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Characterisation of DISC1 Ubiquitination and its potential as a therapeutic intervention for Psychiatric disorders

by

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This thesis is dedicated to my loving parents

Abstract

Since its discovery over a decade ago, DISC1 has become one of the most promising candidate genes for Schizophrenia and associated chronic mental disorders. This notion has been supported by a wealth of evidence from genetic and biochemical studies. With multiple interacting partners, DISC1 acts as a scaffold protein, orchestrating vital signalling pathways that underpin neurodevelopment and signalling. While the aetiology of Schizophrenia is poorly understood, loss of DISC1 protein function remains one of the proposed disease mechanisms. Furthermore, its tendency to form aggregates is reminiscent of neurodegenerative illnesses such as Alzheimer's and Parkinson's disease. C-terminal truncation of DISC1 (TrDISC1) is known to decrease neurite outgrowth and number in the PC12 cell line, abolish protein interaction with proteins such as Ndel1 and also disrupt vital physiological process such as mitochondrial transport. However, very little is known about the underlying disease mechanism at the molecular level.

In order to gain insight in to the role of DISC1 pathway in Schizophrenia and associated mental illnesses, I studied novel post translational modifications of DISC1. The main conclusion of my thesis is that these modifications affect DISC1 turnover and its scaffold function.

In the first part of my thesis, I provided the first direct evidence that DISC1 can be SUMOylated. Peptide array technology was used to SUMOylate and map potential SUMO acceptor lysines on DISC1, *in vitro*. As detecting SUMO conjugates in cells is challenging, I utilised the "Ubc9 fusion directed SUMOylation" method (UFDS) to discover that K643 as the one of the SUMO target sites. Mass spectrometric analysis was employed to corroborate this novel post translation modification on DISC1. Preliminary evidence was also provided that SUMO conjugation of DISC1 obliterates its interaction with DIXIN, while having no effect on Ndel1 binding. This is an interesting finding since DISC1-DIXIN-Nudel form a protein complex to regulate neuronal migration during cortical development. While SUMOylation has been implicated in pathological protein aggregation, a hallmark of many psychiatric illnesses, this work provides an experimental framework to understand DISC1 aggregation which disrupts intracellular mitochondrial transport.

In the second part of my thesis, using biochemical and mass spectroscopy analysis, I demonstrated that DISC1 protein levels are regulated by the ubiquitin proteasome system (UPS). Using an siRNA library screen, Fbxw7 was identified as a key Fbox protein which functions as a key component of an SCF E3 ligase complex that catalyses ubiquitin transfer to the DISC1 protein. Peptide array studies identified a phosphodegron motif on DISC1 which interacts with SCF^{Fbxw7} following a dual phosphorylation event by a yet unidentified kinase. Based on this experimental evidence, a hypothesis was developed that this novel protein – protein interaction may have a great potential for therapeutic intervention to address the loss DISC1 of function, a proposed disease mechanism for SCZ and associated chronic mental disorders. According to my hypothesis, inhibiting SCF^{Fbxw7} mediated ubiquitination of DISC1 stabilises DISC1 which may compensate for the low cellular levels or dysfunctional DISC1 protein.

The proposed hypothesis was addressed in the third section. Using structure activity relation analysis and peptide array studies, a disruptor peptide was developed which interferes with SCF^{Fbxw7} – DISC1 phosphodegron/CPD peptide (Cdc4 phosphodegron). Two lead peptides were identified which successfully stabilised DISC1 protein levels in HEK293 cells. These peptides had no effect on the protein levels of other vital Fbxw7 substrates suggesting their specificity towards DISC1- SCF^{Fbxw7} interaction. I also explored iPS cell technology, which has the potential to provide innumerable patient specific neurons for disease modelling, to test the effect of the lead peptides. My work provides preliminary evidence of DISC1 protein stabilisation following treatment with the lead peptides in the neuronal progenitors differentiated from Schizophrenia patient specific iPS cells.

In the final section, High throughput screens (HTS) were performed to identify non-peptide, small molecule inhibitors of this novel PPI. A quantitative and reliable fluorescence polarization (FP) binding assay was developed and optimized based on SCF^{Fbxw7} – DISC1 phosphodegron/CPD peptide interaction to perform the HTS. The hits identified in these screens require more intense characterisation in secondary screens to validate their effect as modulators of this interaction.

The work described in this thesis has uncovered 2 novel post translational modification and identified the E3 ligase involved in regulating DISC1 turn over. My work has also laid the foundation for the design and discovery of both peptide and non-peptide, small molecule inhibitors of the DISC1-Fbxw7 interaction. These inhibitors can serve as both pharmacological tools and for further investigation of the role of this novel interaction in DISC1 pathway and the vital physiological functions it is involved in. Furthermore, this work also indicates the feasibility of controlled and directed differentiation of patient specific iPS cells in to neurons, which act as a useful tool for disease modelling.

Declaration of Authorship

I declare that, except for where noted, all work contained in this thesis was performed and composed by myself. Where others have contributed to elements of the work, this is stated clearly in the text. No element of this work has been submitted for any other degree of professional qualification.

