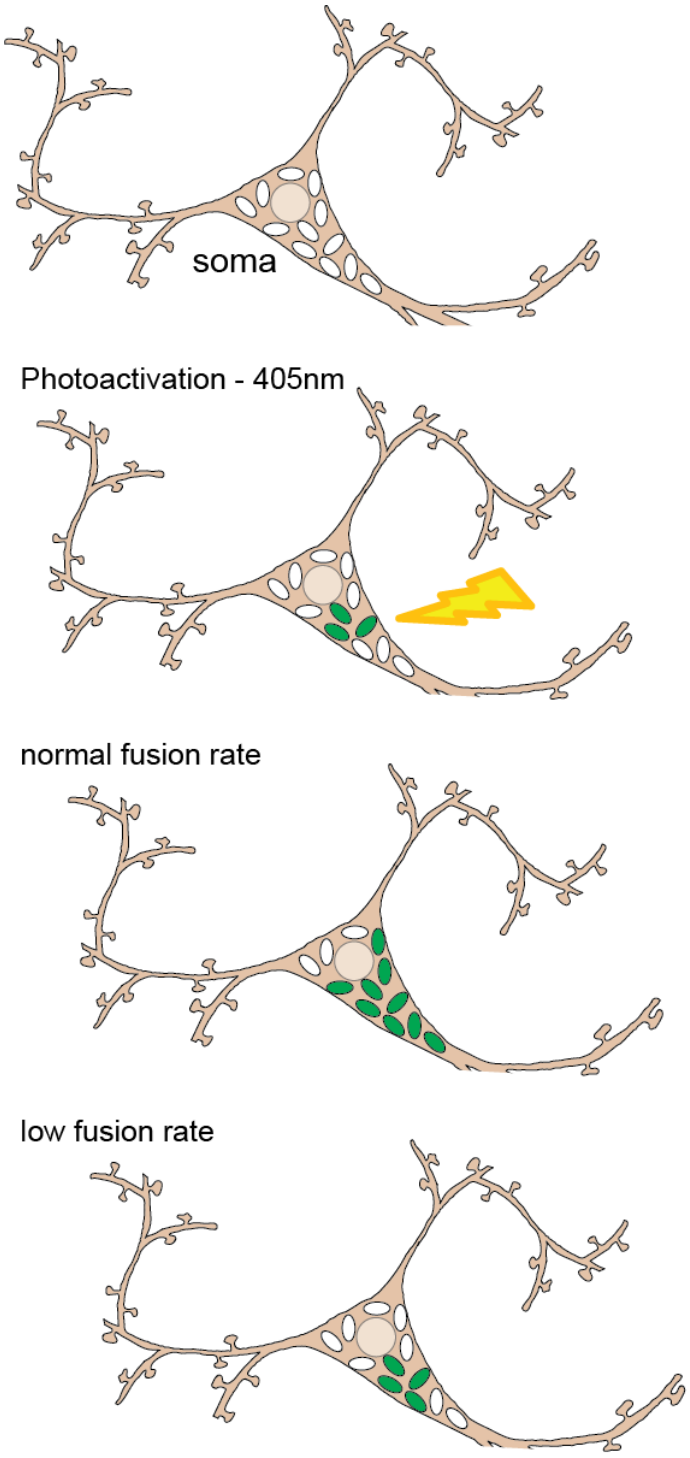


**Figure 4-6 Photoactivation assay to measure mitochondrial fusion**

Schematic demonstrating photoactivation to specifically label mitochondria in a defined region. The spread of the mitochondrial GFP signal gives a readout for fusion rate via exchange of mitochondrial contents. A slower change in area is indicative of a low fusion rate.

In order to confirm that a decrease in fusion would lead to a decreased change in GFP area over time, a positive control experiment was carried out using a dominant negative (DN) Mitofusin2 construct, incapable of effecting mitochondrial fusion (described in the methods section). This has been achieved by mutation of lysine 109 to alanine, rendering the Mitofusin GTPase domain inactive (Ishihara et al., 2004). Cells expressing MtDsRed2 and photoactivatable mitochondrial GFP formed the control condition. Results can be seen in Figure 4-7. The slower increase in GFP area indicates a lesser fusion rate (n=6 control and 8 Mfn DN neurons, final normalised area for Mfn2 DN expressing cells is 56% lower than controls).

$^{HA}$Boymaw was co expressed with these reporters to investigate its effect on mitochondrial fusion. Please see Figure 4-8 part A for example images throughout the experiment. A decrease in spread of GFP signal post photoactivation is seen in $^{HA}$Boymaw expressing neurons, showing a decreased mitochondrial fusion. This can be noted from the decreased slope compared to control in Figure 4-8 part B (n = 17 control and 15 $^{HA}$Boymaw neurons, final normalised area is 55% less in the $^{HA}$Boymaw expressing neurons than the control condition). The comparable outcome of this and the Mitofusin2 dominant negative experiments support a decreased mitochondrial fusion rate upon expression of the DISC1-Boymaw fusion protein.

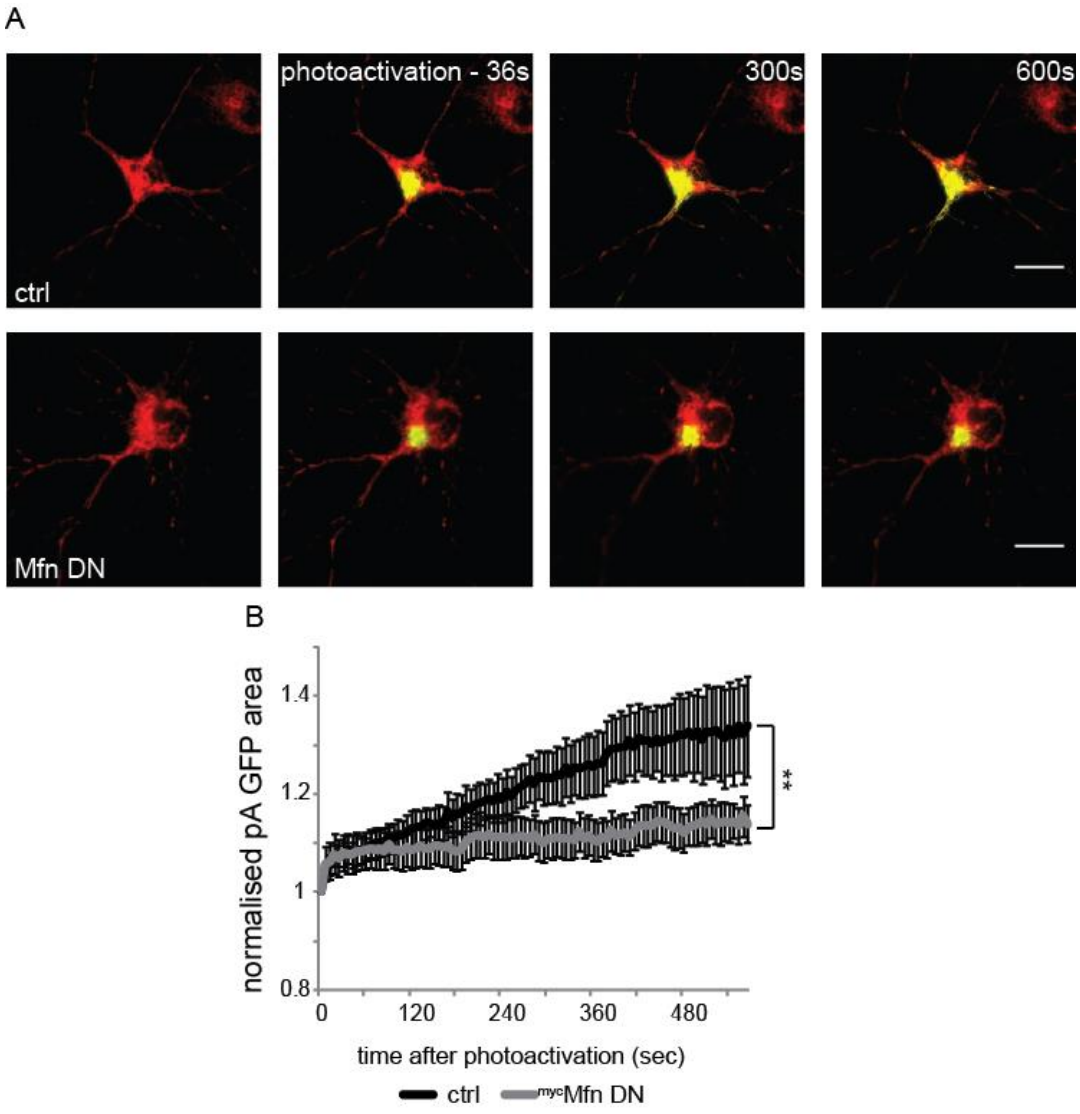


**Figure 4-7 Photoactivation experiment to measure mitochondrial fusion in neurons**

(A) shows representative images of neurons expressing MtDsRed2 and photoactivatable GFP (pAGFP) at time zero, immediately following photoactivation (36 s), 300 and 600 s post-photoactivation. (B) The change in area of GFP signal after photoactivation is decreased in Mfn DN expressing neurons (n = 6 control and 8 Mfn DN expressing neurons, final normalized area control = 1.31 ± 0.1, Mfn DN = 1.12 ± 0.04 AU, p=0.009 unpaired t-test). Scale bar is 20μm.

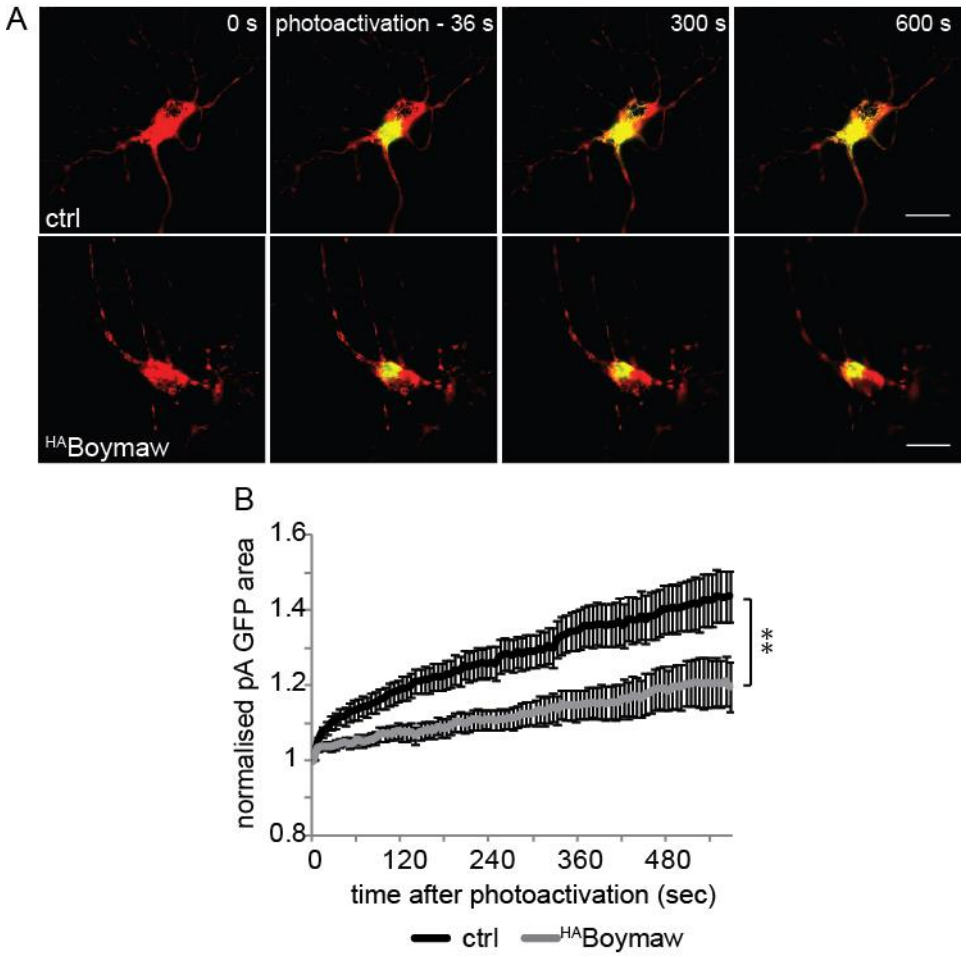


**Figure 4-8 The DISC1-Boymaw fusion protein decreases mitochondrial fusion in neurons**

(A) shows representative images of neurons expressing MtDsRed2 and photoactivatable GFP (pAGFP) at time zero, immediately following photoactivation (36 s), 300 and 600 s post-photoactivation. (B) The change in area of GFP signal after photoactivation is decreased in $^{HA}$Boymaw expressing neurons (n = 17 control and 15 Boymaw expressing neurons, final normalised area control = 1.44 ± 0.07, $^{HA}$Boymaw = 1.20 ± 0.07AU, p=0.02 unpaired T-test). Scale bar is 20μm.

### 4.2.5 The DISC1-Boymaw fusion protein decreases mitochondrial fusion in COS7 cells.

Whilst the photoactivation experiments in neurons are indicative of a decrease in mitochondrial fusion rate, this result could be partially attributable to the decreased mitochondrial transport reported in Figure 3-11 upon DISC1-Boymaw fusion protein expression. In order to overcome this, a second mitochondrial fusion assay was carried out in COS7 cells. In this assay, 2 populations of cells are transfected with different mitochondrially targeted fluorophores, co-cultured, and their respective plasma membranes fused with polyethylene glycol (PEG) 1500 treatment (Chen et al., 2003). Therefore, the two populations of mitochondria can then fuse with each other. Colocalisation analysis between each fluorophore reveals fusion rate. Please see 2.5 for further details and Figure 4-9 for a schematic demonstrating experimental set up.

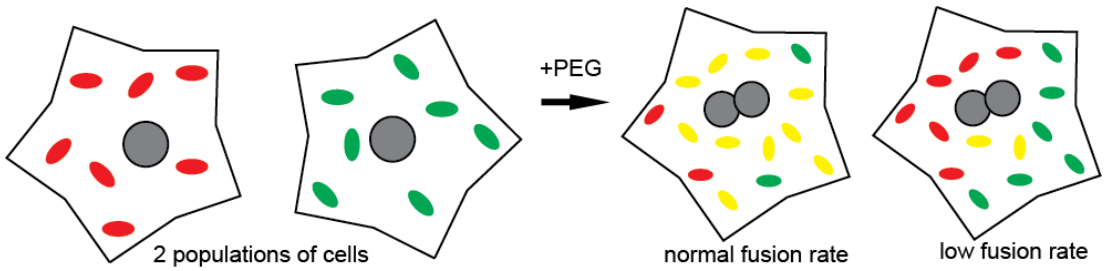


**Figure 4-9 PEG fusion assay schematic**

Schematic demonstrating a mitochondrial fusion assay carried out by PEG mediated-cell fusion. Cells are transfected with one of two mitochondrially targeted fluorophores with different spectra (e.g. MtDsRed2 and $Su9^{GFP}$) and cultured together for 24h to allow plasmid expression. Treatment with poly ethylene glycol fuses plasma membranes allowing mitochondria form two different cells to encounter each other and fuse. A decreased fusion rate is indicated by a lower population of mitochondria positive for both fluorophores.

The advantage of this assay over the photoactivation experiments detailed in 4.2.4 is that only one set of cells expresses the DISC1-Boymaw fusion protein. In the following experiment, $^{HA}$Boymaw was cotransfected with MtDsRed. The su9$^{GFP}$ expressing cells would, therefore, display normal mitochondrial transport. It would follow that any observed decrease in fusion would be less dependent on a decrease in trafficking than in the previous experiments. In this way fusion effects can be observed distinctly from trafficking events. Example cells are shown in Figure 4-10 A where fused mitochondria appear yellow. Quantification of the yellow signal, normalised to MtDsRed2 is shown in B.

Colocalisation analysis between red and green signals revealed a decrease in the percentage of red mitochondria also positive for green signal (yellow signal) upon overexpression of the DISC1-Boymaw fusion protein compared to control (n = 15 fused cells, control = 27% ± 5.2, $^{HA}$Boymaw = 6.4% ± 1.9). This is consistent with the DISC1-Boymaw fusion protein acting in a dominant negative manner for fusion as well as trafficking, as suggested by the decrease in mitochondrial length caused by both DISC1-Miro interacting domain and $^{HA}$Boymaw expression in Figure 4-1. Taken together, these data support a role for DISC1 in mitochondrial fusion as well as trafficking and, potentially, an impairment in this process associated with schizophrenia.

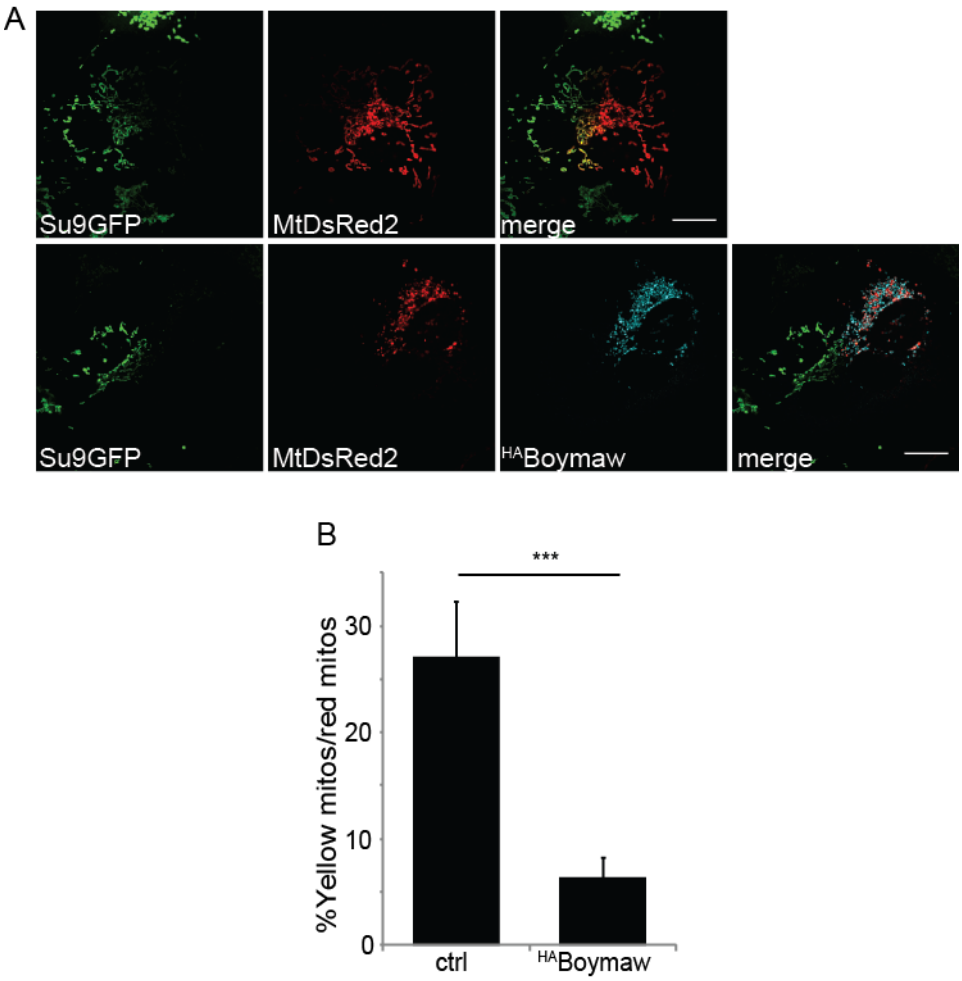


**Figure 4-10 The DISC1-Boymaw fusion protein decreases mitochondrial fusion in COS7 cells**

(A) $^{HA}$Boymaw decreases mitochondrial fusion in COS7 cells compared to control following polyethylene glycol treatment to induce plasma membrane fusion. (B) shows quantification of colocalisation between MtDsRed2 and Su9GFP signals, normalised to MtDsRed2 signal. (n = 15 post fusion cells from 3 individual preparations, p = 0.0009, unpaired T-test). Scale bar = 20μm.

### 4.2.6 The DISC1-Boymaw fusion protein decreases area of ER-mitochondria contacts

Contact sites between the ER and mitochondria are hubs for intracellular signalling. The yeast homologue of Miro (Gem1) is a component of ER mitochondria contact sites (Kornmann et al., 2011). Mitofusin2, has also been proposed to be required to form these tethers. Moreover the GTPase activity was reported to be necessary (de Brito and Scorrano, 2008). The DISC1 interaction with these putative contact site components prompted investigation into the effects of DISC1 and the DISC1-Boymaw fusion protein on ER-mitochondria interface. COS7 cells were used due to their extensive ER network. Cells expressed $Su9^{GFP}$ and $ER^{dsred}$ to label mitochondria and ER respectively, along with $^{HA}$DISC1 or $^{HA}$Boymaw. Confocal imaging with post acquisition deconvolution to improve resolution by reassigning out of focus light was carried out (Vonesch and Unser, 2008). This allows co-localisation analysis to measure contact site area. Representative volume renderings are shown in Figure 4-11 part A. Colocalisation was quantified by Mander's coefficient (as in (de Brito and Scorrano, 2008)) and are shown in the graph in Figure 4-11 B. Coefficients were 0.19 ± 0.04 for control, for $^{HA}$DISC; 0.18 ± 0.03, and $^{HA}$Boymaw; 0.15 ± 0.05. Whilst DISC1 overexpression itself had no effect on contact site area, DISC1-Boymaw fusion protein expression caused a significant decrease in this area compared to control. This would suggest uncoupling of these organelles and subsequently, impairments in intracellular signalling in these cells, for example a decrease in calcium transfer from ER to mitochondria, as a causative factor in schizophrenia.