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Table of Contents

Characterisation of DISC1 ubiquitination and its potential as a therapeutic intervention for psychiatric disorders

List of Figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

APPPENDIX

List of Tables

Abbreviations

Ab	Antibody
AD	Alzheimer's disorder
AKAP	A-Kinase Anchoring Protein
ATF	Activating Transcription Factor
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
bp	Base pairs
BP	Bipolar disorder
BSA	Bovine serum albumin
C	Carboxy terminal
cAMP	Cyclic 3'5'-adenosine monophosphate
CBP	CREB Binding Protein
CNS	Central Nervous System
Co-IP	Co-Immunoprecipitation
CPD	Cdc4 phosphodegron
CREB	cAMP Response Element Binding
DAPI	4',6-diamidino-2-phenylindole
DISC1	Disrupted in schizophrenia 1
DIXDC1	DIX domain containing 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E.coli	Escherichia coli
E18	Embryonic day 18
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
eGFP	Enhanced green fluorescent protein
EGTA	ethylene glycol tetra acetic acid

ENU	N-nitroso-N-ethylurea
Es	Extremely Short Isoform (of DISC1)
Esc	Embryonic stem cells
Esv	Extremely Short Variant Isoform (of DISC1)
FEZ1	Fasciculation of Elongation Factor Zeta 1
FGF2	Fibroblast growth factor 2
FITC	Fluorescein isothiocyanate
FRET	Förster/fluorescence resonance energy transfer
GABA	γ-Amino butyric acid
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GFP	Green fluorescent protein
GSKβ	Glycogen Synthase Kinase 3 Beta
GWAS	Genome Wide Association Study
HD	Huntington's Chorea
HDAC	Histone deacetylase
HECT	Homologous to E6-AP carboxyl terminus
HEK	Human embryonic kidney
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IC_{50}	Half maximal inhibitor concentration
iPSc	Induced Pluripotent Stem cells
Jnk	C-Jun N-terminal kinase
K_d	Dissociation constant for ligand binding
K_i	Dissociation constant for inhibitor binding
K_M	Michaelis constant (Concentration of substrate leading to half maximal enzyme velocity)
KO	Knock Out
L	Long isoform (of DISC1)
LB	Lysogeny broth
LIS1	Lissencephaly 1
Lv	Long Variant isoform (of DISC1)
MAP2	Microtubule Associated Protein 2
MEF	Mouse Embryonic Fibroblasts

MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
MS	Mass Spectroscopy
mTOR	Mammalian Target of Rapamycin
N	Amino terminal
NDE1	Nuclear Distribution Factor Element 1
Ndel1	Nuclear distribution protein nudE-like 1
NGF	Nerve Growth Factor
NLS	Nuclear Localisation Signals
NMDA	N-Methyl-D-Aspartic Acid
NPs	Neuronal Progenitors
NRG1	Neuregulin 1
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	Parkinson's disorder
PDE	Phosphodiesterase
PDE4	Phosphodiesterase-4
PKA	Protein Kinase A
PML	Promyelocytic Leukemia
PPI	Protein-Protein Interaction subunit
PSD95	Post Synaptic Density
PSD95	Post Synaptic Density 95
PTM	Post Translational Modifications
RING	Really Interesting Gene
SAE	SUMO activating Enzyme
SCF	Skp-Cullin-Fbox E3 ligase
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphism
STAT1	Signal Transducers and Activators of Transcription
SUMO	Small Ubiquitin like Modifier
TG	Transgenic
TNIK	TRAF2- and NCK interacting Kinase

Tris	Tris (hydroxymethyl) aminomethane
Ubiquitin	Ubiquitin
UFDS	Ubc9 Fusion Directed SUMOylation
UPS	Ubiquitin Proteasome System

Units

Da	Dalton
gm	Gram
l	Litre
m	Metre
M	Molar
nm	Nano Meters
^{o}C	Degrees Centigrade
V	Volt

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
mD		N methylated Aspartic acid
mE		N methylated Glutamic acid
N	Asn	Asparagine
P	Pro	Proline
	Pip	(S)-N-Fmoc-piperidine-2-carboxylic acid

pS		Phospho Serine
pT		Phospho Threonine
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
	Sar	Sarcosine, L-β-homoalanine
T	Tyr	Tyrosine
	Tic	N-FMOC-L-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid
W	Trp	Tryptophan
βD		β Aspartic acid
βE		β Glutamic acid

1.

Introduction

1.1 Schizophrenia

Schizophrenia (SCZ) is a chronic and debilitating mental illness, affecting nearly 1% of the worldwide population (Perälä et al., 2007). It is mainly characterised by a condition known as psychosis, which is a hallmark of the onset of SCZ. Symptoms are primarily classified as positive symptoms, such as altered behaviour and thoughts, hallucinations and/or delusions and negative symptoms such as lack of motivation and interest, poor speech. On the other hand, it is also believed that other factors such as behavioural and environmental influences may also contribute collectively to the development of this disease.

Behavioural and genetic studies over a wide range of populations have implied that the incidence of SCZ is often prevalent in families, which have a high incidence of other mental illnesses. It has been established the risk of developing SCZ is directly related to the biological association to an affected individual (Middleton et al., 2002). Several studies show that males have a higher incidence of SCZ than females although this difference was not observed at ages younger than the age of 17 (Jablensky et al., 1992, Jablensky, 2000, McGrath et al., 2004, Kleinhaus et al., 2011). This difference may be attributed to the differential neurodevelopmental processes in the sexes, a notion supported by evidence provided by imaging studies (Lenroot et al., 2007). However, nearly 60% of patients with SCZ have neither first nor second degree relative(s) with the

disorder which highlights the complexity of the disease (Gottesman and Erlenmeyer-Kimling, 2001). Linkage studies have established an association between SCZ susceptibility and loci associated with brain development such as 22q11–13, 6p, 13q, and 1q21–22 (Pulver, 2000). This also supports the belief that SCZ is a developmental disease. Although it is inheriTable, so far, no particular pattern of inheritance has been reported , possibly due to the complex interaction between genetic and environment factors. Stress and smoking were also named as possible risk factors for SCZ (Hays, 2000). However, these factors don't explain all the symptoms of the disease. In the absence of a specific diagnostic marker, SCZ is mainly diagnosed by a comprehensive mental health evaluation by health care professionals.