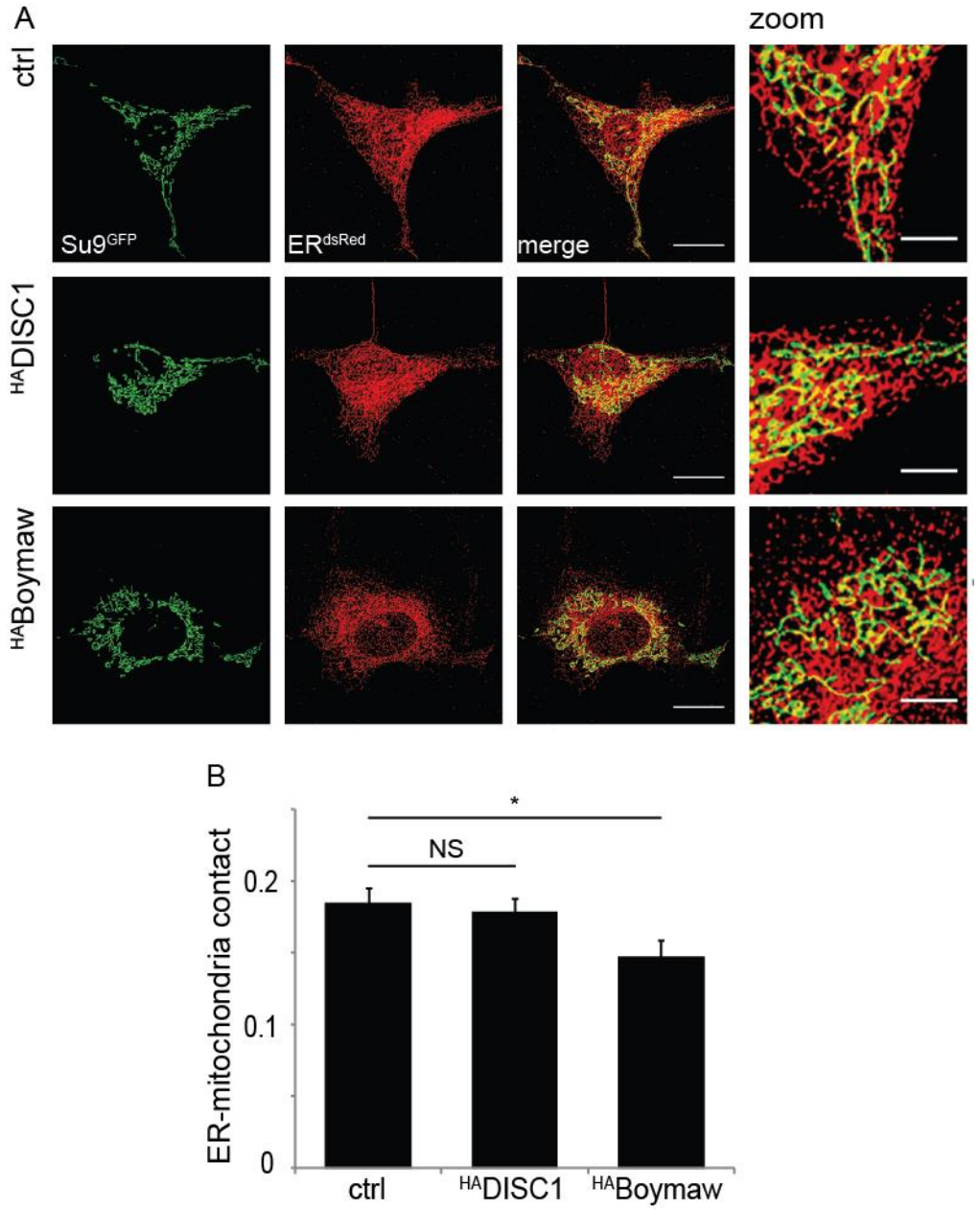


**Figure 4-11 The DISC1-Boymaw fusion protein decreases area of ER-mitochondria contacts**

(A) shows representative volume rendering of cells expressing ERDsRed and Su9GFP as a control, and with $^{HA}$DISC1 or $^{HA}$Boymaw overexpression. Scale bar = 20μm. Zoomed images show ER and mitochondria dense regions of example cells. Scale bar = 5 μm. (B) Mander's coefficients to measure colocalisation between ER and mitochondrial signal in ctrl vs Boymaw expressing cells p=0.03, ctrl vs

DISC and DISC vs Boymaw NS, n=15 cells per condition. One way ANOVA with Tukey's post hoc test.

In addition to investigating the area of the ER-mitochondria interface, it is also of great interest to identify components of these contact sites. To this end, the distribution of both DISC1 and the DISC1-Boymaw fusion protein in comparison to these sites was studied. To carry out these experiments, images of colocalised regions from cells shown in Figure 4-11 were generated to give images showing contact sites exclusively. Colocalisation analysis was carried out between these images and HA signal for DISC1, or the DISC1-Boymaw fusion protein. Representative cells and graph showing Manders colocalisation coefficients are shown in Figure 4-12. Despite the reduced ER-mitochondria interface, a greater fraction of $^{HA}$Boymaw signal was found at contact sites than of $^{HA}$DISC1 (Mander's coefficients; $^{HA}$DISC1=0.07 ± 0.01, $^{HA}$Boymaw=0.14 ± 0.03 p=0.03). In order to measure colocalisation by a second method based on signal intensity rather than on area alone, the Pearson's correlation coefficient was calculated. The Pearson's correlation coefficient between $^{HA}$DISC1 and contact sites is 0.062 ± 0.015, whilst that for the DISC1-Boymaw fusion protein is 0.173 ± 0.032. Both the Manders and Pearsons values are low, indicative of relatively low residence for DISC1 and the DISC1-Boymaw fusion protein at these sites. However, the DISC1-Boymaw fusion protein was consistently found to preferentially localise at these sites compared to wild-type DISC1. This can also be seen from the example image in Figure 4-12. This may indicate that the DISC1-Boymaw fusion protein acts to locally destabilise and decrease these contact sites.

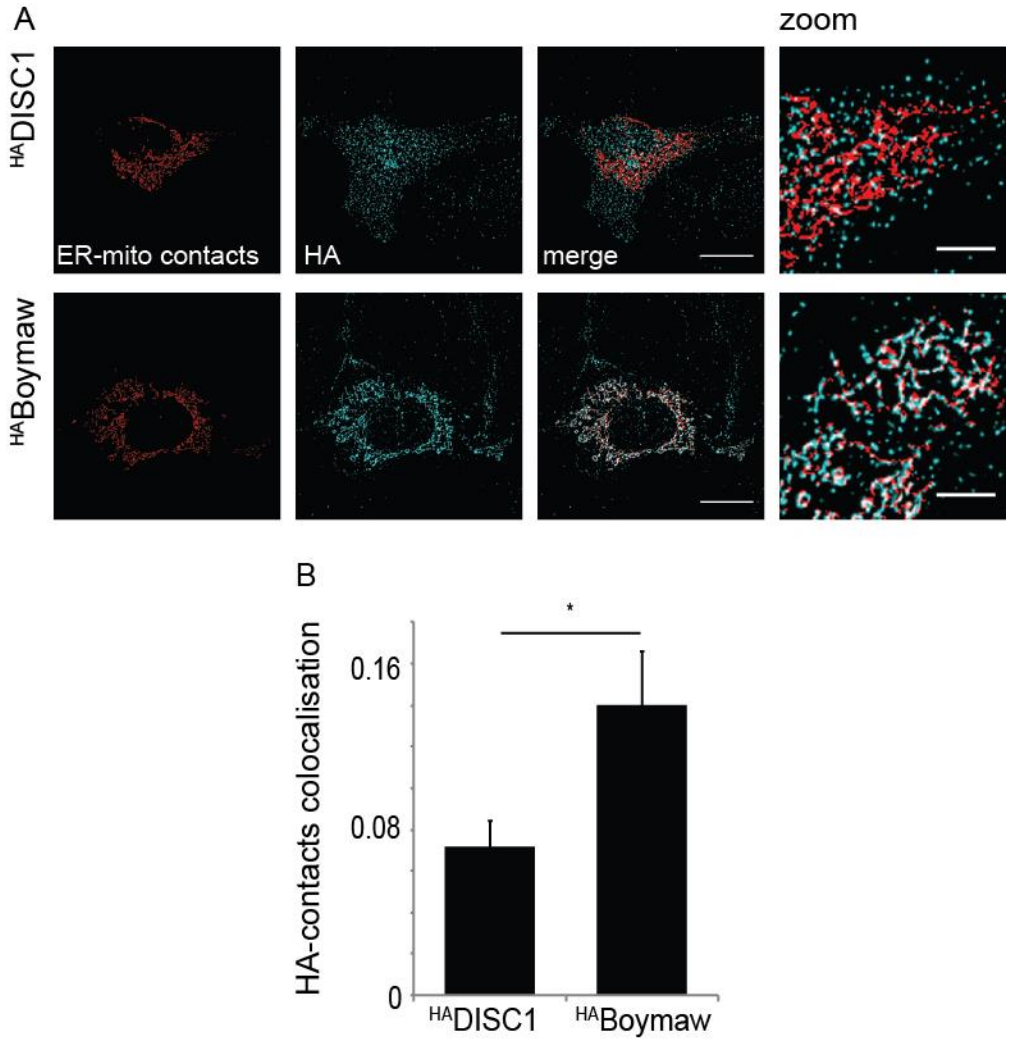


**Figure 4-12 The DISC1-Boymaw fusion protein shows a preferential localisation to ER-mitochondria contact sites over wild-type DISC1**

(A) Representative images of ER-mitochondria contact sites generated from colocalised regions in Figure 4-11 and distribution of wild-type $^{HA}$DISC1 or $^{HA}$Boymaw signals. Scale bar = 20µm. Zoomed images highlight distribution of HA signal in comparison to ER-mito contacts. Scale bar = 5µm. (B) Shows Mander's colocalisation coefficients between HA signal for $^{HA}$DISC1 or $^{HA}$DISC1-Boymaw fusion protein and contact sites, n= 15 cells per condition. $^{HA}$Boymaw residence is significantly higher than wild-type $^{HA}$DISC1, p=0.03, unpaired T-test.

### 4.2.7 DISC1 mediated mitochondrial trafficking is necessary for correct dendritic arborisation.

Both DISC1 and mitochondrial distribution have been linked to dendritic development (please see 1.5 on page 42 and 1.8.2 on page 53). The interaction between DISC1 and dendritically targeted TRAK2 prompted investigation into effects of disrupting DISC1-mediated mitochondrial dynamics on dendritic development. In order to do this, the DISC1-Miro interacting domain and the DISC1-Boymaw fusion protein (shown to act domninant negatively to inhibit mitochondrial trafficking in Figure 3-11) were transfected into hippocampal neurons with GFP to delineate neuronal morphology. Cells were transfected at DIV 7 and analysed at DIV 10. Two markers of complexity were analysed; average dendritic length and average numbers of dendritic branch points per cell. Representative neurons are shown in Figure 4-13.

Neurons expressing the DISC1-Miro interacting domain (DISC1 1-301) to disrupt mitochondrial trafficking showed a decreased dendritic complexity as shown by the representative neurons in Figure 4-13 part A. B shows the total dendritic length per cell was decreased 31% (control = 1669.4μm ± 99, 1-301 = 1148.6μm ± 88, n = 16 cells from 4 preparations) compared to control. Next Sholl analysis was performed to study whether dendrite arbor complexity differed as a function of distance from the soma in Figure 4-13 part C. First demonstrated in 1953, this involves quantification of intersections and branch points of dendrites within concentric circles of evenly increasing distances from the soma (Sholl, 1953). This showed the decrease in the number of intersections – a readout for dendritic length - to be pronounced at 80μm, and 100μm from the soma. Similar analysis was then carried out with the number of dendritic branch points per neuron. As with dendritic length, the number of branch points per cell decreased by 31% upon expression of the DISC1-Miro interacting domain compared to control (Figure 4-13D, E control = 16.6 ± 1.9, 1-301 = 11.5 ± 1.2), with the effect most noticeable at 90μm from the soma. These data show that DISC1-dependent mitochondrial dynamics are essential for normal dendritic development.

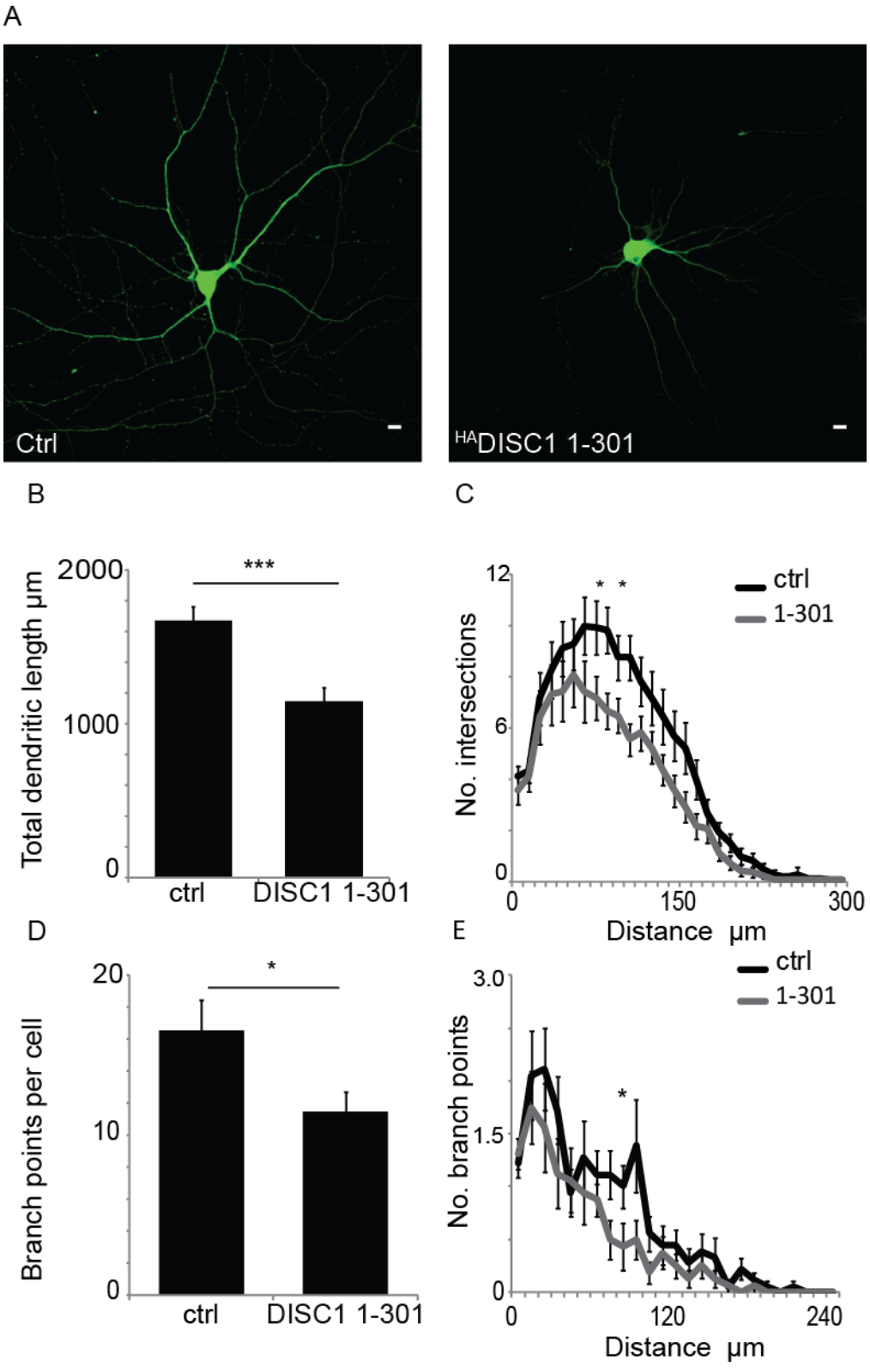


**Figure 4-13 DISC1 1-301 expression decreases dendritic complexity**

(A) Representative images showing control 10 DIV neurons and those expressing the DISC1 Miro binding domain (1-301). GFP was used to

visualize neuronal morphology. Scale bar = 10μm. (B) Total dendritic length per cell was decreased ($p = 0.001$, unpaired T-test), (C) Sholl analysis reveals a decrease in intersections to be significant at 80 and 100μm from the soma ($p < 0.05$, Two way repeated measures ANOVA) (D) Average number of branch points per cell is decreased with DISC1 1-301 expression ($p=0.04$ unpaired T-test). (E) Sholl analysis of branch points shows a decrease specifically at 90μm from the soma ($p < 0.05$, Two way repeated measures ANOVA) (n = 16 neurons from 4 preparations).

The schizophrenia associated DISC1-Boymaw fusion protein impairs mitochondrial dynamics in a comparable fashion to the DISC1 Miro interacting domain (1-301). Dendritic development is also reportedly interrupted in models of psychiatric disease (Kulkarni and Firestein, 2012). Thus it was of great interest to investigate the possibility that the DISC1-Boymaw fusion protein could also mimic the effect of the DISC1 Miro interacting domain in this respect. A decrease in dendritic complexity, and correspondingly, neuronal connectivity, could contribute to the onset of schizophrenic symptoms. Investigation into the impact of the DISC1-Boymaw fusion protein on dendritic development was carried out as for the DISC1 Miro interacting region. Please refer to Figure 4-14 for representative neurons and quantification.

Calculation of total dendritic length revealed a decrease of 35% compared to control, (Figure 4-14 ctrl = 1590.4 ± 142.7μm, Boymaw = 1033.3 ± 101.0μm, n = 15-16 neurons from 4 individual preparations). By Sholl analysis, a significant decrease in the number of dendritic intersections was noted between 50-80μm from the soma (see part C of Figure 4-14). As with dendritic length, the total number of branch points was decreased 33% upon expression of the fusion protein (see part D of Figure 4-14, ctrl = 16 ± 1.7, Boymaw = 10.7 ± 1.5). Concurrently, Sholl analysis revealed this decrease to be most obvious 50μm from the soma (part E). Taken together, these data demonstrate the expression of the DISC1-Boymaw fusion protein to have a significant negative impact on dendritic development, and show this effect to be linked to the impairment in mitochondrial dynamics. These findings provide further evidence for the importance of correct mitochondrial distribution in development and

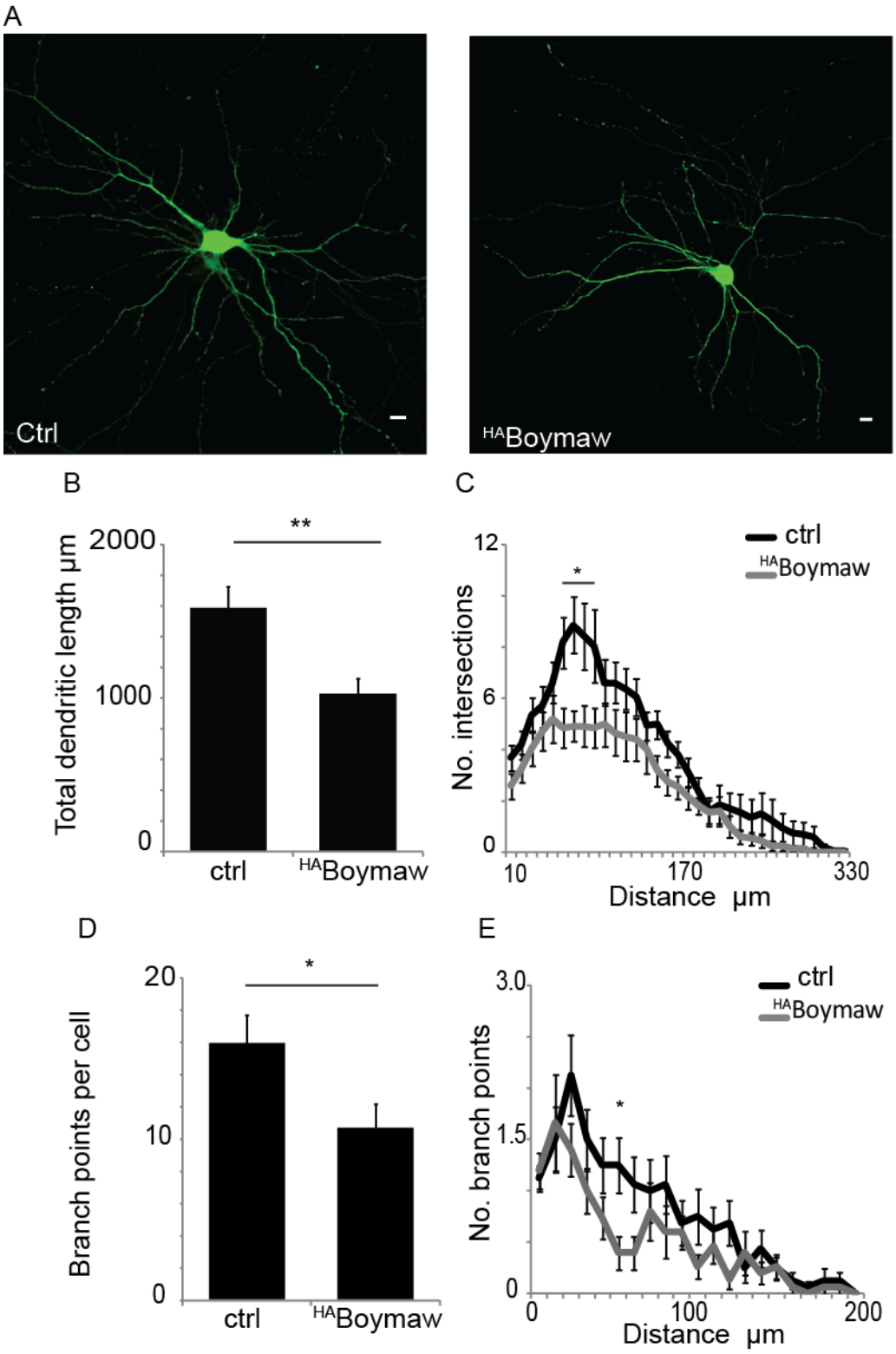


**Figure 4-14 DISC1-Boymaw fusion protein expression decreases dendritic complexity**

(A) Representative images showing control 10 DIV neurons expressing GFP in the presence or absence of $^{HA}$Boymaw. (B) Total dendritic

length is decreased when the DISC1-Boymaw fusion protein is expressed ($p = 0.004$, unpaired T-test). (C) Sholl analysis showing the number of intersections at 10μm intervals. A significant decrease in intersections in Boymaw expressing neurons is seen at a distance of 50-80μm from the ($p < 0.05$ at each point, Two way repeated measures ANOVA). (D) Analysis of branch points reveals expression of the DISC1-Boymaw fusion protein leads to a decrease in the number of branch points per cell ($p = 0.03$ unpaired T-test). (E) Sholl analysis reveals this decrease to be significant at 50μm from the soma ($n = 15$-16 neurons from 4 preparations $p < 0.05$, Two way repeated measures ANOVA).

maintenance of dendritic arbours. Furthermore, these data support a key role for DISC1 in this process, and further dissect the pathways through which this occurs.