Anatomical abnormalities such as reduced relative head circumference was reported at birth in individuals who later developed this disorder (Lewis and Levitt, 2002). Researchers reported significant cortical thinning, reduction in grey matter density and an increase in lateral and third ventricle volumes. However, MRI (magnetic Resonance Imaging) studies failed to reach a statistical significance in the reduction of total brain volume In affected individuals (Shenton et al., 2001). Other structural abnormalities in cerebral volume, glial processes, neurite lengths and synapse number have also been reported in SCZ patient brains (Bertolino et al., 1996). Despite much research, much remains to be discovered about the underlying molecular events that lead to the pathophysiology of this illness.

Several research groups identified many candidate susceptibility genes that confer considerable risk for SCZ and associated psychiatric illnesses. Various hypothesis have been proposed linking SCZ to dopamine receptor signalling (Harrison, 2000, Bertolino, 1999, Grima et al., 2003) and serotonin (Slowik, 1967, Robledo, 1961). To date, nearly 100 genes have been proposed to be possible susceptibility factors, though only very few have been verified in follow-up studies. The first publication identifying 'DISC1' as a possible genetic factor for SCZ triggered much interest in gaining a better understanding of the molecular mechanisms behind its signalling role in neurons (Millar et al., 2000c).

1.2. DISC1 (Disrupted In Schizophrenia)

DISC1 was first discovered as a potential candidate susceptibility gene for SCZ, when a balanced translocation (1; 11) (q42:q14) was identified in a large Scottish family with a number of psychological disorders (Millar et al., 2000a). Chromosomal translocation can be defined as an event in which a segment of a chromosome is detached and interchanged with a different chromosome segment. This can be detected by karyotyping. Of all the family members karyotyped, nearly a third of the members carrying the truncation have been diagnosed with various psychological disorders, including SCZ, bipolar disorder and unipolar depression. LOD (linkage of the odds ratio) of 3.6 and 4.5 has been achieved for SCZ and schizoaffective disorders respectively (Blackwood et al., 2001). However, the disease penetrance is insignificant as certain family members diagnosed with the psychiatric disorder carried a normal karyotype (Blackwood et al., 2001). Another subgroup within the family also included translocation carriers, which showed no abnormality in mental state with no known history of psychiatric illness. This suggests that the translocation may not lead to major disturbances in brain function in all the members (Blackwood et al., 2001).

Published in 2000, Miller and colleagues cloned the gene directly disrupted by the translocation and named it as DISC1 (Disrupted in SCZ). A non-coding antisense RNA to the DISC1 locus was identified and named as DISC2. It was hypothesised that this antisense RNA could be involved in regulating the DISC1 gene expression (Millar et al., 2000c). Numerous studies have confirmed these original observations that link disruption in the DISC1 gene with SCZ (Millar et al., 2001, Chubb et al., 2008, Devon et al., 2001, Millar et al., 2000c, Brandon et al., 2004, Clapcote et al., 2007). The DISC1 locus has been found to be associated with SCZ, bipolar disorder, autism, depression and other associated psychiatric illnesses (Song et al., 2010, Hennah and Porteous, 2009).

Population based case studies identified ultra-rare mutations, which were found to be associated with a 2% risk for SCZ (Song et al., 2008). Three common amino acid variants R264Q, L607F and S704C were identified to be associated with the risk of SCZ and

associated disorders (Chubb et al., 2008, Bradshaw and Porteous, 2012). In particular, the DISC1 SNP S704C has been shown to be associated with SCZ and major depression in several populations (Callicott et al., 2005, Hashimoto et al., 2006). Additionally, studies reported that F607 allele carriers had significant morphological abnormalities, such as reduced grey matter in superior frontal gyrus and anterior cingulate cortex in F607 homozygotes, while S704C variants exhibited severe positive SCZ symptoms. In light of this data, it is surprising that none of the genome wide association studies identified a common DISC1 variants that may independently increase the risk of developing SCZ or its associated illness. However, the precise underlying biology still needs to be elucidated (Nakata et al., 2009).

1.2.1. DISC1 structure

Although DISC1 has been shown to be associated with number of mental disorders, not much information is available about its tertiary structure. Therefore, it becomes increasingly difficult to understand the physiological effects of several reported mutations, truncations and SNPs in DISC1. However, genomic DISC1 structure is well conserved in low organisms including macaque (*Macaca mulatta*), mouse (*Mus musculus*), rat (*Rattus norvegicus*) zebrafish (*Danio rerio*) and pufferfish (*Fugu rubripes*) (Chubb et al., 2008).

Located on chromosome 1, the human DISC1 gene spans over 415 kbp with 13 exons. Exon 9 is the largest and covers about a third of the entire gene in humans. The size of this exon is relatively conserved in other species and this may be the evidence that this exon is functionally significant (Chubb et al., 2008). The Scottish translocation occurs in exon 8, causing exons 9 to 13 to move to chromosome 11. As depicted in Figure 1.1, the full length human DISC1 protein has been hypothesised to have a globular N terminus (encoded by exons 1 and 2), a α helical and coiled-coiled region in the C terminus which carries many protein interaction sites (Millar et al., 2000c, Chubb et al., 2008, Ma et al., 2002b). C terminus is more conserved within species compared to the N terminus. C terminus contains self-association domains which lead to formation of stable dimers (Leliveld et al., 2009). Sequence analysis studies revealed the presence of conserved nuclear localisation signal (NLS) in the N terminus.

Nearly 40 splice variant transcripts of DISC1 have been reported so far. However, no clear information is available about their existence at the protein level. Characterised DISC1 protein isoforms in humans include Long (L) 854 amino acids (100kDa); Long Variant (Lv) 832 amino acids (~98 kDa); Short (S) 678 amino acids (~75kDa). EST (Extension Sequence Tags) studies show that L and Lv forms are conserved across various species (Taylor et al., 2003). RT-PCR analysis of mRNA levels in post mortem brains of adult humans show high levels of full length DISC1 and low levels of other variant transcripts (Lipska et al., 2006). Antibodies raised against different regions of DISC1 detect number of bands at various molecular weights using Western blotting techniques. The bands observed at higher molecular weights have been speculated to be multimers of various isoforms or post transnationally modified isoforms (James et al., 2004b). Oligomerisation of DISC1 was initially proposed by Brandon et al (Brandon et al., 2004). Narayanan and colleagues reported biochemical characterisation of full length DISC1 oligomerisation using size exclusion chromatography and analytical ultra-centrifugation studies (Narayanan et al., 2011). Their studies show that although the common variant S704C, tends to form higher molecular weight oligomers, this SNP has no effect on its interaction with Ndel1 (Narayanan et al., 2011). Residues 403-504 on DISC1 were identified by biochemical studies on region specific DISC1 mutants and were shown to constitute a self-association domain (Kamiya et al., 2005a). Immunoreactive, detergent insoluble DISC1 aggregates were identified in nearly 20% of post mortem brains with chronic psychiatric illnesses (Leliveld et al., 2009).

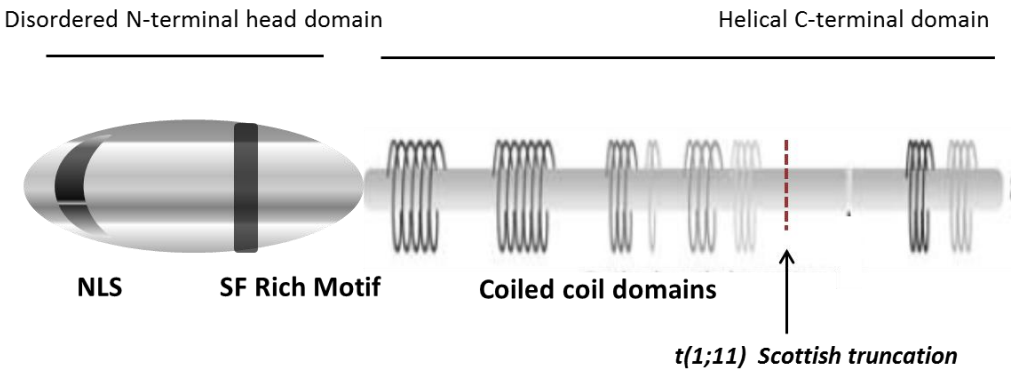


Figure 1.1 Schematic representation of proposed DSIC1 tertiary structure. Adapted from (Chubb et al., 2008, Soares et al., 2011), this picture shows largely disordered N terminal head domain, which harbours a nuclear localisation signal (NLS), SF motif (Ser and Phe rich). The C terminal domain consists of a series of coiled-coiled

domains which were implicated in protein interactions and self-association. Translocation break point t(1;11) is indicated with an arrow (at amino acid position 597).

1.2.2. DISC1 tissue Expression and sub cellular localisation

Though DISC1 is mostly discussed in the context of neuronal signalling, its expression is not just confined to brain but ubiquitously expressed in various other tissues. Placenta, kidneys and heart have the highest expression of DISC1 transcripts. Abundant DISC1 has been detected in the dentate gyrus of adult human brain by James et al (2004), although relatively low expression has been detected in hippocampal pyramidal cells (James et al., 2004a, Millar et al., 2000b). Northern blotting studies revealed the presence of various DISC1 transcripts in various parts of the brain (Millar et al., 2000b). Similar DISC1 transcript expression patterns have been reported in mouse brain (Ma et al., 2002c). The consistent observation of prominent expression of DISC1 transcripts in hippocampus across species is interesting as the hippocampus is associated with a number of neurocognitive and other behavioural disorders (Chubb et al., 2008). DISC1 expression has also been observed in cerebral cortex, sub ventricular zone and olfactory bulb (Ma et al., 2002a, Schurov et al., 2004b, Miyoshi et al., 2004). DISC1 co localises with Nestin and SOX2 in these regions, which are sites for neurogenesis. This suggests a role of adult neurogenesis in the pathophysiology of SCZ (Mao et al., 2009a), (Schurov et al., 2004a, Kim et al., 2012).

DISC1 protein localisation in mammalian cell lines and cultured neurons has also been investigated by several groups. DISC1 was shown to be localised to multiple subcellular compartments such as the centrosome (Miyoshi et al., 2004), microtubules (Morris et al., 2003), mitochondria (James et al., 2004a) nucleus (Sawamura et al., 2005a) and synapse (Camargo et al., 2007). It was observed that the localisation varied with the cell type and the antibodies employed for probing. Co-localisation of overexpressed DISC1 with PSD95 in primary cultures of hippocampal neurons (Bradshaw et al., 2008), presence of 200kDa DISC1 protein in post synaptic density cell fractions and the presence of other major DISC1 interacting proteins in the synaptic fractions strongly supports DISC1 localisation as an important factor for synaptic signalling. Notably, synaptic dysfunction has also been

implicated in SCZ (Harrison and Weinberger, 2005). In spite of the data described above, the two major sites of DISC1 verified by many groups was mitochondria (Atkin et al., 2011, James et al., 2004a, Millar et al., 2005a) and nucleus (Sawamura et al., 2008, Brandon et al., 2005, Ma et al., 2002a). Punctate distribution of endogenous DISC1 has also been reported in mouse cortical neurons, a distribution, which has been replicated by overexpressed C terminally GFP tagged DSIC1 in HeLa cells and mouse cortical neurons (Brandon et al., 2005). Immunocytochemistry studies confirmed the localisation of DISC1 with cytoskeleton proteins α-tubulin and MAP2 (Microtubule Associated Protein 2) in cultured mouse neurons (Brandon et al., 2005). Multi subcellular localisation of DISC1 implicates its involvement in diverse cellular processes mediated by multiple interacting partners.