## 4.3 Discussion

In this chapter, DISC1 has been implicated in the regulation of mitochondrial fusion in addition to trafficking. This has been shown by quantification of a decrease in mitochondrial length upon expression of DISC1 dominant negative or schizophrenia associated DISC1-Boymaw fusion protein expression. Further, the subcellular localisation of DISC1 is altered upon Mitofusin overexpression. DISC1 is recruited to mitochondria, redistributed from its diffuse distribution. This has been shown in two ways, initially by immunocytochemistry and supported by mitochondrial isolation assays, both from COS7 cells. These experiments show the Mitofusins to have a comparable effect on DISC1 as does Miro, and mark the Mitofusin proteins as novel anchors for DISC1 at the outer mitochondrial membrane. This is consistent with the Mitofusin proteins interacting with mitochondrial trafficking complex proteins Miro1 and 2 and TRAKs to regulate normal mitochondrial transport (Misko et al., 2010). Furthermore, DISC1 has been shown here to biochemically interact with both Mitofusin1 and Mitofusin2 in a heterologous expression system. The interaction between DISC1 and Mitofusin1 was confirmed by coimmunoprecipitation assay in rat brain lysates showing DISC1 to be part of a native complex with the fusion machinery. Further investigation in knock down or knock-out samples will be required to

determine the composition of this complex and necessity of each protein. For example, is the interaction between DISC1 and the Mitofusins dependent upon the presence of TRAK or Miro? Alternatively, is DISC1 necessary for the interaction between the transport and fusion complexes, perhaps acting as a scaffold allowing coordination of these processes? There are significant commonalities between Mitofusins and Miros. It is of note these mitochondrial interactors of DISC1 described in this and the previous chapter both contain GTPase domains. This raises the possibility that DISC1 could be regulating activity of these domains. This would likely be indirect, via regulation of a GTPase activating protein (GAP) or Guanine nucleotide exchange factor (GEF) as DISC1 is regarded as a scaffold. Further, both Mitofusins and Miro have been shown to recruit the E3 ubiquitin ligase Parkin to the mitochondria to effect clearance of damaged mitochondria by mitophagy (Birsa et al., 2014; Chen and Dorn, 2013). Therefore, DISC1 could be involved in regulating this initial step in mitophagy.

In addition to a biochemical interaction, this chapter demonstrates a functional effect of the DISC1-Boymaw fusion protein on mitochondrial fusion. Here it is shown that this construct acts to impede the rate of mitochondrial fusion. Experiments in both rodent neurons and COS7 cells support this conclusion and aim to distinguish between trafficking and fusion effects. These data raise the possibility that DISC1 not only acts to regulate normal mitochondrial transport, but also, mitochondrial fusion. It would follow that loss of normal fusion activity by expression of the DISC1-Boymaw fusion protein could be a causative factor in schizophrenia. Mitochondrial fusion is known to be necessary in development (Westermann, 2010), and mitochondrial fragmentation – indicative of loss of fusion activity – has been reported in multiple neurodegenerative disorders including AD and Charcot Marie Tooth 2A (Knott et al., 2008). Data presented here implicate impaired mitochondrial fusion in neuropsychiatric disorders as well. Balance of mitochondrial fission and fusion is known to be crucial for maintaining the integrity of the mitochondrial network via inheritance of mitochondrial DNA, and sequestration of damaged mitochondria for clearance (Westermann, 2010). The deficits in mitochondrial fusion presented here upon DISC1-Boymaw fusion protein expression provide a potential mechanism for the damage to mitochondria reported upon expression of chimaeric DISC1 transcripts (Eykelenboom et al., 2012). In this case, a decrease in mitochondrial membrane potential was detected upon comparison of mitotracker and cytochrome C signals. This accumulation of

damaged mitochondria could be due to impaired clearance of these organelles, implicating a role for DISC1 in mitochondrial turnover.

As well as mitochondrial fusion, data presented in this chapter propose a role for DISC1 in maintenance of associations between the endoplasmic reticulum and mitochondria. This has been shown by co-localisation analysis of lumenal fluorophores localised in the ER or mitochondria. Whilst expression of wild-type DISC1 itself had no effect on contact area, expression of the DISC1-Boymaw fusion protein led to a significant decrease in contact area. This effect could be due to a loss of Mitofusin activity suggested by data in sections 4.2.4 and 4.2.5. Whilst the necessity of Mitofusin2 fusion activity for ER mitochondria contacts is now controversial (Cosson et al., 2012; Filadi et al., 2015), the decreased area could be indirectly related and due to altered mitochondrial morphology described in Figure 4-1 via lower fusion activity. Analysis of DISC1 distribution in comparison to ER-mitochondria contact sites did not reveal strong evidence for DISC1 as a resident at these sites, therefore, it is unlikely that DISC1 could act as a tether to locally stabilise these microdomains. In contrast, The DISC1-Boymaw fusion protein seemed to be preferentially localised to these sites, despite their lower area in these cells. This is consistent with the mitochondrial localisation reported in (Figure 3-10).

The decrease in contact area between the endoplasmic reticulum and the mitochondria would imply a decrease in inter-organellar communication. This could manifest in terms of decreased lipid exchange, but in the case of DISC1, an impairment in calcium transfer would be more probable. Investigation into calcium levels and buffering capacity of mitochondria in cells expressing the DISC1-Boymaw fusion protein would be needed to address this point. DISC1 has been previously linked to mitochondrial calcium uptake via interaction with Mitofilin (localised to the inner mitochondrial membrane) (Park et al., 2010b). In these assays knockdown of either protein resulted in abnormal calcium buffering capabilities of mitochondria upon challenging the cells with ionomycin (a calcium ionophore). Further, DISC1 has been shown to regulate ER calcium dynamics via $IP_3$ receptors – the first described route of calcium transfer from ER to mitochondria. Knockdown of DISC1 resulted in increased calcium release from the ER upon stimulation with ATP in both cell lines and hippocampal neurons from a DISC1 deficient mouse (Park et al., 2015). These findings posit DISC1 as a regulator

of neuronal calcium signalling. Additionally, the ER release deficit could be rescued by treatment of cells with antipsychotic drugs clozapine and haloperidol (Park et al., 2015). Thus, impaired calcium dynamics, potentially via aberrant ER-mitochondria associations, could contribute to the abnormal neuronal signalling associated with psychiatric disease.

Whilst intramitochondrial calcium concentration has been linked to mitochondrial transport as well as function, it is unlikely these two factors are acting in concert in the case of the DISC1-Boymaw fusion protein. Mitochondrial calcium levels and trafficking are inversely correlated. Studies carried out in axons of mouse hippocampal cultures employed a mitochondrially targeted calcium sensor, in conjunction with mitoRFP to label mitochondria, and revealed stationary mitochondria to display increased calcium levels in comparison to motile mitochondria. Further, of the moving mitochondria, the slower moving ones had a higher matrix calcium concentration (Chang et al., 2011). Thus, the decrease in mitochondrial motility presented in the previous chapter is unlikely to be caused directly by a potential decrease in mitochondrial calcium, rather via indirect signalling pathways. A possible example here would be impaired mitophagy via a lesser available area for formation of autophagosome membranes as described in the introduction to this chapter. This would lead to the previously demonstrated accumulation of damaged mitochondria (Eykelenboom et al., 2012) which are hypothesised to be less motile than their healthy counterparts (Miller and Sheetz, 2004; Wang et al., 2011c).

Finally in this chapter, DISC1-dependent mitochondrial dynamics have been linked to dendritic development – a process impaired in psychiatric disease. Impeding mitochondrial trafficking via expression of either the DISC1 dominant negative caused a decrease in dendritic complexity. This was manifested both in terms of dendritic length and dendritic branching. The effects were consistent upon expression of the DISC1-Boymaw fusion protein (please refer to Figure 4-13 and Figure 4-14). Thus, the loss of dendritic complexity in this system is likely due to impairments in mitochondrial dynamics, rather than a gain of function of the novel transcript. The data presented here show a possible mechanism by which DISC1 could be mediating dendrite outgrowth. This mitochondrial transport dependent effect of DISC1 may also be applicable to its role in FEZ1 mediated dendrite outgrowth as indicated in section

1.8.2. FEZ1 is a kinesin adaptor and is implicated in mitochondrial trafficking (MacAskill and Kittler, 2010). DISC1 could act to locally regulate this complex in addition to Miro mediated mitochondrial trafficking and contribute to neurite outgrowth in this way.

Abnormal mitochondrial distribution due to decreased trafficking could contribute to impaired dendritogenesis in multiple ways. Firstly, local energy supply for development and maintenance of dendrites could be impaired. This could be direct or via a decreased provision of energy for other factors. For example, synaptic activity, an energy costly process known to stabilise dendrites (Puram and Bonni, 2013). Further, a decreased local energy supply could contribute to lesser local protein synthesis and turnover or a decrease in trafficking and distribution of other organelles. Both endoplasmic reticulum and Golgi have been deemed essential for dendrite outgrowth (Cui-Wang et al., 2012; Horton et al., 2005). The data presented in this chapter, in conjunction with these previously reported findings suggests collaboration between calcium transfer at ER-mitochondria contact sites and dendritic development or stabilisation could be a promising avenue for further exploration.

The decrease in dendritic arbours could represent a disease mechanism in the pathology of schizophrenia and related psychiatric disease. The loss of the normal structure of the dendritic arbour could contribute to the onset of schizophrenic symptoms via decreased neuronal connectivity. The lesser complexity would decrease the area available to form stable synapses. Further experiments to test this may involve immunocytochemistry for synaptic markers and quantifying numbers or areas of synapses as indicated by this staining. Therefore, network activity, and subsequently, information processing would be negatively impacted. This deficit could contribute to the pathogenesis of schizophrenic symptoms. Indeed, a recent mouse model which expresses the DISC1-Boymaw fusion protein shows cognitive defects consistent with major mental illness (Ji et al., 2014). Experiments from neuronal cultures or organotypic slices from these mice could address the possibility that mitochondrial dynamics and dendrite development are impaired in this more intact system. Further, electrophysiological studies could address any correlated impairments in neuronal activity.

# Chapter 5
# Investigating Mitochondrial Dynamics in Differentiated Human Neurons

## 5.1 Introduction

In previous chapters, mitochondrial dynamics have been investigated in rodent hippocampal neurons. In this chapter, these findings will be extended to studies in differentiated human neurons and similarities and differences between mitochondrial trafficking in these two culture systems will be addressed. The differentiated human neurons were derived indirectly from patient fibroblasts. The fibroblasts were first reprogrammed into iPSCs – induced pluripotent stem cells. The generation of iPSCs from patient fibroblasts is a Nobel prize winning technology. Fibroblasts or other somatic cells harbouring a mutation, or mutations, of interest can be reprogrammed to take on a pluripotent state. This is defined as the ability to differentiate into almost any cell type. Acquisition of this state can be achieved by retroviral transduction of four factors. This was simultaneously demonstrated in human cells by two groups; one using a C myc, SOX2, Klf4 and Oct3/4 cocktail (Takahashi et al., 2007), and the other, Lin28, NANOG, Oct3/4 and SOX2 (Yu et al., 2007).

This approach aims to advance mechanisms derived from studies in model systems towards the situation in human patients. In the case of neurological disease, patient samples are available only post mortem, and so, studies of early phases of disease are not possible. Indeed, in the study of neurodegenerative disease, the affected cell types have been lost (Dolmetsch and Geschwind, 2011). Moreover, live imaging studies are not possible in these samples. Differentiation of iPSCs holds the enormous advantage

of being able to study disease causing mutations in the relevant cells (Yamanaka and Blau, 2010). Rodent models are not always sufficient in this respect; for example dopaminergic neurons can be generated from iPSCs to study Parkinson's disease, but cultures of this type of neurons are not feasible from rodents. Further, the prefrontal and temporal cortices of rodents are less complex than in humans, marking these structures as insufficient to accurately model neurological disease (Dolmetsch and Geschwind, 2011). Moreover, iPSC-derived neurons allow the study of disease causing mutations at relevant expression levels – without reliance on overexpression or knock-in models. This factor is important for work presented so far in this thesis which has been dependent on overexpression.

Crucially, onset of multiple neurological diseases is caused by unique combinations of genetic variation rather than mutations in single genes, thus modelling this aspect of disease is not practical in transgenic models. Examples of these diseases include sporadic Alzheimer's disease, and major mental illnesses including schizophrenia. By using iPSCs and derived neurons, it is possible to model diseases according to phenotype. For example, Young and colleagues generated iPSCs and, subsequently, neurons, from fibroblasts of sporadic Alzheimer's patients to investigate effects of differing SORL1 genotypes (an endocytic trafficking factor involved in APP processing, please see Figure 5-8). They found variation in expression levels of SORL1 according to genotype – haplotypes conferring protection from Alzheimer's had higher expression levels than haplotypes conferring risk for AD. These expression levels correlated with amyloid beta generation (Young et al., 2015). Further, iPSCs from schizophrenic patients have been generated and investigated independent of genotype. In neurons derived from these cells, a decrease in neurite length and synapse density was reported in schizophrenic samples compared with wild-type. This corresponded to a decrease in neuronal connectivity (Brennand et al., 2011). Microarray and proteomic data from these cells revealed potential differences in cAMP, WNT and glutamate receptor signalling, consistent with previously proposed phenotypes for schizophrenia. Gene expression profiles indicated these cells were most similar to human foetal brain tissue, thus these cells can accurately model early development of human disease (Brennand et al., 2015; Brennand et al., 2011). Additionally, there is a great interest in this technology to generate transplantable cells for regenerative medicine and for high throughout compound screening during drug

development. This is of exceptional importance in neurological diseases as neurons are post mitotic and so, primary cultures do not expand as do other cell types (Dolmetsch and Geschwind, 2011).

There are key disadvantages to this technology. They lie in the cost of maintaining stem cells and the tendency of these cells to spontaneously differentiate, giving rise to heterogenous cultures with a decreased differentiation efficiency and homogeneity. To overcome this for neuronal culture, a novel method to generate an intermediate stable population of neural progenitor cells (NPCs) from iPSCs was recently described. This population has been derived using small molecules and has a higher efficiency than retroviral transduction based methods where each cell can express different levels of each gene, or not receive the full cocktail. Therefore, the population is more homogeneous than if derived by integrating methods. These cells expand immortally, marking them as good candidates for repeated culture, and, crucially, can be differentiated into human neurons more quickly than can iPSCs – over a time frame of 1-2 weeks in comparison with 6 weeks or more. These NPCs have been confirmed to undergo directed differentiation into dopaminergic neurons and motor neurons – examples of ventral and posterior CNS lineages respectively. Immunocytochemistry and qPCR was performed to determine expression levels of specific markers, e.g. tyrosine hydroxylase (dopaminergic neuron marker) or choline acetyl transferase (motor neuron marker). Further, the derived dopaminergic neurons have been characterised elctrophysiologically. These cells were shown to have a membrane potential of -35mV and to generate spontaneous action potentials with similar kinetics to those of human neurons (Reinhardt et al., 2013). Therefore, these cells appear to rival neurons derived from iPSCs in physiological relevance, with the advantage of more efficient culture methods.

In this chapter, the novel system of neural progenitor derived human neurons will be harnessed to examine the effects of pathogenic mutations in DISC1 and Presenilin1. The efficiency of the differentiation paradigm will be assessed for individual cell lines via immunocytochemistry for neuronal markers. Mitochondrial transport in these human derived neurons will be assayed by live imaging. Results will be compared to outcomes of comparable assays employing rodent hippocampal neurons as in previous

chapters. The mutations to be studied are a 4 base pair deletion in DISC1, causing a frameshift mutation and a point mutation in Presenilin1 (A246E).

This DISC1 mutation was first described in 2005 (Sachs et al., 2005), and is further described in Figure 5-1, but has yet to undergo extensive functional study. However, iPSCs from patients harbouring this mutation have been generated (Chiang et al., 2011), representing the first iPSC lines from schizophrenia patients. Further, glutamatergic neurons differentiated from these cultures display decreased synaptic vesicle glycoprotein (SV2) puncta number – indicative of fewer mature presynaptic vesicles. Consistent with this, spontaneous currents in mutant DISC1 cells were less frequent, though of comparable amplitude, to controls. This is consistent with the DISC1 4bp deletion causing a presynaptic deficit (Wen et al., 2014).

The Presenilin mutation A246E was first described with the original discovery of Presenilin1 as a genetic risk factor for Alzheimer's disease – the most common neurodegenerative disease (Sherrington et al., 1995). Presenilin has since been shown to be an aspartyl protease with numerous substrates. The most renowned, APP, is a transmembrane protein which can be cleaved by beta secretase (BACE1) and the gamma secretase complex (containing Presenilin1, 2 and nicastrin among other factors). Dependent upon the sites of proteolysis of APP, this cleavage can generate amyloid beta peptides. These are mostly comprised of 40 or 42 amino acids, with 42 amino acids being the more pathogenic species. The exact mechanisms contributing to spine loss and cell death have yet to be determined, but increased amyloid beta levels give rise to disrupted calcium homeostasis and excessive reactive oxygen species production, contributing to spine loss and apoptosis (Pozueta et al., 2013).

## 5.2 Results

### 5.2.1 A four base pair deletion in DISC1 perturbs mitochondrial dynamics in rodent hippocampal neurons.

As described in Chapter 3, multiple mutations in DISC1 confer a decrease in mitochondrial trafficking. These experiments will investigate the effects of a 4 base pair deletion in the DISC1 gene on mitochondrial trafficking. This 4 base pair deletion (4bp deletion from here on) has been described in an American kindred with a high incidence of schizophrenia and schizoaffective disorder – which manifests as schizophrenia and a mood disorder such as depression or bipolar disorder. Please refer to Figure 5-1 for an illustration of the affected region of the DISC1 locus. The 4bp deletion causes a frameshift, giving rise to a transcript lacking 52 amino acids at the DISC1 C-terminus. 9 novel amino acids are fused to the C-terminus. This interruption of the DISC1 C-terminus is similar, although less severe, than in the case of the DISC1-Boymaw fusion protein. This transcript was shown in chapter Chapter 3 to mimic a loss of function by behaving as does the DISC1 dominant negative to decrease mitochondrial motility.

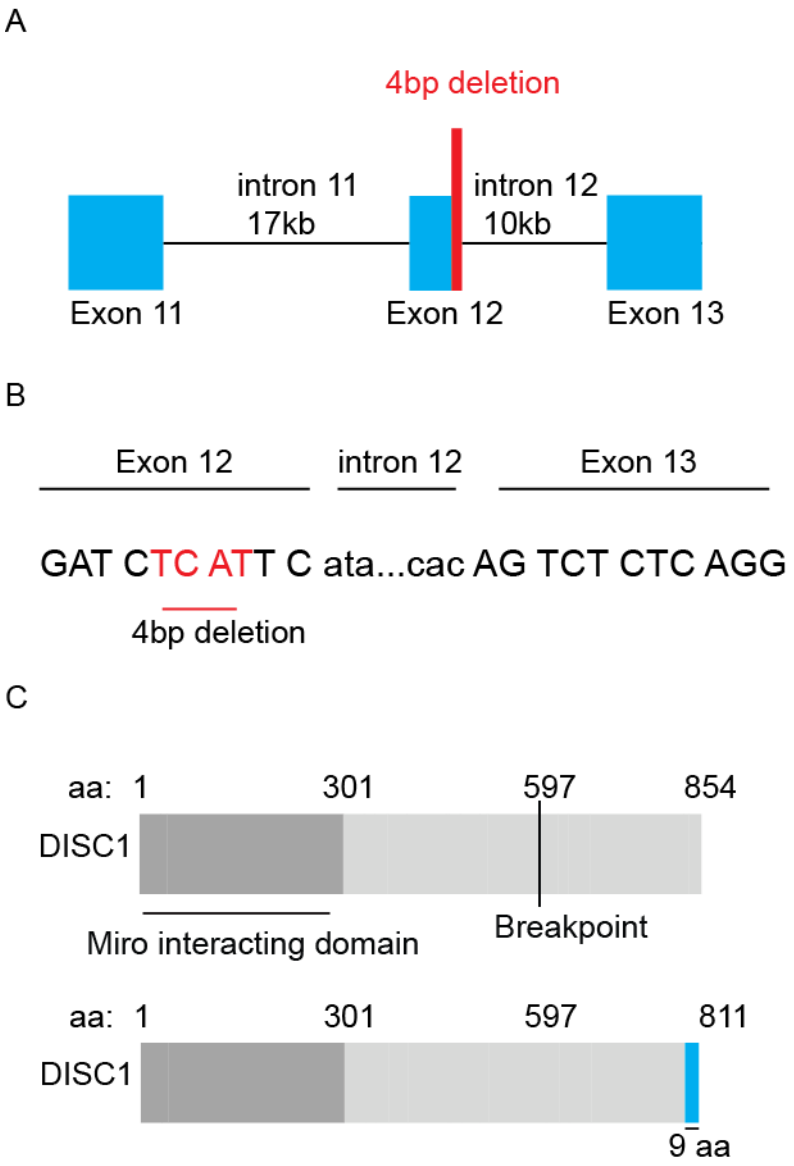


**Figure 5-1 A 4bp deletion in DISC1 in an American kindred**

(A) Shows the site of the four base pair deletion in the DISC1 locus. (B) Shows DNA sequence at the site. The bases highlighted in red are deleted in one allele of members of an American kindred. (C) Shows a schematic of wild-type DISC1 (top) and the novel transcript generated by the frameshift mutation. Adapted from (Wen et al., 2014).

The similarities between the DISC1-Boymaw fusion protein and the DISC1 4bp deletion prompted investigation into any effects on mitochondrial trafficking with this mutation. To this end, a construct encoding the sequence generated by the DISC1 4bp deletion, carrying an HA tag, was overexpressed in rat hippocampal neurons. As previously, MtDsRed2 was used to label mitochondria and single transfection of this construct formed the control condition. Initially, immunocytochemistry experiments were carried out to confirm expression and investigate localisation of this transcript. As seen in Figure 5-2, the DISC1 4bp deletion shows a partial colocalisation with mitochondria. This is demonstrated by the covariance in the signals in the line scan. However, it is notable that the DISC1 4bp deletion is also present throughout the processes and not exclusive to mitochondria. This distribution is comparable with that described for wild-type DISC1 (Ogawa et al., 2014) and contrasting to that described for the DISC1-Boymaw fusion protein in Chapter 3. Trafficking assays demonstrated that, in comparison to the control cells, those expressing the DISC1 4bp deletion displayed a significantly decreased fraction of motile mitochondria (ctrl = 16.8% motile ± 2.1, 4bp deletion = 9.8% motile ± 1.9, a 40% loss of moving mitochondria). Figure 5-3 shows representative kymographs and quantification of moving mitochondria. Therefore, the DISC1 4bp deletion hinders mitochondrial trafficking in this overexpression system, consistent with data presented in Chapter 3 regarding the DISC1 1-301 region and the DISC1-Boymaw fusion protein.

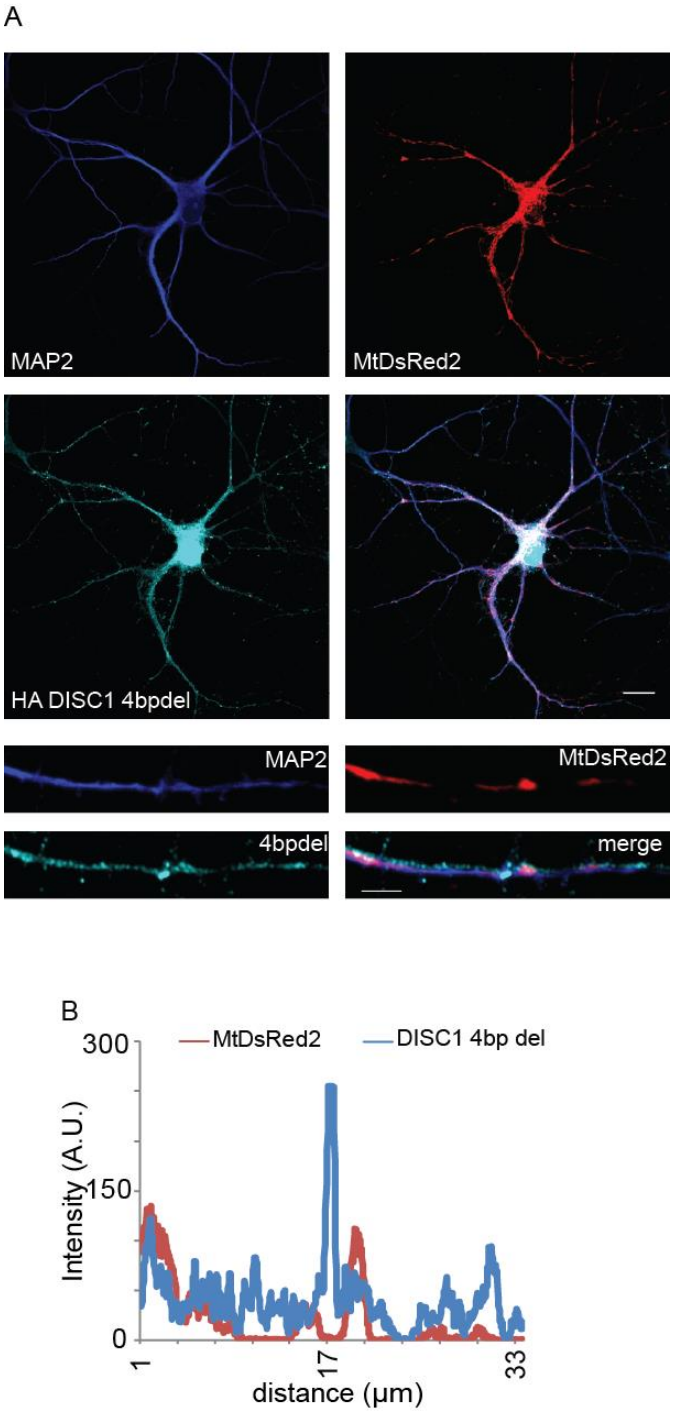


**Figure 5-2 DISC1 4bp deletion partially localises to mitochondria in processes of rodent hippocampal neurons**

(A) Immunocytochemistry in 10DIV hippocampal neurons showing localisation of the DISC1 4bp deletion. Scale bar = 20µm, 5 µm on zoomed image. (B) Line scan of the zoomed process showing a partial colocalisation between mitochondrial signal and HA staining for the DISC1 4bp deletion.

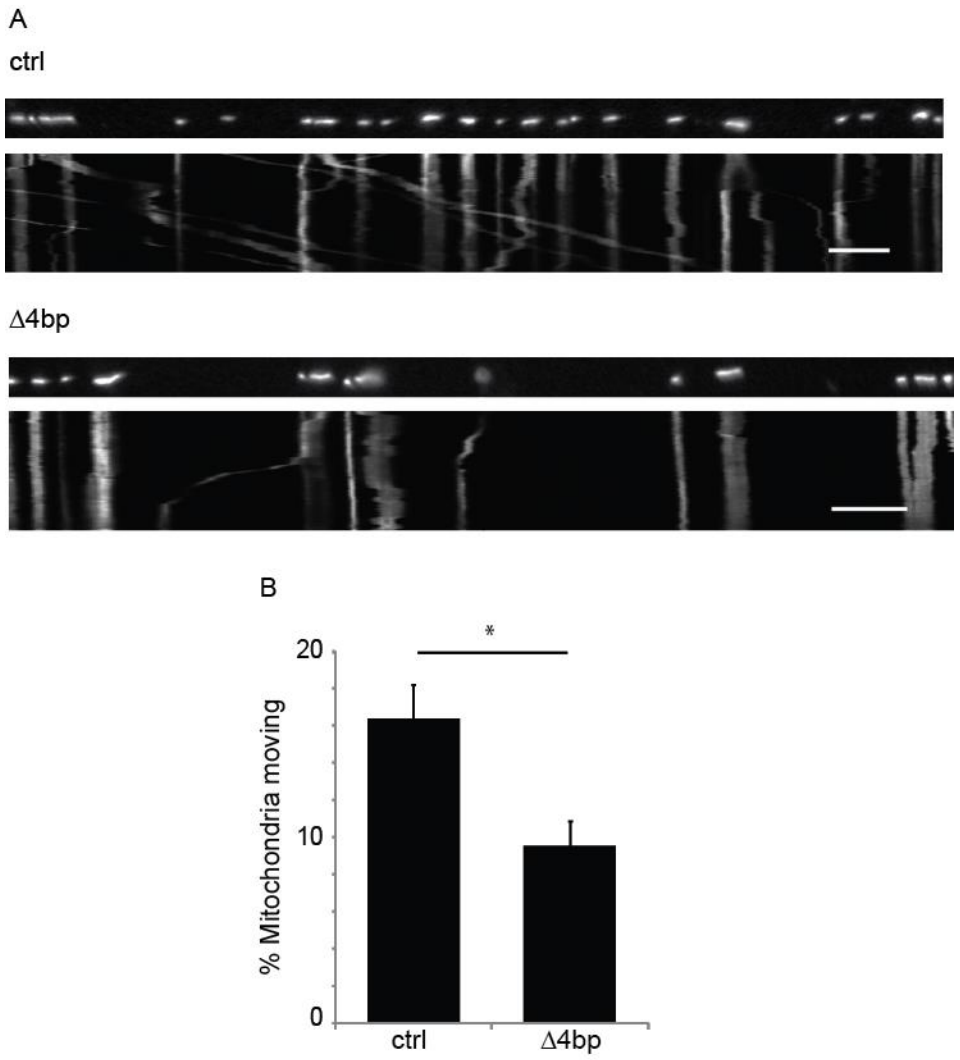


**Figure 5-3 A 4 base pair deletion in DISC1 impairs mitochondrial trafficking in rodent hippocampal neurons**

(A) Shows representative kymographs depicting mitochondrial trafficking in axons of rodent hippocampal neurons expressing MtDsRed2 (ctrl) or coexpressing a construct encoding the transcript generated by the DISC1 four base pair deletion. Scale bars 10μm (B) Shows pooled data from axons and dendrites quantifying percentages of moving mitochondria. There is a significant decrease upon expression of the DISC1 four base pair deletion (p=0.02, unpaired T-test. n= 22-24 cells from 3 preparations).

### 5.2.2 Differentiation of patient derived DISC1 neural progenitor cells into glutamatergic neurons.

In order to more thoroughly investigate the prospect that the DISC1 4bp deletion might impair mitochondrial dynamics, trafficking assays were to be carried out in differentiated neurons. This system carries numerous advantages as detailed in the introduction of this chapter. Of note, these cells are proposed to express the DISC1 mutation at physiological levels. Consequently, patient derived neural progenitor cells were differentiated into glutamatergic neurons. For a description of each of the lines used here please see Table 5-1. A control line has been derived from fibroblasts from an individual unrelated to the cohort carrying the DISC1 4bp deletion (C1-1). Two patient derived lines have been generated from fibroblasts of two individuals carrying the 4bp deletion – D3-1 and D3-2. Additionally, two control lines have been derived from the D3-2 line. Zinc finger nuclease technology has been utilised to correct the mutation in Z1. Thus the genotype of these cells has gone from heterozygous for the DISC1 4bp deletion to wild-type. This method is a gold standard control – any difference in phenotype between these two lines is due to the genotype rather than an artefact of inconsistencies in the reprogramming process. Z3 is derived from D3-2 in a similar way, but the 4bp deletion has been introduced into the other (wild-type) DISC1 allele. This line, in conjunction with D3-2 and Z1, can give an indication of a dose dependency of the DISC1 mutation on any observed phenotype.

In order to investigate differentiation efficiency, expression of neuronal markers was assayed by fluorescence microscopy. The protocol for this differentiation is detailed in 2.3.3. Neurons were fixed and immunocytochemistry was carried out to investigate the numbers of cells in each culture expressing neuron specific markers Beta III tubulin, MAP2, TAU and glutamatergic marker vGlut2. Each of these proteins is expressed throughout the brain, rather than specific to any region. Beta III tubulin is a neuron specific component of the microtubule skeleton. MAP2 and TAU are neuron specific microtubule associated proteins. In rodent hippocampal cultures they are targeted to dendrites and axons respectively. Therefore, these proteins serve not only as markers for these compartments, but indicate polarity of a neuron. vGlut2 is a vesicular glutamate transporter responsible for loading glutamate into presynaptic vesicles. Thus this protein acts as a marker of the presynapse. DAPI was used to stain

the nucleus and is not neuronal specific. Instead, this will label every cell in the culture to give the total number of cells. Calculation of the percentage of cells expressing each of these markers (compared to the DAPI signal) gives a readout for differentiation efficiency. Please see Figure 5-4 for representative images of each cell line and Table 5-2 for values of cells positive for each of these markers. Values are taken from 10 images from 2 experiments. The majority of the cells in each culture were positive for both βIII tubulin and vGlut2 as is evident from the graphs. Therefore the differentiation paradigm has been successful. Further, whilst a slight variation can be seen between each line, no significant differences are observed correlating with genotype. Therefore, this DISC1 mutation does not impair differentiation of these neural progenitors into neurons and so these lines can be readily compared in downstream experiments.

| Line | Derivation | Genotype | Notes |
|---|---|---|---|
| C1-1 | Control fibroblasts | WT | |
| D3-1 | Schz patient fibroblasts | 4bp deletion HETEROZYGOUS | |
| D3-2 | Schz patient fibroblasts | 4bp deletion HETEROZYGOUS | |
| Z1 | Isogenic correction of D3-2 | WT | DISC1 4 bp deletion corrected to give two copies of WT allele |
| Z3 | Isogenic introduction of 4bp deletion into WT DISC1 allele of D3-2 | 4 bp deletion HOMOZYGOUS | DISC1 4 bp deletion introduced into WT DISC1 allele. Thus, both DISC1 alleles harbour the 4 bp deletion |

**Table 5-1 Description of DISC1 cell lines used**

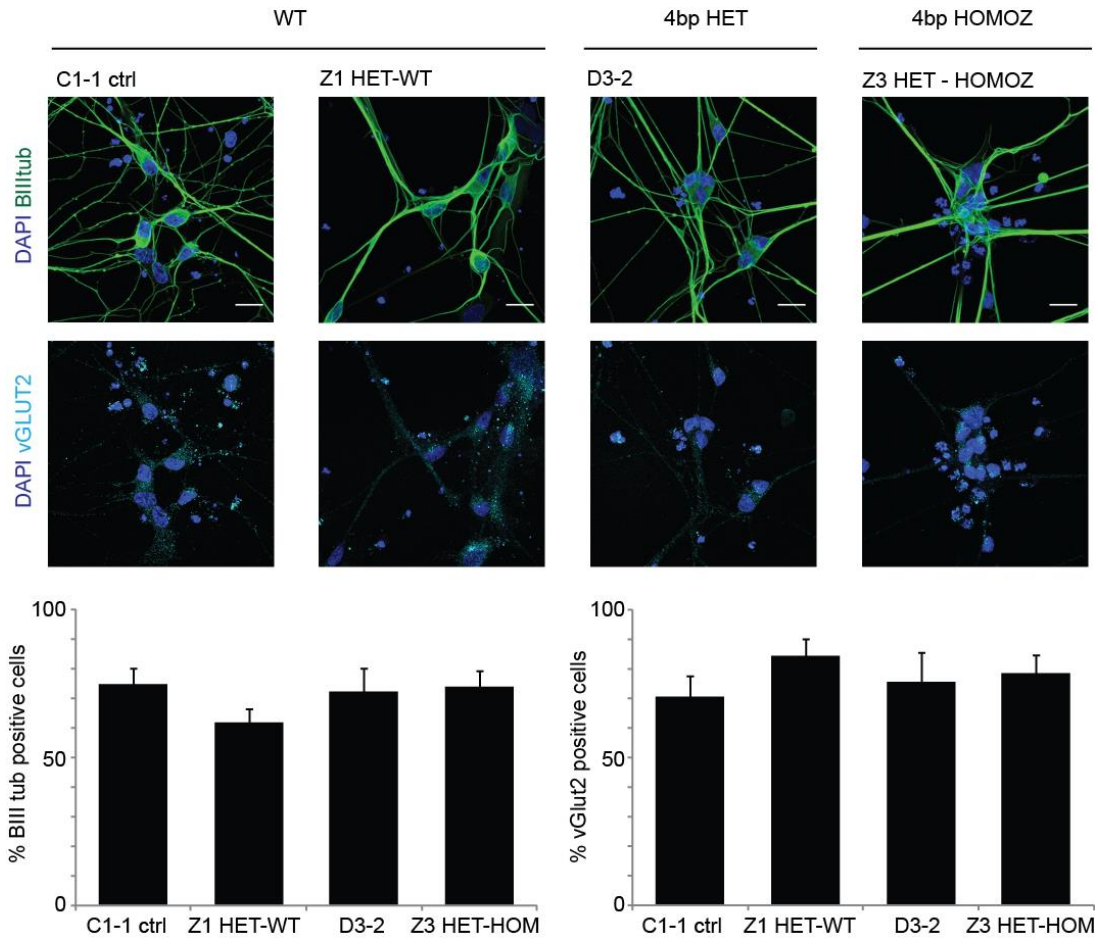


**Figure 5-4 Characterising differentiation of DISC1 mutant neural progenitors into glutamatergic neurons**

Neural progenitors were differentiated into neurons, fixed and stained for neuronal marker βIII tubulin and for glutamatergic neuronal marker vGlut2. Panels show merged images of DAPI and βIII tubulin (green) or vGlut2 (cyan). Scale bar = 20μm. Graphs show percentage of DAPI positive cells that also express Beta III tubulin or vGlut2. Values are calculated from 10 images from 2 independent differentiations.

**Table 5-2 Quantifying differentiation efficiency of DISC1 mutant lines; Beta III tubulin and vGlut2**

| Line | % cells per image positive for Beta III tubulin | % cells per image positive for vGlut2 |
|---|---|---|
| C1-1 | 75.5 ± 5.5 | 70.5 ± 7.1 |
| Z1 | 61.7 ± 4.6 | 84.4 ± 5.8 |
| D3-2 | 72.2 ± 7.9 | 75.4 ± 10.1 |
| Z3 | 73.9 ± 5.5 | 78.5 ± 6.0 |

**Table 5-3 Quantifying differentiation efficiency of DISC1 mutant lines; MAP2 and TAU**

| Line | % cells per image positive for MAP2 | % cells per image positive for TAU |
|---|---|---|
| C1-1 | 83.3 ± 5.5 | 83.3 ± 5.5 |
| Z1 | 84.6 ± 7.1 | 84.6 ± 7.1 |
| D3-2 | 88.9 ± 5.3 | 90.4 ± 4.2 |
| Z3 | 77.0 ± 3.9 | 77.0 ± 3.9 |

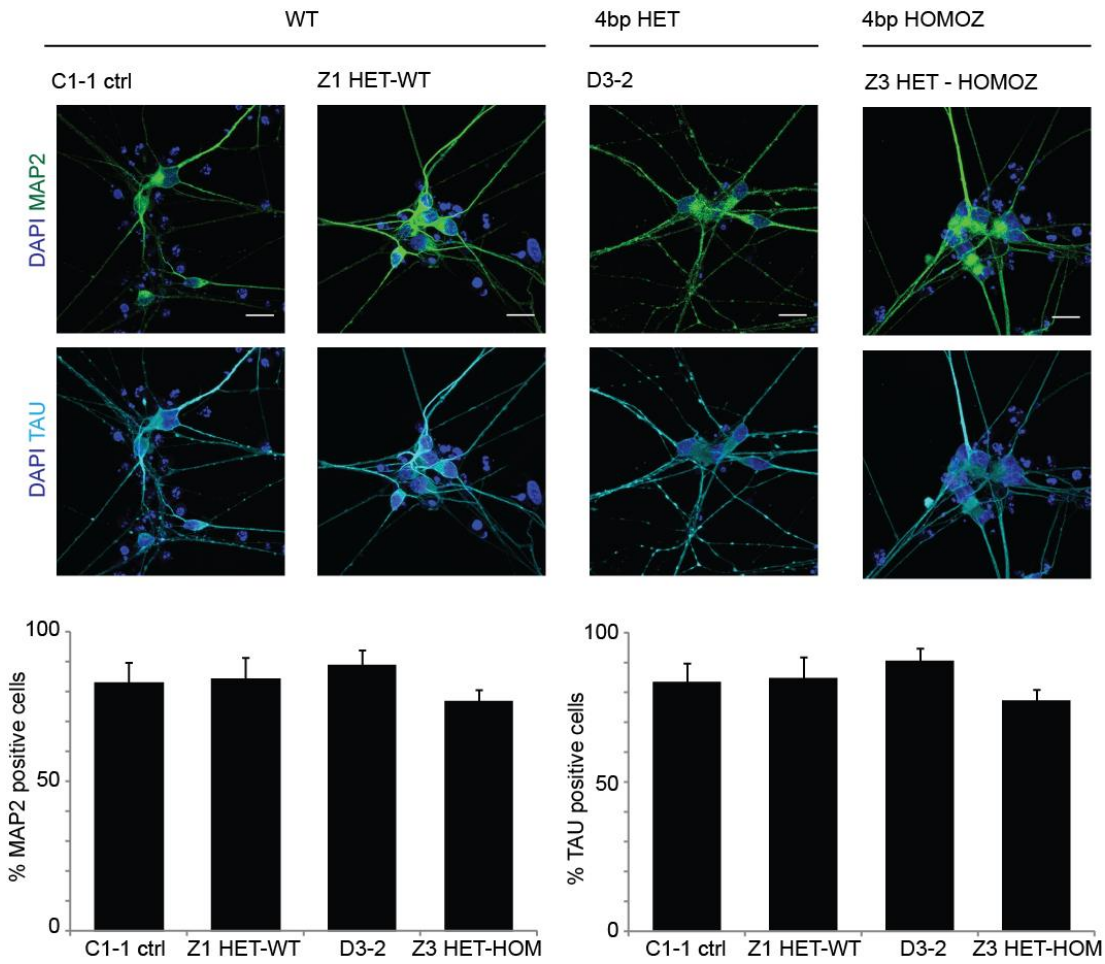


**Figure 5-5 Characterising differentiation of DISC1 mutant neurons; axonal and dendritic markers**

Neural progenitors were differentiated into neurons, fixed and stained for dendritic marker MAP2 and for axonal marker TAU. Panels show merged images of DAPI and MAP2 (green) or TAU (cyan). Scale bar = 20μm. Graphs show percentage of DAPI positive cells that also express MAP2 or TAU. Values are calculated from 10 images from 2 independent differentiations.