1.2.3. DISC1 in Neuronal signalling and development

DISC1 possess multiple interacting partners that demonstrate its pathological relevance and represent potential for therapeutic intervention. Initial studies that identified a number of putative DISC1 interacting partners utilised yeast -2-hybrid screens (Ozeki et al., 2003a) (Camargo et al., 2007). Many of these interacting partners have been validated by several groups using a range of biochemical techniques.

Most of the interacting partners can be now be categorised in groups of proteins involved in: cytoskeleton regulation, cell cycle control, signal transduction and central nervous system signalling. DISC1 forms immune complexes with F actin (Miyoshi et al., 2003b), alpha tubulin (Brandon et al., 2004), MAP2 (Brandon et al., 2005) suggesting its association with cytoskeleton. Upon analysis of the DISC1 sequence, it was found to be similar to those involved in cytoskeleton and transport. Many cytoskeletal proteins are involved in signalling pathways at the synapse, thereby increasing the chances of DISC1 playing a role in neuronal signalling pathways and suggesting a contribution of DISC1 to neurological disorders. DISC1 and mitofilin association has been noted in human cell line and culture mouse neurons (Park et al., 2010). DISC1 knockdown directly effects mitofilin stability and activity of mitochondrial enzymes like NADH dehydrogenase (Park et al., 2010).

Several groups independently characterised interacting partners of DISC1 such as LIS1, Ndel1 and Nde1. These are crucial centrosomal proteins, which are involved in neuronal migration, neurogenesis and neurite outgrowth (Bradshaw et al., 2009, Kamiya et al., 2005a). Dishevelled axin (DIX) domain containing 1 (DIXDC1) is another DISC1 interacting partner and DISC1-DIXIN complex is known to modulate neuronal proliferation and migration via Wnt/GSK3β/ β-catenin signalling (Mao et al., 2009b, Singh et al., 2010). While knockdown of DISC1 decreases hippocampal progenitor proliferation, DISC1 over expression has the opposite effect. This effect was also known to be mediated by Wnt signalling pathway of which GSK3β is one of the major component. The kinase activity of GSK3β is inhibited upon its interaction with the C terminus region of DISC1, leading to stabilisation of β catenin protein which translocates in to the nucleus to modulate translocation of various neurodevelopmental genes (Bradshaw and Porteous, 2010, Ming and Song, 2009). This has pharmacological relevance as lithium chloride, a GSK3β inhibitor has been widely employed in management of bipolar disorder (Ming and Song, 2009).

The DISC1 interactome contains a number of proteins specifically localised at the synapse. TNIK (TRAF2- and NCK interacting Kinase) is one of the PSD (Post Synaptic Density) localised DISC1 interacting partners whose activity is inhibited when in complex with DISC1 (Bradshaw and Porteous, 2012). Kal7 which encodes multifunctional Rho GDP/GTP exchange factor, is one of the candidate genes whose transcripts were found at reduced levels in SCZ patients (Hill et al., 2006) and an independent study identified KALRN locus (includes all KAL isoforms) to be associated with SCZ in a GWAS in Japanese population (Mandela et al., 2012),(Ikeda et al., 2011).

DISC1 role in cAMP signalling is emphasised by its interaction with PDE4enzymes. PDEs, a large family of phosphodiesterases are the only means of degrading cAMP, a crucial secondary messenger in many physiological pathways (Houslay et al., 2005). Mammalian PDE4 enzyme family consists of four gene subtypes with multiple isoforms constitute 4 sub family (PDE4A, PDE4B, PDE4C, and PDE4D)(Houslay, 2001). PDE4 inhibitor, rolipram

was elucidated to exhibit anti-psychotic effects in mice. In the absence of PDE4 subtype specific inhibitors, knockout mice were generated to understand the contribution of each subtype to the rolipram mediated effects (Zhang et al., 2002). Deficiency of PDE4D gene produced antidepressant-like effects suggesting that this subtype mediates the effects of PDE4 inhibitor (Zhang et al., 2002). Biochemical studies also established a direct interaction between PDE4 isoforms and DISC1. PDE4 isoforms were co-immunoprecipitated with 100kDa isoform of DISC1 and using peptide array studies, PDE4 binding sites on DISC1 have been mapped (Murdoch et al., 2007). A more recent study elucidated that ATF4-DISC1 act as repressor complex and regulates PDE4D9 transcription (Soda et al., 2013). Using ChIP-PCR, it was determined that DISC1 binds to PDE4D gene locus via ATF4, as it lacks DNA binding domain. Increased levels of PDE4D9 transcripts and protein levels were reported in embryonic mice brains in which ATF4 and DISC1 were knocked down using shRNA technology (Soda et al., 2013). PDE4D9 was also shown to co-localise with DISC1 in the cytosol in cultured hippocampal neurons, emphasising the involvement of DISC1 in cAMP regulated pathways (Soda et al., 2013). DISC1 interacting partners in the context of neural signalling and development are discussed in depth in this review article (reviewed in Bradshaw and Porteous (2012)). As described by Costas et al, DISC1 can be best termed as a 'hub' protein forming a centre point for the orchestration and regulation of several cellular pathways and exerting its function by protein-protein interactions (Costas et al., 2013). Interactors of DISC1 and epistatic gene regulation between them have also been shown to be associated with SCZ risk (Millar et al., 2005b, Burdick et al., 2008).