Repeating characterisation experiments for axonal and dendritic markers serves first to expand upon results described in Figure 5-4 Characterising differentiation of DISC1 mutant neural progenitors into glutamatergic neurons. Consistent with these data, immunocytochemistry for MAP2 and TAU showed the majority of cells in these cultures were positive for each marker. Data are presented in Figure 5-5 and desbribed in Table 5-3. This is supportive of the differentiation paradigm being effective. Further, these data also show no trend towards variation according to genotype. Thus the DISC1 4 bp deletion does not impair neuronal differentiation. However, it is notable from the images in Figure 5-5 that MAP2 and TAU signals colocalise instead of being confined to their expected compartments (dendrites and axons respectively). Therefore, these differentiated neurons are non-polarised. This is consistent with vGlut2 signals in Figure 5-4. In these cells, vGlut2 is not seen to localise in a specific compartment or adopt a punctate distribution as seen in cultured rodent neurons. However, this is not dependent upon genotype and is instead a feature of these cultures.

### 5.2.3 Mitochondrial trafficking in wild-type and DISC1 4bp deletion human neurons

In order to extend the findings in Chapter 3 and section 5.2.1, mitochondrial trafficking assays were carried out in these human neurons. In this way, the effect of the 4bp deletion in DISC1 can be explored without overexpression and any artefact induced here. Mitochondria were visualised with cell lights MitoRFP (Invitrogen) – a baculovirus based method of introducing a mitochondrially targeted red fluorescent protein - as detailed in the methods section. Percentages of moving mitochondria and representative kymographs can be seen in Figure 5-6. From the kymographs, it can be seen that, in these cultures, mitochondria are motile in the processes in a comparable manner to rodent hippocampal cultures. They undergo rapid bidirectional transport. The quantification according to genotype (where the 2 control lines and 2 heterozygous lines are pooled) shows no significant difference in moving mitochondria between wild-type and heterozygous lines (wild-type = 23.5% ± 3.6, Heterozygous = 22.5% ± 4.1). In contrast to data presented in Figure 5-3, the DISC1 4bp deletion does not confer an impairment on mitochondrial dynamics in this system. Upon comparison of data obtained from wild-type and homozygous lines, it can be seen that there is a trend towards a decrease in percentages of moving mitochondria (percentage of mitochondria moving in the homozygous line Z3 = 15.6% ± 3.2). The decrease is approximately 30% compared to either heterozygous or wild-type neurons. However, this does not represent a significant decrease (WT vs Homozygous p=0.1, unpaired T-test). The data are also presented as percentages of moving mitochondria according to line in Figure 5-6. It is evident that the two control lines behave very similarly to each other in this respect (C1-1 = 24.7% ± 4.6, Z1 = 22.7% ± 3.7). This highlights that the reprogramming and differentiation processes have not introduced inconsistencies in this case and these results are reliable. Upon comparison of the two heterozygous lines, a much greater difference is observed; approximately 56% fewer mitochondria are moving in processes of D3-1 compared to D3-2 (D3-1 = 9.0% ± 3.8, D3-2 = 20.8% ± 4.5). In this case, these two lines are behaving differently from each other. Since these cells have the same DISC1 genotype, this could be a factor introduced in the reprogramming process. Of these two lines, data obtained from D3-2 are more reliable. This is due to the derivation of two isogenic lines from this line, forming optimal controls. Comparison of data obtained from D3-2 to that from Z1 shows no clear

difference in terms of mitochondrial motility (percentage of moving mitochondria in D3-2 is 9% lower than in Z1). Upon comparison between D3-2 and the homozygous condition (Z3), there is once more a trend towards a decrease in mitochondrial trafficking. The value obtained for Z3 is 25% lower than that obtained for D3-2 (not significant, p=0.36, unpaired T-test). Therefore, the DISC1 4bp deletion could contribute to less obvious defects in mitochondrial trafficking, for example, in response to a specific signalling pathway rather than under basal conditions. Altogether, these data show that the 4bp deletion in DISC1 does not severely impair mitochondrial dynamics in this system. This is in disagreement with data obtained from overexpression studies in rodent hippocampal neurons in Figure 5-3. Further, the high degree of variability between the two heterozygous lines suggests this study would benefit from inclusion of many other patient derived lines. This would minimise contributions of inconsistencies in the reprogramming process to the data set. Ideally, generation or further isogenic lines could be carried out (e.g. from heterozygous line D3-1) and would differentiate between effect of the mutation or reprogramming artefact.

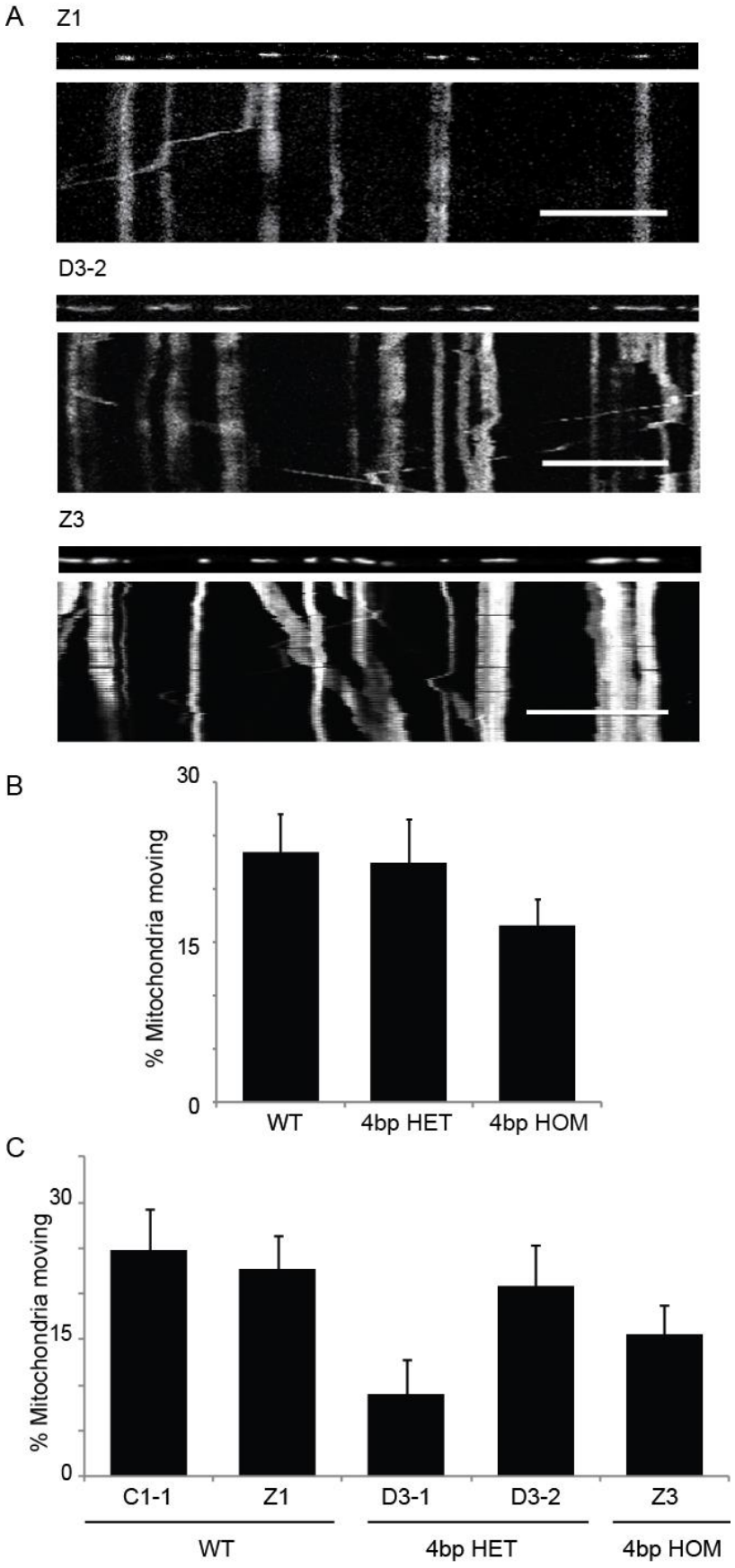


**Figure 5-6 Mitochondrial trafficking in DISC1 mutant human neurons**

(A) Shows representative kymographs of mitochondrial motility in processes for each genotype, WT (Z1), 4bp HET (D3-2), 4bp HOMOZ (Z3). Scale bars are 20μm. (B) Shows quantification of percentages of moving mitochondria, pooled according to genotype. (C) Shows quantification of percentages of moving mitochondria in each line. (n=6-19 cells from 4 different preparations).

### 5.2.4 Effects of cAMP levels on mitochondrial motility in DISC1 mutant human neurons.

The previous data do not support a role for the DISC1 4bp deletion in impairing mitochondrial dynamics under basal conditions. However, this system represents a physiologically relevant way to investigate the contribution of intracellular signalling pathways to mitochondrial trafficking and further, any alterations dependent on DISC1 mutation. Of particular interest in this case are cyclic adenosine monophosphate (cAMP) levels. This is due to the interaction of DISC1 with phopshodiesterases (PDEs – please see 1.9.1 on page 54), enzymes responsible for the degradation of cAMP. Moreover, cAMP levels have previously been reported to alter mitochondrial trafficking (Rui et al., 2006). To this end, mitochondrial trafficking assays were carried out as in the previous section, but with pharmacological manipulation of cAMP levels by inhibiting adenylate cyclase. Cells were treated with 10uM SQ22536 (Sigma-Aldrich) for 30 min prior to and during imaging. In order to decrease the contributions of any effects of reprogramming, these experiments were carried out with the heterozygous line D3-2 and the derived wild-type line Z1 as a control. Results are shown in Figure 5-7. Values for DMSO solvent controls can be seen to be comparable for wild-type vs heterozygous neurons, consistent with data presented in Figure 5-6. Inhibition of adenylate cyclase confers a significant increase in mitochondrial motility of approximately 30% in wild-type neurons (Z1) (18.3% ± 3.2 for DMSO treated cells compared with 27.4% ± 2.7). However, no change in percentages of moving mitochondria is detected in DISC1 4bp deletion heterozygous neurons (percentage of moving mitochondria = 16.5% ± 5.1 for DMSO treated cells compared to 19.2% ± 4.3).

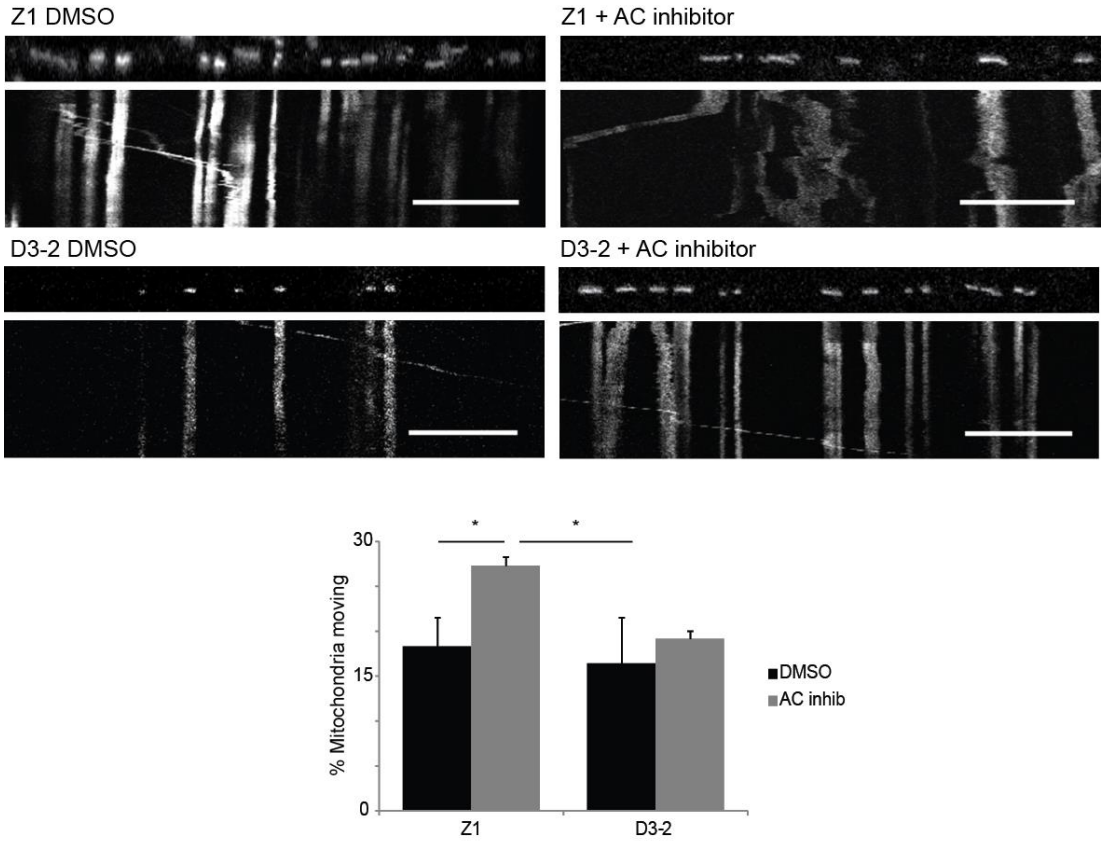


**Figure 5-7 Effects of cAMP levels on mitochondrial trafficking in DISC1 mutant human neurons**

(A) Shows representative kymographs of mitochondrial motility in processes for WT (Z1) and 4bp HET (D3-2) upon treatment with DMSO as a vehicle control. (B) Shows representative kymographs upon treatment with 10uM SQ22536 – adenylate cyclase (AC) inhibitor. Scale bars are 20μm. (C) Shows quantification of percentages of moving mitochondria. n=10-15 neurons from 4 experiments. An increase in moving mitochondria is seen in wild-type neurons (p=0.04, unpaired T-test), but not in 4bp HET neurons.

These data suggest mitochondrial trafficking can be modulated by cAMP levels in human neurons. Further, the neurons harbouring a DISC1 mutation are rendered insensitive to this signal and are unable to effect this change. Thus, DISC1 could be a crucial factor in regulating this signalling.

### 5.2.5 Amyloid beta perturbs mitochondrial dynamics in rodent hippocampal neurons.

The amyloid beta peptide is widely thought to be a causative factor in the onset of Alzheimer's disease (Shankar et al., 2008). The generation of the amyloid beta peptide from APP and the role of gamma secretase component presenilin 1 are depicted in Figure 5-8. Whilst many mechanisms have been put forward for the contribution of this peptide to neurodegeneration, the cause remains elusive (Pozueta et al., 2013). One potential mechanism contributing to synapse loss and neuronal death is a decrease of mitochondrial trafficking. Treatment of hippocampal neurons with 20µM amyloid beta peptide causes a decrease in moving mitochondria as determined by live imaging experiments (Rui et al., 2006). Additionally, trafficking assays have been carried out from cultured hippocampal neurons from mice carrying an AD mutation in Presenilin1 (M146L). Mitochondria in these cells display reduced velocity compared to controls, consistent with amyloid beta dependent defects in trafficking (Trushina et al., 2012). In order to confirm these findings, mitochondrial trafficking assays were carried out in rodent hippocampal neurons transfected with MtDsRed2 and treated with freshly prepared recombinant amyloid beta peptide for 1 hour prior to imaging, preventing inconsistencies between oligomeric species. Results are shown in Figure 5-9. In the DMSO treated control cells, 26% ± 3.9 of mitochondria were moving compared to 6% ± 2.0 in the cells treated with recombinant amyloid beta peptide. This is consistent with previous reports (Rui et al., 2006; Trushina et al., 2012) and so, amyloid beta has a negative effect on mitochondrial trafficking in this system. 20µM amyloid beta peptide was used to be consistent with previous reports (Rui et al., 2006). However, it is important to note that this peptide concentration is too high to be physiologically relevant. Soluble amyloid beta concentrations in AD patient cerebrospinal fluid samples have been calculated to be around 40pM (Lue et al., 1999), and so this concentration would be more appropriate in future experiments.

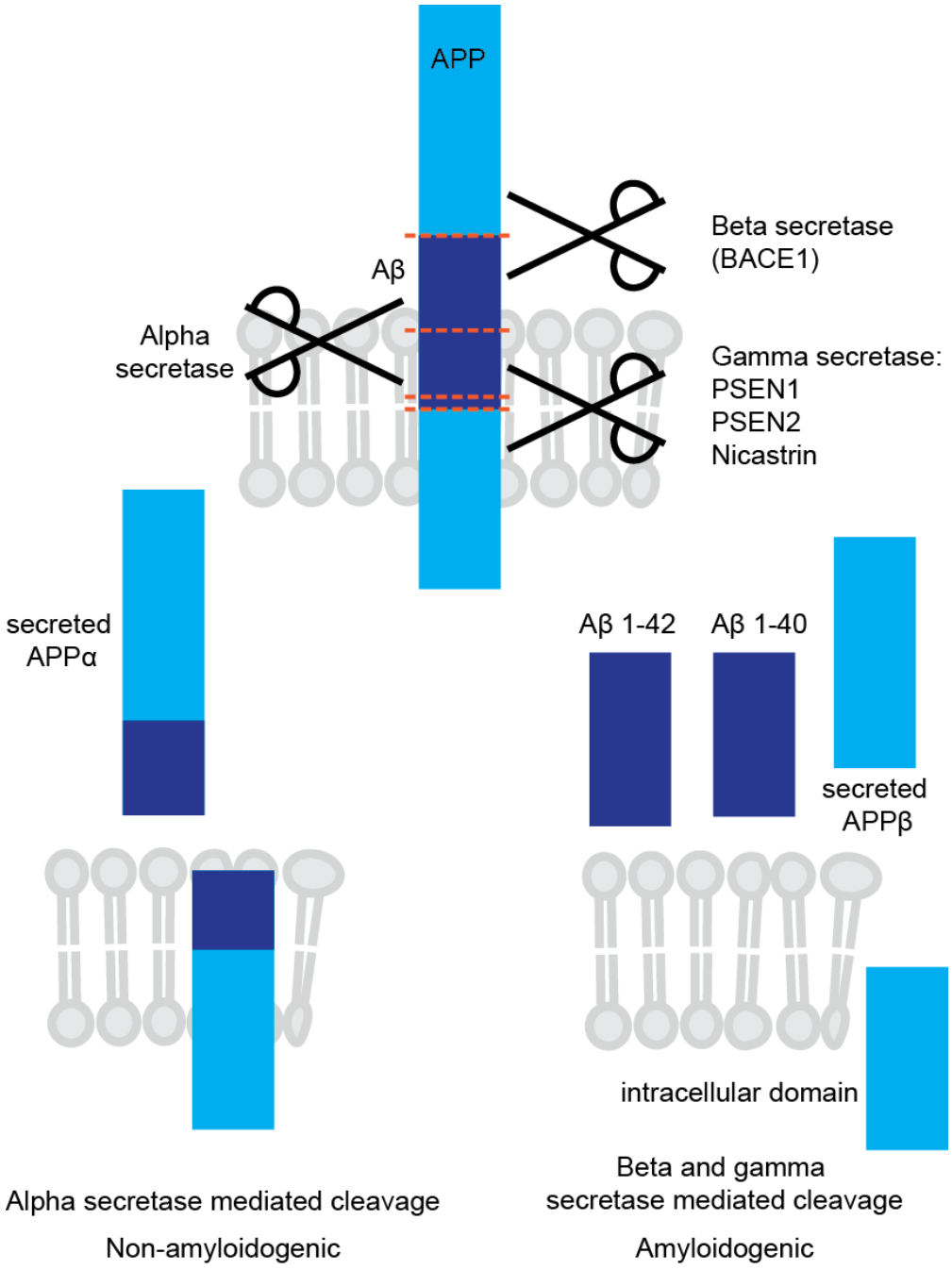


**Figure 5-8 Generation of the amyloid beta peptide from APP by beta and gamma secretase activity**

APP (blue) undergoes proteolytic cleavage by alpha, beta and gamma secretases. Cleavage by beta and gamma secretase gives rise to amyloid beta peptide species (Aβ – purple). Alternative processing by alpha secretase opposes amyloid beta generation, creating non-amyloidogenic peptides.