The role of DISC1 in psychiatric illness has also been highlighted by a number of genetic studies in mice. Although it is impossible to model positive, negative and cognitive symptoms entirely in a mouse, models based on single endophenotype can be developed. To date, seven different mouse models have been generated to investigate the outcome of altered DISC1 function. A study by Clapcote et al, reported depression-like behaviour in mice with ENU induced Q31L mutation, while mice with the L100P mutation exhibited SCZ-like behaviour. Notably, both had reduced brain volumes, even though they all expressed normal levels of all DISC1 isoforms (Clapcote et al., 2007). It is interesting to note that both the above mutations fall in the region on DISC1 known to be involved in

PDE4B interaction (Murdoch et al., 2007). Both the mutants have shown reduced binding to PDE4B while lower PDE4B enzyme activity was reported only in Q31L mutants (Clapcote et al., 2007). PPI (prepulse inhibition) deficit was observed in both these mutants, as seen in patients with certain psychiatric illness.

A mutant mouse strain carrying two termination codons in exon 7 and 8 and a premature polyadenylation site in intron 8 were developed (BAC-DC Tg mice). This led to complete elimination of the 100kDa and 70kDa DISC1 isoforms, while only a low level of the truncated DISC1 protein product was detected using Western blot analysis (Kvajo et al., 2008). Histological studies confirmed reduced cerebral cortex volume and enlarged lateral ventricles, while reduced neuronal proliferation and neurogenesis was also reported in this model (Jaaro-Peled, 2009). Three other models have been generated in which the N terminal DISC1 peptide (1-597 residues, TrDISC1) was overexpressed, potentially replicating t(1;11) translocation (Pletnikov et al., 2008). A mouse model which expresses the C terminal DISC1 peptide (671-852 residues) carrying crucial binding sites for Ndel1 and LIS1 has also been generated (DISC1-cc transgenic mice) (Li et al., 2007). All these mouse models were intensely characterised for behavioural, histological and biochemical abnormalities, as summarised in following review papers - (Jaaro-Peled, 2009, Kellendonk et al., 2009). However, the simplest approach, a knockout DISC1 mouse, has proven difficult to produce due to the complexity involved in DISC1 isoforms which are not fully characterised yet.

1.3. Post Translational Modification (PTM)

Post-translational modification (PTM) is the covalent attachment of additional chemical groups, lipids, sugars or polypeptides to a protein mediated by various enzyme cascades (Walsh et al., 2005). Translated proteins are folded and subjected to certain enzyme catalysed modifications on their amino acid side chains or peptide backbone. Nearly 5% of the genome encodes for enzymes that catalyse PTMs such as ligases, kinases, phosphatases, and hydrolases (Walsh et al., 2005). PTM may involve covalent additions of a co-factor or a co substrate. Examples include ATP-dependent phosphorylation and

acetyl CoA dependent acetylation (Walsh et al., 2005). The other type of PTM involve covalent conjugation of 8 – 10 kDa such as ubiquitin or ubl (ubiquitin like) to the side chain of amino acids on the target substrates, examples include SUMOylation and Ubiquitination which are reviewed under sections 1.4 and 1.5 respectively.

These modifications individually or collectively increase the functional diversity within the proteome. They most often involve a cascade of enzymes, which in turn are tightly regulated by either another post translational modification, spatial or temporal separation. They are critical in determining the protein fate – conformational changes, stability, activity (for enzymes) and sub cellular localisation. However, it should be noted that PTMs can occur at any stage during or at the end of the life time of a protein. In most of the cases, they also regulate the cellular signalling cascades responding to extracellular stimulus, as in the case of the CNS where synaptic communication between neurons is tightly regulated (Wilkinson et al., 2010). PTMs have been implicated in cardiovascular disease, psychiatric disorders and cancer (Anderson et al., 2009, Ciechanover and Iwai, 2004).

To date, phosphorylation was the only PTM, reported on DISC1 using mass spectroscopy studies. Thr50, Ser58 and Ser713 are three phosphorylation sites on DISC1 identified, in which the latter two sites are conserved in mouse (Ser54 and Ser710 respectively) (Ishizuka et al., 2011c). Site directed mutagenesis and *in vitro* phosphorylation assays found that PKA and Cdk5 phosphorylated hDISC1 fragment at Ser713 (Ser710 in mouse). This phosphorylation has also been reported to influence the progression of progenitor proliferation to post-mitotic neuronal migration. Unphosphorylated form of DISC1 preferentially bind to GSK3β and modulates Wnt signalling. In converse, phosphorylated DISC1 forms specifically co-localise with BBS proteins (Bardet-Biedl syndrome) at the centrosome (Ishizuka et al., 2011a). A previously identified PKA mediated phosphorylation site on hDISC1, Ser58 (Ishizuka et al., 2011c) was known to influence nuclear localization of DISC1 in HeLa cells (Soda et al., 2013, Ishizuka et al., 2011c). Phosphorylated WT DISC1 Ser58 was predominantly cytosolic, while the mutant DISC1

S58A was nuclear in the presence of okadaic acid, a serine/threonine phosphatase. This phosphorylation event also reduced DISC1 interaction with ATF4 (Soda et al., 2013).

1.4. SUMOylation

SUMOylation is a post translational modification which involves a covalent attachment of SUMO proteins (Small Ubiquitin like Modifier) on to substrate. SUMO (also known as GMP1, UBL1, PIC1) proteins are present in all eukaryotes with and their homologues in yeast are called Smt3 and Pmt3 (Hay, 2001). Human genome encodes 4 SUMO proteins (SUMO 1 – SUMO 4). So far, only three of the SUMO proteins have been characterized. SUMO1 is the best-studied isoform of this family. SUMO 2 and 3 isoforms differ only by three residues at their C terminus and so collectively they are referred as SUMO 2/3. The functional role of SUMO4 has not yet been elucidated. SUMO proteins are about 10 kDa in size and resemble ubiquitin in their three dimensional structures, however, sequence alignment shows less than 20% similarity with the ubiquitin. A flexible 20 amino acids tail is absent in ubiquitin and over all surface charge distribution is also significantly different (Hay, 2005). Like ubiquitination, SUMOylation involves an active enzymatic cascade where the SUMO moiety (single or as chains) is/are covalently added on to ε-amino group of lysine residues that present on the surface of substrate proteins.

Figure 1.2, represents a schematic of SUMO conjugation cycle. SUMO proteins are expressed in immature forms, which are processed by the protease activity of SUMO specific isopeptidases (SNEPs). In this processing step, variable stretch of the C terminus (4, 11 and 2 amino acids from SUMO 1, 2, 3 respectively) is cleaved off, to expose the glycine –glycine domain. Following this action, the protein is considered as the matured SUMO moiety. This SUMO maturation step is followed by its activation by a heterodimeric E1 enzyme (SAE1–SAE2 in humans/Uba2–Aos1 in S. cerevisiae) that uses ATP to adenylate the C-terminal Gly residue of SUMO. This results in formation of a thioester bond between the C-terminus of SUMO and a Cys residue in active site of SAE2, releasing AMP (Hay, 2001, Meulmeester and Melchior, 2008). Next step involves the

transfer of SUMO from E1 enzyme, SAE1-SAE2 to catalytic Cys residue on Ubc9 (SUMO E2 enzyme) resulting in a thiol-ester bond formation. Ubc9 then transfers the SUMO to the substrate in the final step. An iso peptide bond is formed between the ε amino group of the target lysine and the C terminal glycine of SUMO. This transfer is sometimes facilitated by SUMO E3 ligases which act as adaptor proteins and docking stations (Wilkinson and Henley, 2010) that increase the fidelity of the SUMO conjugation. The exact role of SUMO E3 ligases in this step has been the subject of debate and a firm consensus has not been reached. SUMO E3 ligases are mainly characterised by the presence of a SP-RING (SUMO Protease- Really Interesting Gene of Interest) motif, which is similar to the Ubiquitin RING E3 ligases (discussed under ubiquitin E3 ligase). SP-RING E3 ligases bind to Ubc9 and SUMO non-covalently via SIM/SBM (SUMO Interacting motif/SUMO Binding Motif), functioning as adaptor molecules that act to position the SUMO loaded Ubc9 and the substrate protein in a complimentary position for SUMO transfer (Wilkinson and Henley, 2010, Meulmeester and Melchior, 2008).

One distinct group of SUMO E3 ligases is the PIAS family proteins (Protein Inhibitor of Activated STAT (Signal Transducer and activator of Transcription), which are well characterised. Five of these enzymes have been found in mammals (PIAS1, PIAS3 and the splice variants PIASα, PIASβ and PIASγ (Wilkinson et al., 2010). Nucleoporin RanBP2 also functions as SUMO E3 ligase (Pichler et al., 2002) which has many substrates including Sp100, HDAC4 and PML (Wilkinson et al., 2010). Unlike ubiquitination, where E3 ligases confer substrate specificity, SUMO-1 conjugation to IκBα and RanGAP1 can occur in a purified system only in the presence of SUMO-1, SAE, Ubc9 and the substrate. This suggests that E3 ligase may not be required for SUMO conjugation *in vitro* (Blomster et al., 2010, Hay, 2001).