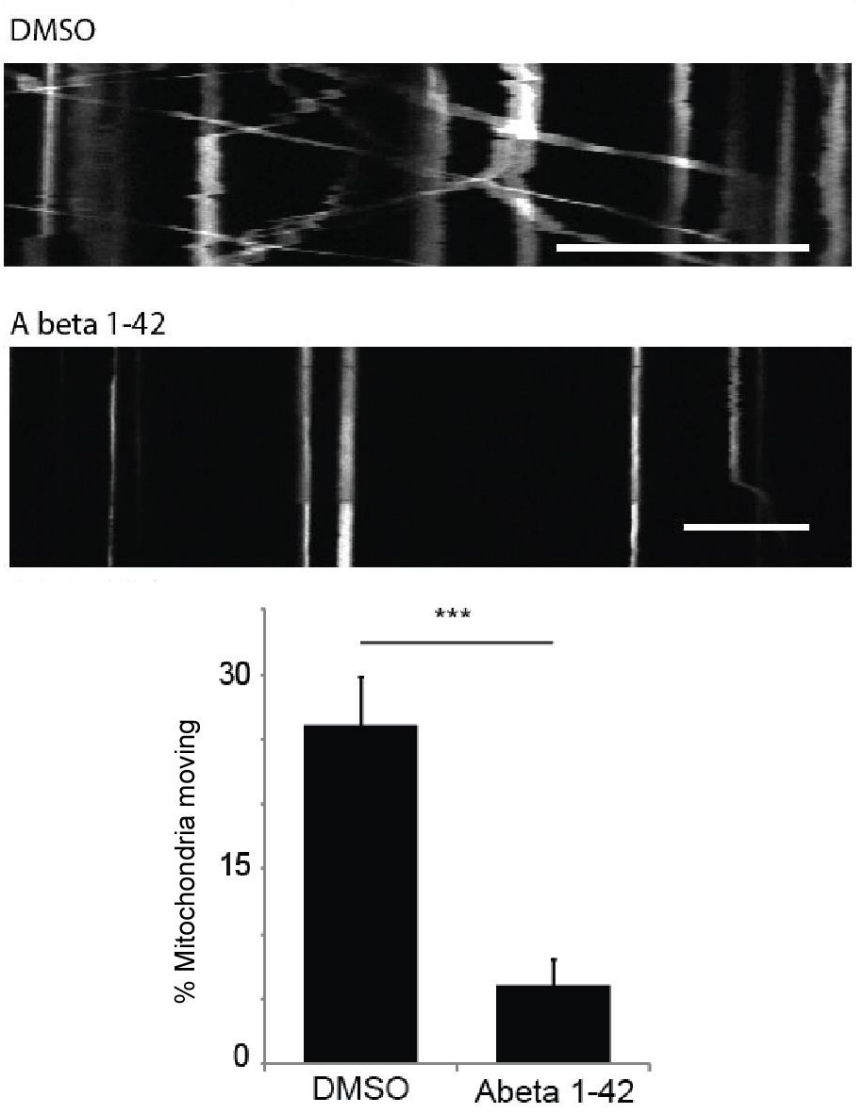


**Figure 5-9 Recombinant amyloid beta impairs mitochondrial trafficking in rodent hippocampal neurons**

Representative kymographs depict mitochondrial trafficking in rodent hippocampal axons. Pooled data from axons and dendrites show a decrease in motile mitochondria following a 1 hour exposure to 20μM amyloid beta peptide (1-42). DMSO was used as a vehicle control. Scale bar = 20μm. n=8-9 neurons from 2 experiments. p=0.0003. unpaired T-test.

### 5.2.6 Differentiation of PSEN1 mutant neural progenitor cells into glutamatergic neurons.

The previous experiment demonstrates mitochondrial trafficking can be impaired by amyloid beta peptide, and so could be impaired in AD models. However, this has yet to be investigated in a more physiologically relevant system. The approach carried out here involves use of neural progenitor cells derived from patient fibroblasts carrying a Presenilin1 mutation (A246E). Thus, the contribution of this AD causing mutation to mitochondrial transport deficits in differentiated human neurons can be determined. This mutation confers an increase in amyloid beta 1-42 levels in comparison to amyloid beta 1-40 in these cells (J. Kasuboski, pers communication, April 2015). Prior to carrying out mitochondrial trafficking assays, the efficiency of the differentiation paradigm was tested in these cells by immunocytochemistry as detailed in the methods section. Initially, differentiated cells were immunostained for neuronal marker βIII tubulin and glutamatergic marker vGlut2. DAPI served to label nuclei of all cells in the culture, and so indicate total numbers of cells per image. Results can be seen in Figure 5-10. Consistent with the DISC1 cells, there is variability in the expression of βIII tubulin between each line. Importantly, a decrease in βIII tubulin positive cells is noticeable comparing PSEN1 A246E line 7768 with the wild-type lines DET and 08249. However, this difference is not correlated with genotype of the cells. The other PSEN1 A246E line (6898) has a comparable number of βIII tubulin positive cells compared to control and is in fact, significantly higher than the value for 7768. In this case, these cultures show highly consistent results in terms of vGlut2 expression. Wild-type and PSEN1 A246E cells express vGlut2 to similar levels. For values of cells expressing each marker please see Table 5-4.

| Line | % Cells expressing Beta III tubulin | % Cells expressing vGlut2 |
|---|---|---|
| DET | 86.7 ± 9.7 | 93.3 ± 6.7 |
| 7571 | 73.7 ± 5.9 | 91.1 ± 3.3 |
| 08249 | 91.9 ± 3.3 | 96.0 ± 2.0 |
| 7768 | 51.9 ± 6.7 | 87.6 ± 5.9 |
| 6898 | 89.3 ± 6.9 | 96.0 ± 4.0 |

**Table 5-4 Quantifying differentiation efficiency of PSEN1 mutant lines; Beta III tubulin and vGlut2**

| Line | % Cells expressing MAP2 | % Cells expressing TAU |
|---|---|---|
| DET | 89.7 ± 6.7 | 89.7 ± 6.7 |
| 7571 | 70.0 ± 3.8 | 70.6 ± 3.6 |
| 08249 | 90.6 ± 2.9 | 90.6 ± 2.9 |
| 7768 | 59.2 ± 6.7 | 61.1 ± 6.4 |
| 6898 | 70.3 ± 3.5 | 70.3 ± 3.5 |

**Table 5-5 Quantifying differentiation efficiency of PSEN1 mutant lines; MAP2 and TAU**

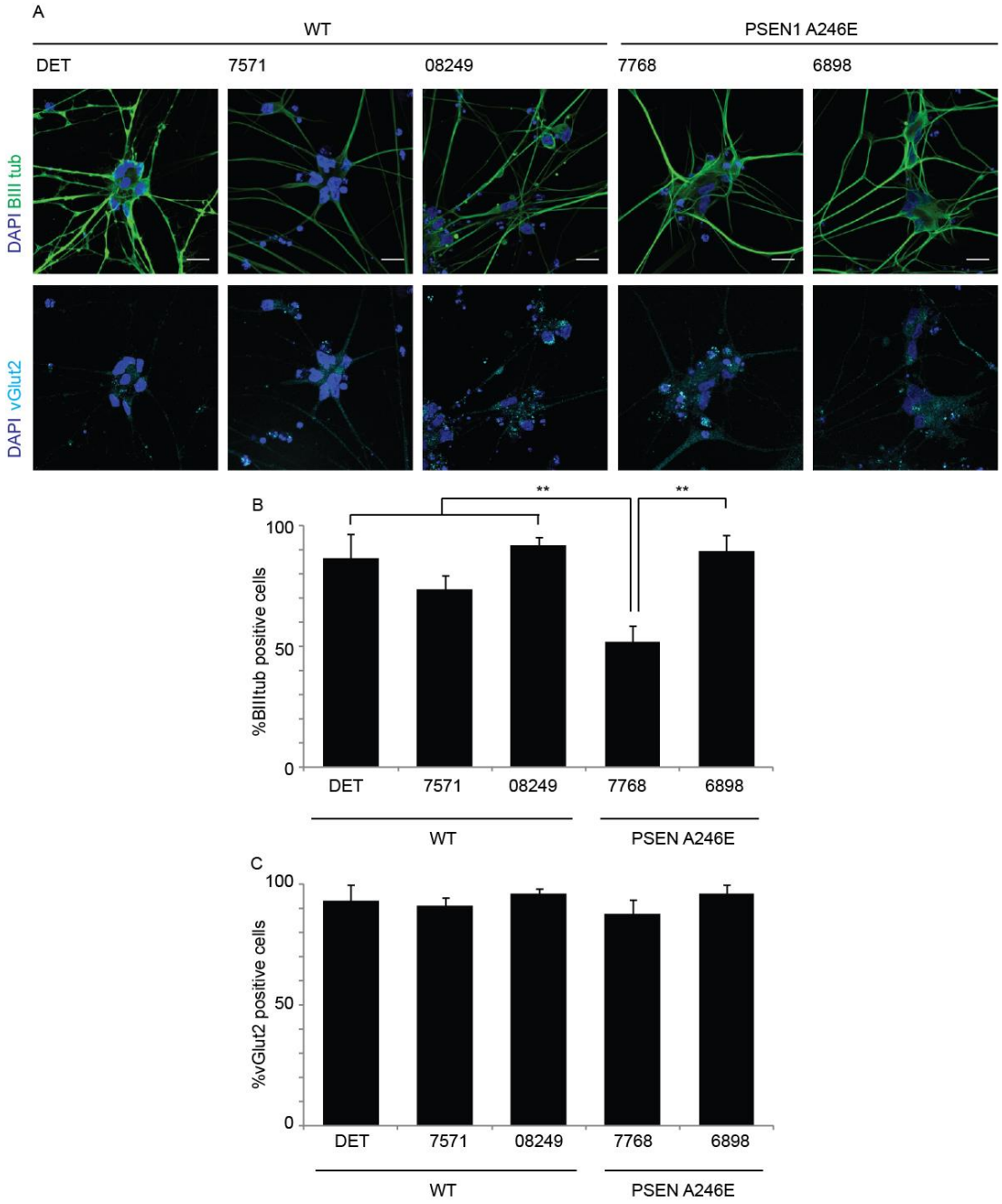


**Figure 5-10 Characterising differentiation of PSEN1 mutant neural progenitors into glutamatergic neurons**

(A) Neural progenitors were differentiated into neurons, fixed and stained for neuronal marker Beta III tubulin and for glutamatergic neuronal marker vGlut2. Panels show merged images of DAPI and Beta III tubulin (green) or vGlut2 (cyan). Scale bar = 20µm. Graphs show percentage of DAPI positive cells that also express Beta III tubulin (B) or vGlut2 (C). Values are calculated from 10 images from 2

independent differentiations. ** $p<0.01$, One way ANOVA with Tukey post hoc test.

These analyses were also carried out for axonal marker TAU and dendritic marker MAP2. Values are shown in Table 5-5 and results shown in Figure 5-11. The majority of cells in each culture are expressing both neuronal markers, thus the differentiation paradigm has been successful. As with data for βIII tubulin, the PSEN1 mutant line 7768 displays a significantly reduced percentage of cells positive for these neuronal markers in comparison to two of the three control lines. However, this decrease is not dependent upon the genotype of the cells as the values for the other PSEN1 mutant line (6898) are not significantly different than any control line. Whilst the decreased differentiation efficiency for this line is problematic, it did not affect the ability to perform mitochondrial trafficking assays as neurons in the culture can be readily distinguished from other cells by their obvious morphology. As with the DISC1 lines, the MAP2 and TAU signals are seen throughout the cell rather than confined to specific subcellular compartments. Therefore, whilst expressing these neuronal markers, these cells are failing to target these proteins correctly. Thus functionally distinct axons and dendrites cannot be concluded to be present in these cultures.

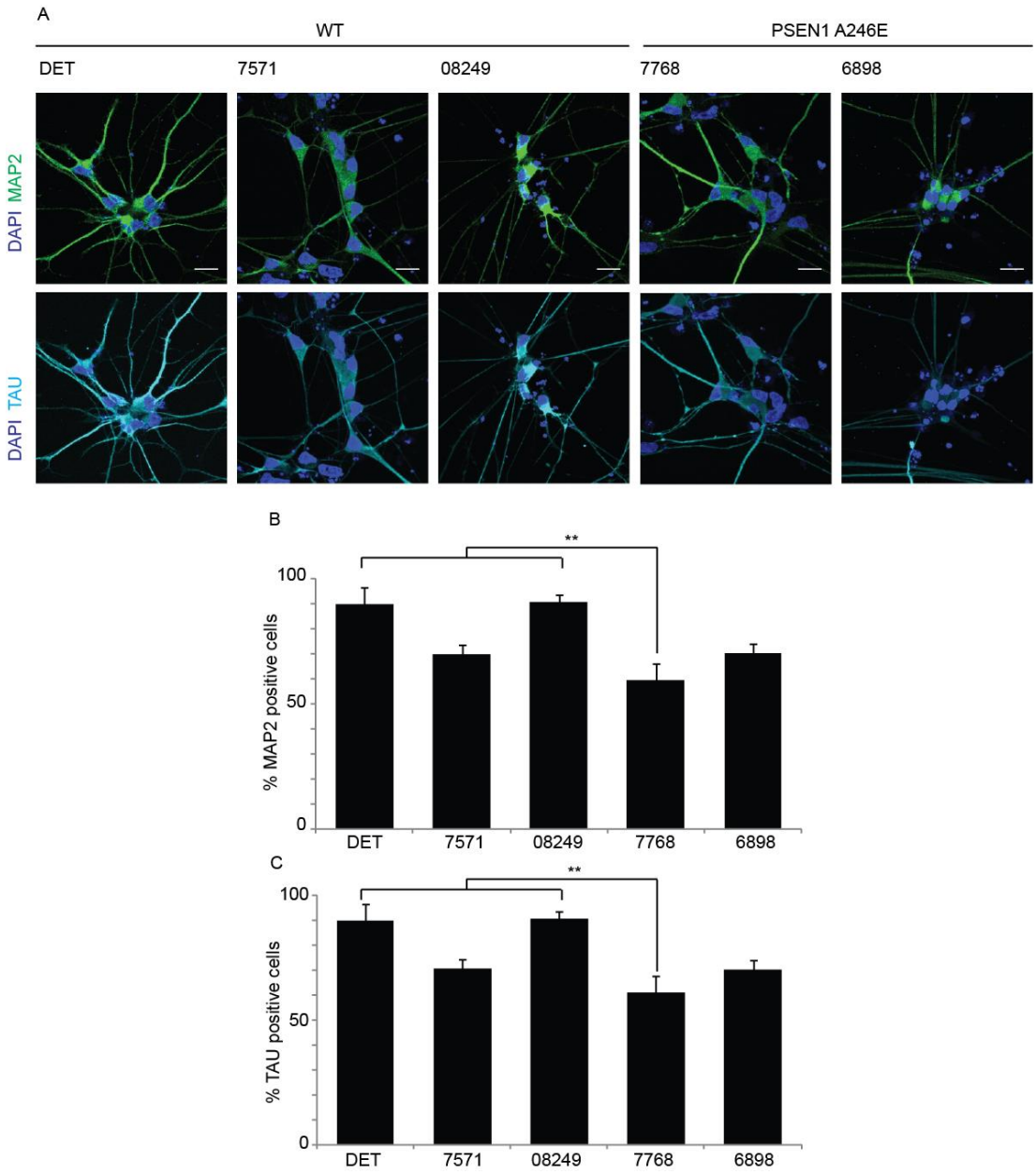


**Figure 5-11 Characterising differentiation of PSEN1 mutant neurons; axonal and dendritic markers**

(A) Neural progenitors were differentiated into neurons, fixed and stained for axonal marker TAU and for dendritic marker MAP2. Panels show merged images of DAPI and MAP2 (green) or TAU (cyan). Scale bar = 20μm. Graphs show percentage of DAPI positive cells that also express MAP2 (B) or TAU (C). Values are calculated from 10 images from 2 independent differentiations. ** $p<0.01$. One way ANOVA with Tukey post hoc test.

### 5.2.7 Mitochondrial trafficking in wild-type and PSEN1 mutant human neurons

Cultures of these cells have been demonstrated to express neuronal markers in their majority. Consequently, mitochondrial trafficking assays could be carried out as previously to investigate the consequence of amyloid beta peptide upon these dynamics. Cells were treated with Cell lights mitoRFP (Invitrogen), as detailed in the methods section, to label mitochondria. Representative kymographs for each genotype and quantification for each line can be seen in Figure 5-12. No significant difference in mitochondrial motility is seen between wild-type and PSEN1 mutant neurons (B; percentage of mitochondria moving in wild-type = 22.2% ± 2.9, in PSEN1 A246E = 26.3 ± 2.0). In fact, a slight trend towards an increase in motility in mutant cells is discernible. Analysis according to cell line rather than genotype is supportive of a lack of effect of this PSEN1 mutation on mitochondrial transport. In contrast to the DISC1 lines, data obtained across these lines are more consistent. The two PSEN1 mutant lines display a similar percentage of moving mitochondria (7768 = 26.9% ± 2.5, 6898 = 24.5% ± 3.0). Two of the wild-type lines also behave alike in this respect (DET = 25.3% ± 6.9, 7571 = 25.9 ± 4.3). One wild-type line – 08249 – shows roughly a 25% decrease in percentages of moving mitochondria compared to each of the other lines. However, this does not represent a significant decrease. Further, the difference in homogeneity of the 7768 cultures described in section 5.2.6 does not alter percentages of motile mitochondria. Thus presence of non-neuronal cells in the culture does not seem to impact mitochondrial dynamics in these cultures. These data are inconsistent with multiple reports in other systems and it would appear that the PSEN1 mutation, conferring increased amyloid beta 1-42 levels, does not contribute to a decrease in mitochondrial transport in this system.

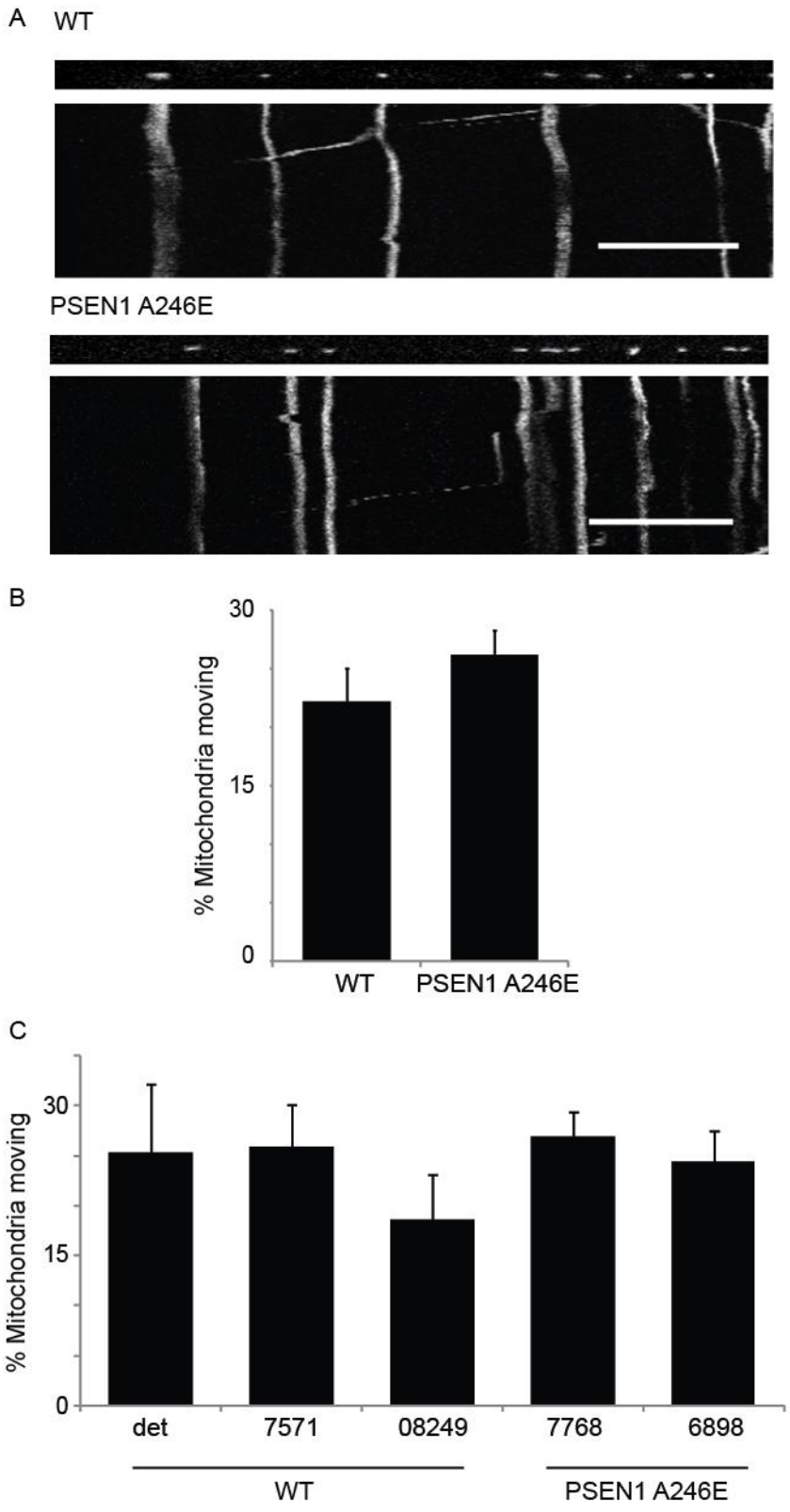


**Figure 5-12 Mitochondrial trafficking in PSEN1 mutant human neurons**

(A) Shows representative kymographs for mitochondria moving in processes of wild-type and PSEN1 A246E differentiated neurons. Scale

bar = 20μm. (B) Shows pooled data quantifying percentage of mitochondria moving according to genotype. (C) Shows percentages of moving mitochondria according to cell line (n=5-25 cells from 4 individual preparations).

## 5.3 Discussion

In this chapter, mitochondrial trafficking has been compared in rodent hippocampal cultures and neural progenitor derived neurons. Specifically, the contributions of two disease causing mutations have been studied; namely, a point mutation in PSEN1 (A246E) and a frameshift in DISC1, causing neurodegenerative disease (AD) and psychiatric disease (schizophrenia and schizoaffective disorder) respectively. This was performed in separate ways for each mutation in rodent cultures. In terms of the DISC1 frameshift, a construct encoding the resultant amino acid sequence was overexpressed in rodent neurons. The effect of the Presenilin 1 mutation was mimicked by acute treatment of hippocampal cultures with synthetic amyloid beta peptide. In both cases, a decrease in mitochondrial trafficking was found. In the case of amyloid beta, this acts to confirm effects previously reported in the literature (Rui et al., 2006; Trushina et al., 2012). In the case of the DISC1 mutation, previous work has yet to investigate mitochondrial dynamics. The findings reported here are consistent with the effect reported in section 3.2.6 for the DISC1-Boymaw fusion protein and similar to effects reported for DISC1 R37W and L607F (Atkin et al., 2011; Ogawa et al., 2014). Moreover, this DISC1 mutation has been shown to decrease soluble levels of DISC1, without altering mRNA levels of the wild-type transcript (Wen et al., 2014). This is comparable to the effect of DISC1 aggregation and aggresome formation. In these experiments, aggregated, insoluble DISC1 was shown to recruit cytosolic DISC1, decreasing levels in the soluble fraction. This led to a decrease in mitochondrial transport (Atkin et al., 2012). Additional potential mechanisms for the DISC1 4bp deletion effect may include loss of regulation of PDE4 or a loss of the interaction between DISC1 and Ndel1 – a dynein component necessary for microtubule based transport by this motor (Vallee et al., 2012). The consistency between DISC1 and amyloid beta experiments shows correct mitochondrial trafficking to be a critical feature in neuronal function, as aberrances are a feature in both psychiatric and degenerative disease models.

A disadvantage of experiments carried out in this thesis so far is the dependence upon overexpression. In order to address this, the revolutionary technology of iPSCs and derived neurons was used to investigate effects of endogenous mutations on mitochondrial trafficking. In order to carry out comparable trafficking assays in human neurons, neural progenitor cells were differentiated into glutamatergic neurons. To determine the efficiency of this process, immunofluorescence based characterisation of the cultures was performed to investigate the percentages of cells expressing four neuronal markers; βIII tubulin, vGlut2, MAP2 and TAU. The DISC1 lines differentiated comparably to one another. Variability between the lines did not correspond to the genotype of the cells. Therefore, the DISC1 mutation does not interfere with the differentiation paradigm. Cultures contained glutamatergic neurons in their majority, and could be used for trafficking assays. Examination of axonal and dendritic markers TAU and MAP2 revealed an improper formation of axons and dendrites, or at least an improper targeting of these two proteins. This was discernible due to overlap of signals for these two proteins. Once more, the genotype of the cells had no bearing on this phenomenon – each line showed this phenotype. Thus it is likely that these cells are not polarised. This finding may imply the cells are incapable of electrophysiological activity. This is supported by the indiscriminate vGLUT2 staining; not contained to discrete puncta in the axon. However, electrophysiological experiments carried out in neural progenitor cell (NPC) derived dopaminergic neurons show comparable activity to human neurons (Reinhardt et al., 2013). Alternatively, this may be due to the neurons not yet being fully mature. Prolonged culture may allow improved synapse formation, and thus activity. Alternatively, varying culture conditions may act to favour neuronal function. One such condition could involve glial coculture (Wen et al., 2014). Additionally, in a recent report, Bardy and colleagues describe BrainPhys medium, which supports neuronal activity much more effectively than classical culture milieu, as determined by calcium imaging and electrophysiological techniques (Bardy et al., 2015). In this case, these differentiated neurons may be more functionally relevant.

These findings are comparable to those presented for the PSEN1 A246E lines. In most cases, these cells can be described as neurons based upon the immunostaining data. The majority of the cells express each of the four markers tested. However, one PSEN1 A246E line (7768) displayed a decreased differentiation efficiency compared to the

control lines. In terms of the βIII tubulin expression, percentage of positive cells was also decreased in comparison to the other PSEN1 A246E line (6698). Therefore, this phenotype does not correspond to the genotype of the cells, but rather may be attributed to variability between lines. The source of this variability likely lies in the reprogramming process and subsequent culture or in the original fibroblasts. The variability could be attributed to genomic variation, such as differences in chromosome number, copy number variations or epigenetic variations. Any of these factors could lead to changes in expression levels of genes required to take on a neuronal fate, such as neurogenin2. Epigenetic variation is the more likely cause in this case as this landscape can be changed with prolonged culture. Cells were karyotyped to identify chromosomal abnormalities after reprogramming, and all lines were determined to be normal at this stage. Therefore, this is an unlikely source of the described variability (Liang and Zhang, 2013).

Upon comparison of mitochondrial trafficking assays carried out in rodent and human neurons, it is evident that effects are not equivalent. This is shown by the decrease in trafficking in the rodent neuron experiments, but no obvious effect in differentiated human neurons. In terms of the DISC1 lines, a decrease in mitochondrial transport was observed between each control line (C1-1 and Z1) and one of the schizophrenia patient derived lines (D3-1). This is consistent with results presented for overexpression experiments in rodent cultures. This result may be supportive of DISC1 mutations conferring decreased mitochondrial trafficking capabilities at endogenous levels in human neurons. However, this cell line behaves differently to the other schizophrenia patient line (D3-2). This result could be an effect of heterogeneity between patients, e.g. the DISC1 4bp deletion, in conjunction with other undetected mutations (specific to D3-1) causes a decrease in mitochondrial trafficking. Alternatively, this effect could be due, once more, to heterogeneity introduced during reprogramming or culture protocols. One such source of variability lies in the mitochondrial genome. The reprogramming process introduces mutations to this genome which could confer bioenergetic variations between cell lines (Prigione et al., 2011). These, in turn, could contribute to variations in mitochondrial transport. In order to differentiate between these two possibilities, it would be beneficial to generate isogenic controls from this line – D3-1 – for example, a correction of the DISC1 mutation. Therefore, any

difference in phenotype between these two lines would be more likely due to the DISC1 genotype rather than anomalies in the reprogramming and culture processes.

The results obtained for line D3-2 are more reliable since this line was used to generate two controls – Z1 (wild-type) and Z3 (4bp deletion homozygous). D3-2 behaves comparably to its corrected version Z1. Therefore, there is no evidence to support a contribution of the DISC1 4bp deletion to aberrant mitochondrial trafficking under basal conditions. However, the results obtained from homozygous line Z3 show a trend towards a decrease in mitochondrial trafficking. Whilst this line does not represent a physiological scenario (no patients were homozygous for the 4 bp deletion), this result may suggest a subtler contribution of this DISC1 mutation to mitochondrial trafficking defects. This is supported by data presented in Figure 5-7 concerning the effect of cAMP levels on mitochondrial trafficking. In these experiments, the DISC1 mutant line failed to respond to changes in cAMP levels, whereas the wild-type line responded by increasing mitochondrial trafficking. This effect suggests DISC1 may act to sense cAMP levels at the mitochondrial trafficking complex and initiate a response – in this case an increase in trafficking. Mutation in DISC1 appears to confer a loss of function on this respect. This could be due to an inability to regulate PDE4 activity via labile interaction. The interaction between DISC1 and PDE4 isoforms has been shown to be dependent upon PKA activity via cAMP levels, with an increase in cAMP causing a dissociation of this complex (Murdoch et al., 2007). This result also extends findings concerning cAMP levels and mitochondrial trafficking. Whilst little is known in this respect, it has been demonstrated that mimicking an increase in cyclic AMP by loading cells with a non-hydrolysing analogue (Dibutyryl-cAMP) increases mitochondrial trafficking. However, this has only been reported following treatment of cells with amyloid beta peptide. Similarly, activating PKA (protein kinase A whose activity is increased with cAMP levels) has the same rescue effect (Rui et al., 2006). Taken together, it seems that both decreasing and increasing cAMP levels may have a positive effect on mitochondrial trafficking. Further work will be crucial to identify if this effect reported in human neurons applies also to rodent culture methods where the majority of trafficking assays have been carried out. Moreover, it is of great interest to dissect the contributions of cAMP levels to regulation of trafficking. Further work could investigate the signalling pathways responsible, for example, PKA activity and potential substrates. Additionally, the role of DISC1 in this process should be

addressed. This is of great importance as data presented here suggests an effect of the mutation only under specific conditions, rather than a constitutive decrease in trafficking. Therefore, loss of DISC1 function in terms of influencing mitochondrial trafficking in response to cAMP levels may be a key contributor to onset of psychiatric disease.

Mitochondrial trafficking assays carried out in PSEN1 mutant neurons show no alteration according to genotype. As with the DISC1 result, this effect is inconsistent with assays in rodent neurons both in this work and previously published data. This may be because amyloid beta levels have not yet reached concentrations sufficient to impair mitochondrial trafficking. Longer culture times could address this issue. Another possibility is that overexpression experiments in rodent culture systems are not sufficiently physiological to model disease. Thus, widely reported mitochondrial trafficking defects may not represent a pathological feature of psychiatric or neurodegenerative disease. However, post mortem samples from AD and schizophrenia brain tissue oppose this possibility. AD brain tissue revealed decreased levels of mitochondrial fission and fusion regulators (Wang et al., 2009). This is supportive of impairments in mitochondrial dynamics as a cause or effect of Alzheimers disease. Further, abnormalities in mitochondrial distribution – consistent with changes to mitochondrial transport - have been reported in schizophrenia post mortem samples. In these neurons, there was a decrease in presynatpic mitochondria in the caudate nucleus and putamen of schizophrenia patients compared to control (Somerville et al., 2011). Therefore, the lack of effect detected here in human NPC derived neurons may not represent a physiological scenario.

The inconsistencies between systems could be explained by differences in culture conditions between rodent and human neurons. These cultures are maintained in higher levels of FGF2, BDNF and GDNF than typical rodent cultures. Further, the neural progenitor cells are maintained in a GSK3 beta inhibitor to allow proliferation. This may override any potential defects in mitochondrial trafficking. GSK3 beta activity has been shown to negatively regulate kinesin based activity via phosphorylation of kinesin light chain (Morfini et al., 2002). Therefore, inhibition of GSK3 beta may increase the baseline for mitochondrial trafficking. Additionally, FGF2, BDNF and GDNF signalling can activate Akt, inhibiting GSK3 beta

(Downward, 2004). This disagreement between stem cell derived cultures and rodent cultures has been reported elsewhere. Cooper and colleagues carried out mitochondrial trafficking assays in iPSC derived neurons from patients carrying Parkinsons causing mutations. Mutations G2019S and R1441C in the kinase LRRK2 caused an increase in mitochondrial motility in these cells (Cooper et al., 2012). However, G2019S and R1441C impaired mitochondrial dynamics in other cell types. Wang and colleagues investigated mitochondrial fusion in SH-SY5Y cells and rodent neurons. They show an imbalance in fission and fusion events dependent upon LRRK2 mutations with a decrease in the rate of mitochondrial fusion. This decreased fusion rate implicates a decrease in trafficking (Cagalinec et al., 2013). Taken together, these data suggest neural progenitor derived neurons do not accurately mimic primary neurons in terms of mitochondrial trafficking. Continued efforts to improve culture and differentiation methods will allow generation of disease, and even, patient specific cells which very accurately mimic features of primary neurons. These improvements will allow compromise between the higher throughput method of neural progenitor derived cells, the elegant iPSC derived methods and physiological relevance of primary neurons. In this way, contribution of organellar trafficking to neurological disorders may be studied in a large variety of highly disease relevant conditions.

# Chapter 6
# General Discussion

## 6.1 Summary

Mitochondrial distribution is essential to the intricate workings of the neuron due to their complex architecture and high localised energy demands. This distribution is achieved via tight regulation of microtubule based transport, allowing delivery of mitochondria to necessary sites such as the growth cone, pre synapse and post synapse. At these specialised sites, mitochondria can provide ATP, buffer calcium and act as signalling hubs. The dependence of correct neuronal function on mitochondrial dynamics is highlighted by alterations in these processes in both disease models and post mortem tissue from patients of neurodegenerative and psychiatric disorders.

The findings presented here show the schizophrenia associated scaffold protein, DISC1, to be part of the native Miro/TRAK mitochondrial trafficking complex. Moreover, the presence of DISC1 in this complex is essential as elucidation of the DISC1-Miro interacting domain, and subsequent trafficking assays, revealed this DISC1 region acted as a dominant negative to impair trafficking. Thus, DISC1 can act to locally regulate mitochondrial trafficking. Additionally, it is required to orchestrate normal mitochondrial fusion – highlighting the role of DISC1 not just as a trafficking regulator, but as a mediator of multiple categories of mitochondrial dynamics. This is further supported by the studies on ER-mitochondria associations reported here.

These findings were extended to investigate the impact of a disease associated chromosomal translocation in the DISC1 locus on mitochondrial dynamics – expression of a DISC1-Boymaw fusion protein. Here it has been demonstrated that this chimaeric transcript acts as a dominant negative, impairing DISC1-mediated mitochondrial transport, fusion and communication with the ER. In order to address contributions of these impairments to psychiatric disease, the morphology of the

dendritic arbour was investigated. Correct formation and maintenance of this compartment is essential for normal neuronal communication. Upon impairment of mitochondrial dynamics, either by expression of the identified dominant negative, or the DISC1-Boymaw fusion protein, the complexity of the dendritic tree was significantly decreased. These data show DISC1 dependent mitochondrial dynamics are essential for normal neuronal development, supporting a role for impaired mitochondrial dynamics in the onset of psychiatric disease.

Finally, mitochondrial trafficking was investigated in neurons derived from fibroblasts of Alzheimer's and schizophrenia patients. Whilst these findings were not consistent with those in rodent cultures, they serve to demonstrate the ability to investigate mitochondrial dynamics in this system – for which there is currently only one published report. Experiments carried out in this system suggest DISC1 may be able to subtly augment mitochondrial trafficking at physiological levels as a decrease in trafficking was noted only in the mutant homozygote condition. Further, this data suggests DISC1 acts in response to certain cues rather than driving a large scale change in transport under basal conditions. This was shown by modulating cAMP levels and observing a subsequent increase in mitochondrial trafficking only in the wild-type condition, whereas the heterozygote mutant condition failed to respond.

Overall, the data presented in this thesis demonstrate that DISC1 is a crucial component of the mitochondrial trafficking complex, acting to regulate transport and fusion. Additionally, evidence supporting a disease mechanism in schizophrenia is proposed, whereby impairing mitochondrial dynamics and inter-organellar communication impairs dendrite outgrowth. This leads to smaller, less complex dendritic arbours, known to have a decreased information processing ability and so contribute to the onset of psychiatric disease. A schematic of these findings is presented in Figure 6-1.

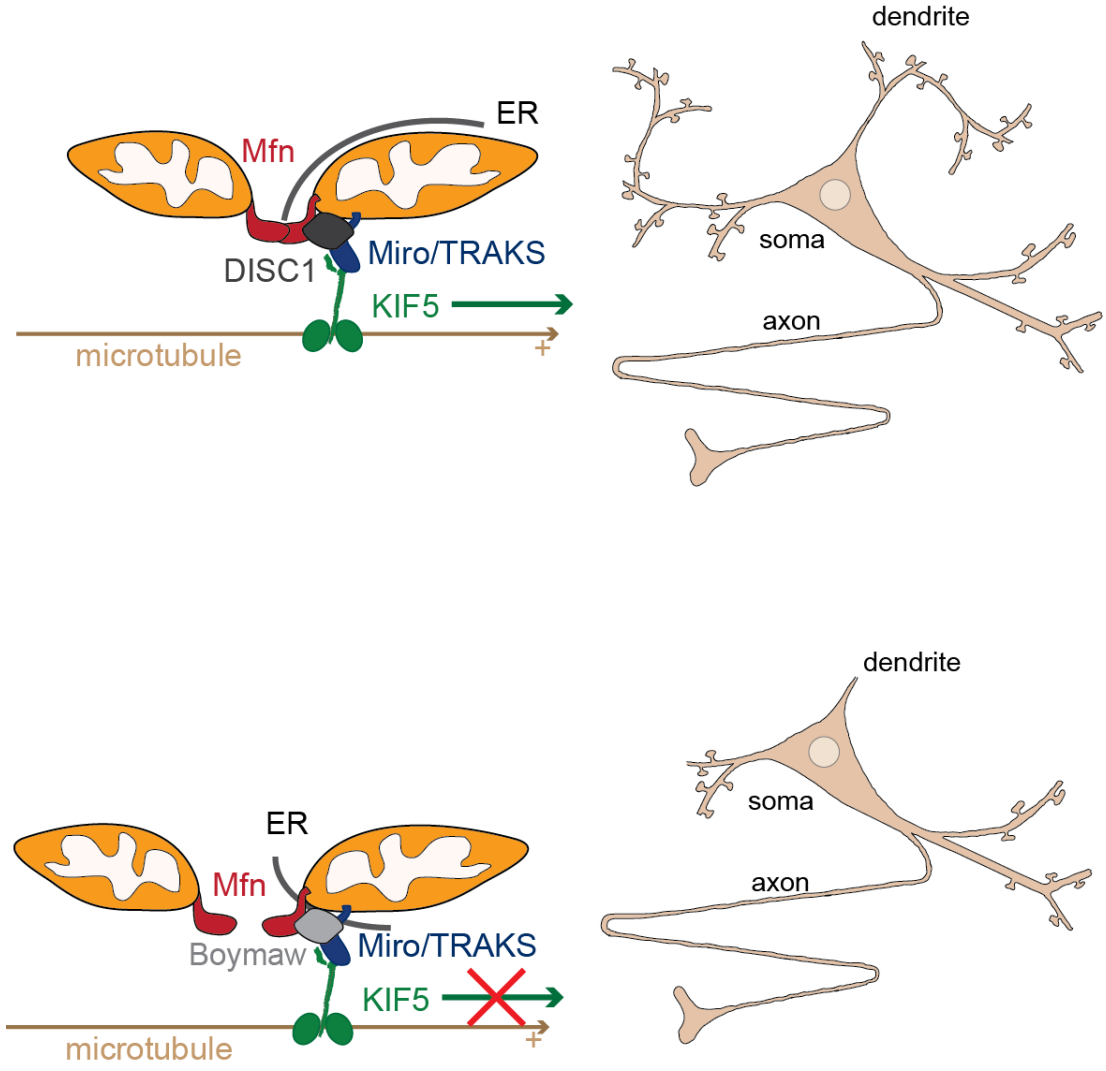


**Figure 6-1 Schematic summarising effects of DISC1 and the DISC1-Boymaw fusion protein on mitochondrial dynamics and dendritic development**

DISC1 is a component of the mitochondrial trafficking complex, allowing mitochondrial transport, fusion and ER-coupling. Under these conditions dendrites are formed normally. Upon expression of the schizophrenia associated DISC1-Boymaw fusion protein, trafficking and fusion are decreased, ER-mitochondria contacts are decreased and dendritic outgrowth is impaired.

## 6.2 DISC1 as a regulator of mitochondrial dynamics

From work presented here, in conjunction with other studies, DISC1 can now be conclusively regarded as a mitochondrial trafficking protein (Atkin et al., 2012; Atkin et al., 2011; Ogawa et al., 2014). Previous work has shown DISC1 to be a positive regulator of mitochondrial trafficking (Atkin et al., 2011). Data presented here support this role for DISC1; loss of DISC1 function by expression of a dominant negative severely decreased mitochondrial transport (Figure 3-7). Data presented in Chapter 3 extend our understanding of DISC1-mediated mitochondrial trafficking by highlighting the presence of this protein within the native mitochondrial trafficking complex. This has been previously suggested in a recent study, showing *in vitro* interaction between DISC1/Miro, and DISC1/TRAK1, and a TRAK1-dependent recruitment of DISC1 to the mitochondria (Ogawa et al., 2014). Therefore, DISC1 could act to locally regulate trafficking of individual mitochondria, rather than influencing a signalling pathway that would impact the whole mitochondrial network. Furthermore, the work presented here investigates the roles of the DISC1-Boymaw fusion protein and a DISC1 4bp deletion - DISC1 mutations yet to be studied in the context of mitochondrial trafficking. These mutations appear to act as dominant negatives by inhibiting mitochondrial trafficking in the presence of endogenous DISC1. Previous DISC1 mutations investigated in this way appear to have a subtler phenotype. The L607F point mutation fails to rescue mitochondrial trafficking on a knockdown background, and the R37W point mutation impairs a preference for anterograde transport rather than decreasing the mobile population of mitochondria (Atkin et al., 2011; Ogawa et al., 2014). Therefore there is heterogeneity in the effects of DISC1 mutations on mitochondrial trafficking. This is supported by the DISC1 S704C mutation rescuing mitochondrial trafficking similarly to wild-type DISC1 in a knockdown experiment (Atkin et al., 2011).

It remains to be determined how DISC1 is positively regulating mitochondrial transport. Due to its role as a scaffolding protein, it may be coordinating the assembly of the mitochondrial trafficking complex. This was suggested by Ogawa and colleagues (Ogawa et al., 2014). Mitochondrial isolation assays revealed an increase in kinesin-1 in this fraction dependent on TRAK1. This effect was enhanced upon presence of wild-type DISC1, and even more so upon presence of DISC1 R37W

(Ogawa et al., 2014). However, this mutant did not favour kinesin-based mitochondrial transport and no effect on the TRAK/kinesin-1 interaction was observed by coIP. Therefore, other mechanisms may be at play concerning DISC1's ability to influence multiple signalling pathways, such as cAMP levels and GSK3 beta activity – themselves known to regulate mitochondrial trafficking. In the case of GSK3 beta activity, a mechanism has been proposed concerning phosphorylation of kinesin light chain and disassembly of motor-cargo complexes (Morel et al., 2010; Morfini et al., 2002). Therefore, DISC1 may upregulate mitochondrial trafficking by local inhibition of GSK3 beta (Ishizuka et al., 2011; Mao et al., 2009). If GSK3 beta regulation is indeed the mechanism by which DISC1 mediates intracellular trafficking, how is this affected during synaptic activity? Further, it would be of great interest to investigate mitochondrial dynamics in dopaminergic neurons. Aberrant activity in this class of neuron, along with glutamatergic neurons, is reported in schizophrenia (Beaulieu, 2011), and signalling in these neurons is highly dependent on GSK3 beta downstream of dopamine signalling (Chen et al., 2008). As well as mitochondria, GSK3 beta regulated kinesin-1 proteins traffic other cargoes including AMPA and $GABA_A$ receptor subunits (Setou et al., 2002; Twelvetrees et al., 2010). Might DISC1 play a role in regulating trafficking of these cargoes via interaction with other specific adaptors?

Less is known concerning cAMP levels and mitochondrial trafficking. It has been shown that upregulating these levels can rescue trafficking upon amyloid beta dependent decrease (Rui et al., 2006). Conversely, increasing cAMP levels has been shown to have an inhibitory effect on trafficking (Chen et al., 2008). This is more consistent with data presented in Chapter 5 where decreasing cAMP levels increased mitochondrial trafficking in differentiated neurons. It is also apparent that DISC1 has a role in this effect as this increase was occluded in cells heterozygous for the 4bp deletion. A well studied effect of increasing cAMP levels is an increase in protein kinase A activity (PKA) (Nicol and Gaspar, 2014). This protein is a putative DISC1 kinase (Ishizuka et al., 2011). Therefore, could PKA-mediated phosphorylation of DISC1 influence mitochondrial trafficking? This may account for the result reported in Figure 5-7. Phosphorylated DISC1 is permissive of GSK3 beta activity, perhaps accounting for lower fraction of motile mitochondria with higher cAMP levels. Upon decrease of cAMP concentration, and thus PKA activity, equilibrium would shift

towards a non-phosphorylated pool of DISC1. This would in turn inhibit GSK3 beta activity, upregulating mitochondrial transport. In this way, DISC1 could be acting to integrate cAMP and GSK3 beta signalling to mediate mitochondrial trafficking. Please see Figure 6-2. Indeed, via modulation of PDE4 activity, it would seem there is crosstalk between GSK3 beta activity and cAMP levels. GSK3 beta activity itself is necessary for tonic PDE4 activity, whereas DISC1 modulates its activity in response to fluctuations on cAMP levels (Carlyle et al., 2011).

It has yet to be determined how mutations in DISC1 might lose the ability to correctly regulate mitochondrial trafficking. It is possible that the DISC1-Boymaw fusion protein, or DISC1 4bp deletion lose the ability to inhibit GSK3 beta, but retain the ability to localise to the trafficking complex, displacing wild-type DISC1. Therefore, the mitochondrial trafficking complex might be more prone to disassembly downstream of kinesin light chain phosphorylation. Alternatively, DISC1 aggregation and recruitment of cytosolic DISC1 into aggresomes could play a role with the mutations described here. Atkin and colleagues showed this feature of DISC1 to cause a decrease in mitochondrial trafficking (Atkin et al., 2012). This is a possibility with the mutations described in this thesis as both mutant proteins have been previously shown to be insoluble, consistent with a propensity to aggregate (Wen et al., 2014; Zhou et al., 2010). Could these mutant transcripts recruit wild-type DISC1 away from the trafficking complex – where it must be to exert its transport effects as shown in Chapter 3 – and into the insoluble fraction where it can no longer carry out its trafficking role? This would be consistent with the dominant negative activity described in this work.

In addition to a loss of trafficking ability, the DISC1-Boymaw fusion protein also interferes with mitochondrial fusion as shown in Chapter 4. It remains to be determined if this loss of fusion activity is dependent on the loss of mitochondrial trafficking as previously suggested (Cagalinec et al., 2013), or indeed, the inverse. A decrease in fusion could decrease the function or health of the mitochondrial network, potentially decreasing trafficking readiness (Miller and Sheetz, 2004). Moreover, the cues promoting mitochondrial fusion are less well studied than those regulating trafficking. Could DISC1 be mediating fusion in the same way as

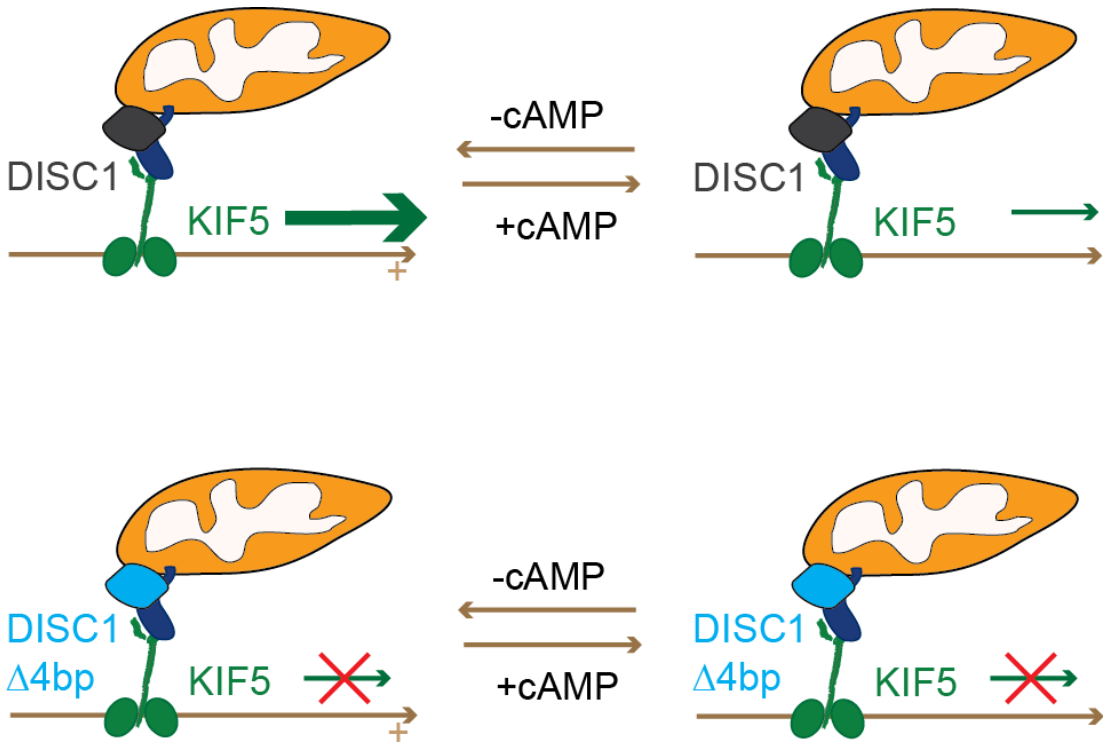


**Figure 6-2 DISC1 regulates mitochondrial trafficking in response to cAMP**

Wild-type DISC1 upregulates mitochondrial trafficking upon a decrease in cAMP. Mutant DISC1 is insensitive to this signal.

trafficking? Or could DISC1 influence a different signalling cascade to regulate mitochondrial fusion separately from trafficking? The prior option may be more likely as Mitofusins are known interactors of Miro and TRAK, thus the trafficking and fusion machineries at the outer mitochondrial membrane are likely present in one complex, containing DISC1 as a regulatory component.

The final aspect of mitochondrial dynamics investigated in this thesis was potential communication with the ER (please see 4.2.6). Expression of the DISC1- Boymaw fusion protein decreased the area of these contacts. This may contribute to impaired calcium communication within the cell – a previously suggested role for DISC1 (Park et al., 2015; Park et al., 2010b). However, the downstream effects of this structural change have yet to be investigated. Calcium imaging with ER and mitochondrially targeted calcium indicators will shed light on any aberrant signalling occurring in cells expressing the DISC1-Boymaw fusion protein. The lesser area of contact sites in these cells could reflect the decrease in mitochondrial transport. The yeast homologue of Miro – Gem1 – has been shown to regulate size of these sites (Kornmann et al., 2011). Perhaps by decreasing Miro-dependent mitochondrial transport, ER coupling is also dysregulated. In addition to transport, ER-mitochondria contact sites are linked to mitochondrial fission – the ER has been shown to localise to mitochondria and form scission sites prior to recruitment of Drp1. How might this decrease in ER-mitochondria contact area contribute to the reported imbalance between fission and fusion? A decrease in ER-mitochondria interface would suggest a lesser area available for Drp1 recruitment, and lower fission rather than decreased fusion activity shown in Chapter 4. Perhaps the equilibrium between fission and fusion cannot be correctly modified upon expression of the DISC1-Boymaw fusion protein.

Taken together, the data presented in this thesis show DISC1 to regulate multiple facets of mitochondrial dynamics, not exclusively trafficking. Moreover, the work described here shows that these dynamics are impaired by a schizophrenia associated mutation of DISC1, raising the possibility that mitochondrial dysfunction is a characteristic of psychiatric disease.

## 6.3 Contribution of DISC1-mediated mitochondrial dynamics to dendritic development

In Chapter 4 it was demonstrated that DISC1-mediated mitochondrial dynamics were essential for dendrite outgrowth. An important question to address is if loss of dendritic complexity is due to insufficient mitochondrial trafficking, or if the decreased mitochondrial trafficking is due to a lesser need for distribution of these organelles in smaller dendritic arbours. The data presented here suggest the former to be the case. Not only was dendritic complexity decreased upon expression of the DISC1-Boymaw fusion protein, but also upon expression of the DISC1-Miro interacting region. This construct acts to inhibit mitochondrial transport via interaction with Miro and TRAK, thus lesser dendritic arborisation is more likely a direct effect of impaired mitochondrial dynamics than distal signalling effects of DISC1. These less complex dendrites may account for the impaired information processing noted in DISC1 mouse models (Kuroda et al., 2011; Kvajo et al., 2008).

DISC1 may also contribute to dendrite outgrowth via multiple other mechanisms, such as microtubule organisation and regulation of protein expression level, as shown for FEZ1 and dysbindin (Lee et al., 2015; Miyoshi et al., 2003). Moreover, DISC1 has a recently characterised role in mRNA trafficking (Tsuboi et al., 2015), and so contributing to local translation, essential for dendritic maintenance (Puram and Bonni, 2013). As described in the introduction, DISC1 is known to facilitate trafficking of other cargoes, for example, presynaptic vesicle precursors. Correct distribution and assembly of these vesicles is essential to normal neuronal, facilitating transmitter release. This is consistent with the presynaptic role for DISC1 proposed by Wen and colleagues (Wen et al., 2014). Therefore, DISC1 may additionally regulate dendrite outgrowth via extrinsic factors if it contributes to synaptic activity, either at the pre or post synapse. From data presented in Figure 3-12, it would seem this synaptic vesicle trafficking aspect of DISC1 function is not altered by DISC1-Byomaw fusion protein expression.

In particular, it would be of great interest to investigate the mechanism by which the DISC1/FEZ1 interaction promotes dendrite outgrowth. FEZ1 has known roles in mitochondrial trafficking (Fujita et al., 2007b). Perhaps DISC1 can regulate

mitochondrial trafficking via FEZ1 to promote dendritic development. Further, might this process be independent of Miro mediated trafficking? If this is indeed the case, DISC1 dependent mitochondrial distribution could be considered a major mechanism for dendritic development and maintenance. Yet, the question remains; what is the role of mitochondria in dendrite outgrowth? Is this process dependent on their local ATP production? Or does local calcium buffering or reactive oxygen species signalling play a role? Recent data suggest ATP production to be a key factor – supply of ATP via creatine was able to promote dendritic development in the absence of mitochondria.

## 6.4 Study of mitochondrial dynamics in iPSC derived neurons

For the last decade there has been intense interest in the development of iPSCs and subsequent disease relevant, human cells. This is of exceptional importance in neurological disease due to the increased complexity of the human brain in comparison to that of the rodent models most often utilised. For example, knockout of neurexin 1 (another genetic candidate susceptibility factor for schizophrenia) gives a mild phenotype in mice. Yet, ablation of this gene in human embryonic stem cell derived neurons shows a severe loss in excitatory currents – highlighting a potential disease mechanism which was not identifiable in mouse models (Pak et al., 2015). There are now two iPSC models of DISC1 mutation. One investigates the effect of the DISC1 4bp deletion, showing deregulation of genes concerned with pre and post synaptic function and a specific presynaptic defect by decreased vesicle release (Wen et al., 2014). DISC1 mutations have also been induced in control iPSCs to decrease DISC1 expression. Subsequent differentiation experiments show subtle differences in fate of mature cells, with a lower tendency to adopt cortical characteristics, such as vGLUT1 expression (Srikanth et al., 2015). These observations mark these cells as useful tools for studying neurodevelopment – crucial to understanding pathophysiology of psychiatric disease. Using schizophrenia patient-derived iPSCs, cytoskeletal remodelling and mitochondrial function have been recently shown to be dysregulated in neural progenitors and derived neurons (Brennand et al., 2015). This finding marks these cells as a promising model system for further investigation of impaired trafficking of mitochondria, or indeed other cargo, as a major contributory factor to neurological disease.

However, there are currently limited data regarding organelle dynamics in iPSC derived neurons. The preliminary data presented in this thesis serve to extend studies by Cooper and colleagues (Cooper et al., 2012), by demonstrating the ability to study mitochondrial dynamics in this novel cell type. This could likely be of substantial significance in future work, as a subtle phenotype has been detected in these cells with regards to DISC1 and cAMP levels. Mutant DISC1 occludes a response to decreased cAMP in terms of mitochondrial trafficking (please refer to Figure 5-7). This observation may have been obscured in the rodent experiments due to the, possibly exaggerated, effect of overexpression. However, polarised neurons have not been generated in this case. Upon further characterisation and improvement of differentiation paradigms, it will be possible to fully understand contributions of a host of psychiatric and neurodegenerative disease factors to aberrant trafficking. Moreover, these cells provide an ideal candidate for drug screens to improve these phenotypes as they can be generated in large quantities, without the need for long term maintenance of rodent colonies (Reinhardt et al., 2013).

Of further interest arising from the experiments presented in Chapter 5 is the possibility of coordination between DISC1 and APP-dependent mitochondrial dynamics. DISC1 affects APP trafficking (Shahani et al., 2015), and interacts with APP during cortical development (Young-Pearse et al., 2010). Additionally, expression of the APP processing enzyme, presenilin1, is regulated by the transcription factor ATF4 – a known DISC1 interactor. Might DISC1 influence amyloid beta generation and so, affect mitochondrial trafficking?

## 6.5 Future Directions

A major question to be determined regards the crosstalk of DISC1 as a mitochondrial trafficking regulator and DISC1 as a regulator of neuronal migration and integration. Aberrant neurodevelopment is a characteristic of psychiatric disease. This has been recently shown to be relevant in the case of the Scottish pedigree with a DISC1 translocation – carriers had a decreased cortical thickness compared to unaffected individuals (Doyle et al., 2015). As described in the introduction, DISC1 has known roles in maintaining the neural progenitor pool – the niche of cells which undergo asymmetric division to provide mature pyramidal neurons of the cortex. This is

dependent on DISC1 phosphorylation, which is higher at E18 than E14 (Ishizuka et al., 2011). The subcellular distribution of phosphorylated DISC1 has yet to be investigated – does the phosphorylation state of DISC1 affect its mitochondrial localisation or trafficking ability?

How might DISC1 influence the trafficking of mitochondria during this neuronal maturation process? And how could this mitochondrial distribution affect neuronal migration and polarity. There is evidence for DISC1 as a mitochondrial trafficking regulator in more mature neurons *in vitro* presented here and in other works (Atkin et al., 2012; Atkin et al., 2011; Ogawa et al., 2014). However, mechanisms regulating mitochondrial trafficking in immature neurons or neural progenitors are yet to be determined and little is known regarding mitochondrial distribution in neuronal migration. DISC1 would be an ideal candidate to mediate mitochondrial dynamics during this phase of development, potentially via interaction with Nde1 – a protein essential to cortical development via centrosome duplication (Bradshaw et al., 2011; Feng and Walsh, 2004). Further, there may be roles for mitochondrial distribution in establishing neuronal polarity (Ikuta et al., 2007), as distribution of these organelles is essential for maintenance of processes (Chen et al., 2007a; Takihara et al., 2015) and organelle distribution influences neuronal polarity. The increased stability of the microtubule cytoskeleton in the developing axon favours kinesin-1 trafficking (Kapitein and Hoogenraad, 2015) conferring differential organelle trafficking, and the centrosome and Golgi are known to influence polarity, promoting axon and dendrite formation respectively (de Anda et al., 2010; Horton and Ehlers, 2003). Additionally, these compartments are not equivocal in terms of mitochondrial trafficking mechanisms; there is growing evidence for differential mitochondrial trafficking regulation in axons versus dendrites. For example, the activity dependent static anchor, syntaphilin, seems to be present exclusively in axons. Furthermore, two independent studies show the adaptor proteins TRAK1 and TRAK2 to be targeted into axons and dendrites respectively (Loss and Stephenson, 2015; van Spronsen et al., 2013), and independently regulate trafficking in this way. Thus, asymmetric mitochondrial trafficking, mediated by DISC1 could contribute to the polarisation and integration of neurons. This would be of great interest to investigate by *in utero* electroporation of the DISC1 dominant negative during migration and examination of the position, structure and mitochondrial distribution of derived pyramidal neurons.

Secondly, it is of great importance to thoroughly investigate aberrations in these processes in psychiatric disease. Data presented in Chapter 4 show an impairment in dendrite outgrowth upon expression of the schizophrenia associated DISC1-Boymaw fusion protein, and show this to be dependent upon mitochondrial trafficking by utilising the identified DISC1 dominant negative. This likely relates to synapse formation and maintenance. This aspect of neurodevelopment is of great importance to study in schizophrenia models as changes in brain circuitry have been frequently reported in psychiatric disease (Brandon and Sawa, 2011). Are these stunted dendrites also depleted of mature, functioning synapses? If this is indeed the case, is this due to insufficient mitochondrial trafficking to these sites, conferring lesser ability to power synaptic communication and buffer calcium? To address the possibility of altered synaptic activity, it would be of great interest to investigate spine size and density, and presynaptic opposition as a measure for neuronal communication ability in cultures expressing the DISC1 dominant negative or DISC1-Boymaw fusion protein. These experiments could be furthered by electrophysiological studies of cultures or slices expressing the DISC1-Boymaw fusion protein. Therefore, how DISC1 contributes to the onset of psychiatric disease via mitochondrial trafficking, and how altered trafficking might give rise to other neurological disease remains a crucial question.

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