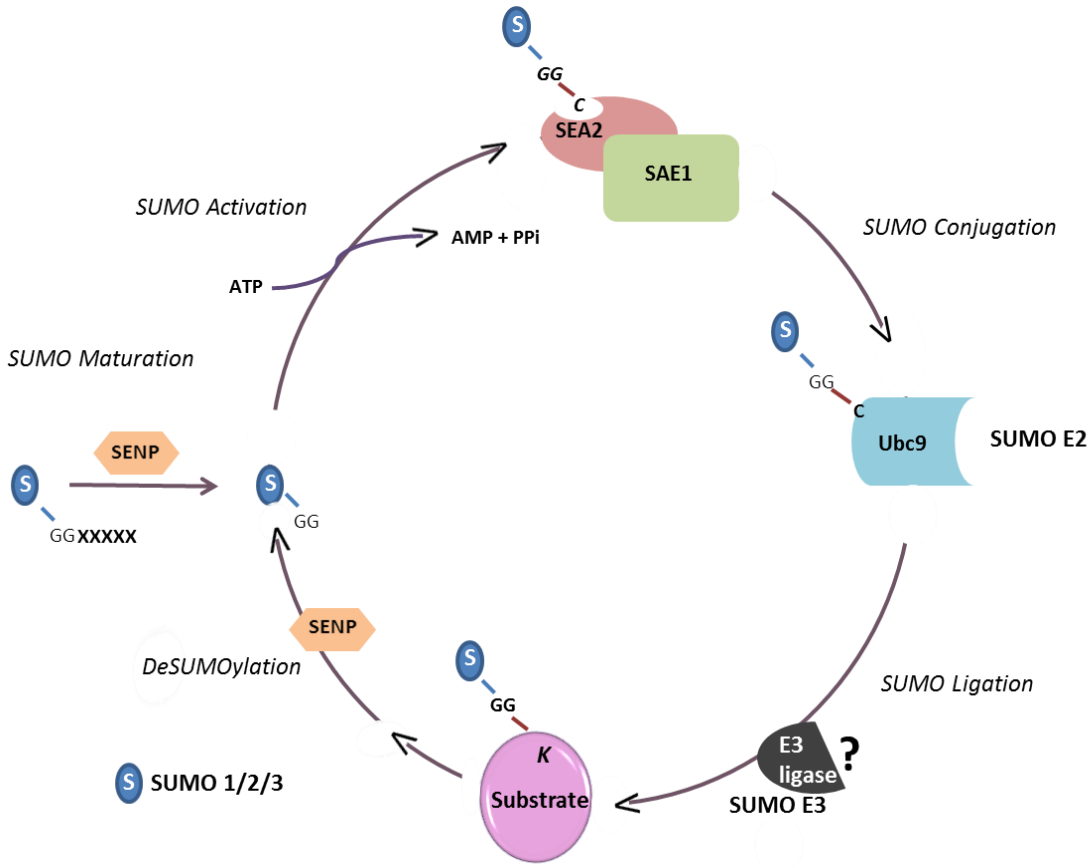


Figure 1.2 Schematic diagram representation of SUMO conjugation cascade. SUMO moieties are processed by removal of an inhibitory C-terminal extension, known as maturation, catalysed by SUMO-specific proteases (belong to enzyme class-hydrolases). With the C-terminal carboxyl groups cleaved, SUMO is then attached to ε-amino group(s) on Lys residues on the target substrate proteins by an active, ATP-dependent enzymatic cascade. SUMO is activated by the E1 enzymes SAE2–SAE1 and is then transferred to the E2 enzymes, Ubc9. The target specificity of SUMO may be dictated by the presence of a ψKxE/D motif in the substrate that is recognized by the E2–SUMO conjugate. The role of E3 ligases in SUMO cascade is still a debate unlike in ubiquitination (represented as ?). Deconjugation reactions are mostly catalysed by SUMO-specific proteases that cleave catalyse the maturation step of SUMO moieties.

Unlike the case for ubiquitination, SUMOylation generally occurs at a well-defined consensus sequence. The SUMO acceptor site has been identified after mapping a number of target SUMOylation sequences like RanGAP1, PML, C-Jun and P53 (Rodriguez et al., 2001). The SUMOylation consensus motif has been identified as 'ψKxD/E' (ψ is a hydrophobic amino acid such as lysine, Isoleucine, Valine or Phenyl alanine, x is any amino acid) (Rodriguez et al., 2001). Nearly 75% of the known substrates are known to be SUMO conjugated within this consensus site. However, some substrates get

SUMOylated at lysine residues outside this consensus motif by “atypical” SUMO acceptor lysines (Hoege et al., 2002). Hence, the presence of ‘ψKxD/E’ can be used as a predictor but is not a definitive indicator of SUMOylation (Wilkinson et al., 2010). In addition to the covalent SUMO conjugation to an acceptor lysine, recent studies have identified motifs on substrates that mediate non covalent interactions with SUMO proteins (Minty et al., 2000, Hecker et al., 2006). These motifs are known as SIMs (SUMO Interacting Motifs) which are found to contain a hydrophobic core flanked by acidic residues within the general consensus sequence V/I-x-V/I-V/I or V/I-V/I-x-V/I/L, where position two or three can be any amino acid (V,I and L are single letter representation of Valine, Isoleucine and Leucine respectively) (Song et al., 2004). Since the present study focuses on the covalent SUMO interactions, it has not been possible to detail SIMs in this review. SIMs have been reviewed in Song et al. (2005).

Initially on the basis of immunocytochemical studies, it was suggested that SUMOylation occurs primarily in the nucleus or perinuclear space (Scheschonka et al., 2007). Subsequently, most of the SUMOylated proteins were found localised in the nucleus while the SUMO machinery was found distributed both in the nucleus and cytoplasm. Nuclear SUMO conjugates include transcription factors, nuclear pore proteins and proteins involved in maintaining genome integrity. Recent evidence of reported SUMOylation of plasma membrane proteins such as ion channels (K^+ channel) and GPCRs (G-protein-coupled receptors) also suggests the presence of SUMO components at the plasma membrane (Scheschonka et al., 2007).

1.4.1. Molecular consequences of protein SUMOylation

Covalent linkage of a 10kDa SUMO protein moiety to the protein substrate may have one or more consequences (schematic in Figure 1.3) - (a) SUMOylation may block interaction sites on the target protein either by directly competing with the lysine residue which is also targeted by acetylation, methylation or acetylation cascade or by stearic hindrance caused by the SUMO moiety (Pichler et al., 2002), (b) SUMO conjugation may add additional binding surface (sometimes including its own residues) to substrates therefore

attracting new interacting partners in a SUMOylation dependent manner (Matunis et al., 1996). (c) SUMOylation may cause conformational changes to the target protein uncovering previously masked binding sites (Hay, 2001). The downstream effects of protein SUMOylation (in neurons), mediated by the above mechanisms are detailed in the next section.

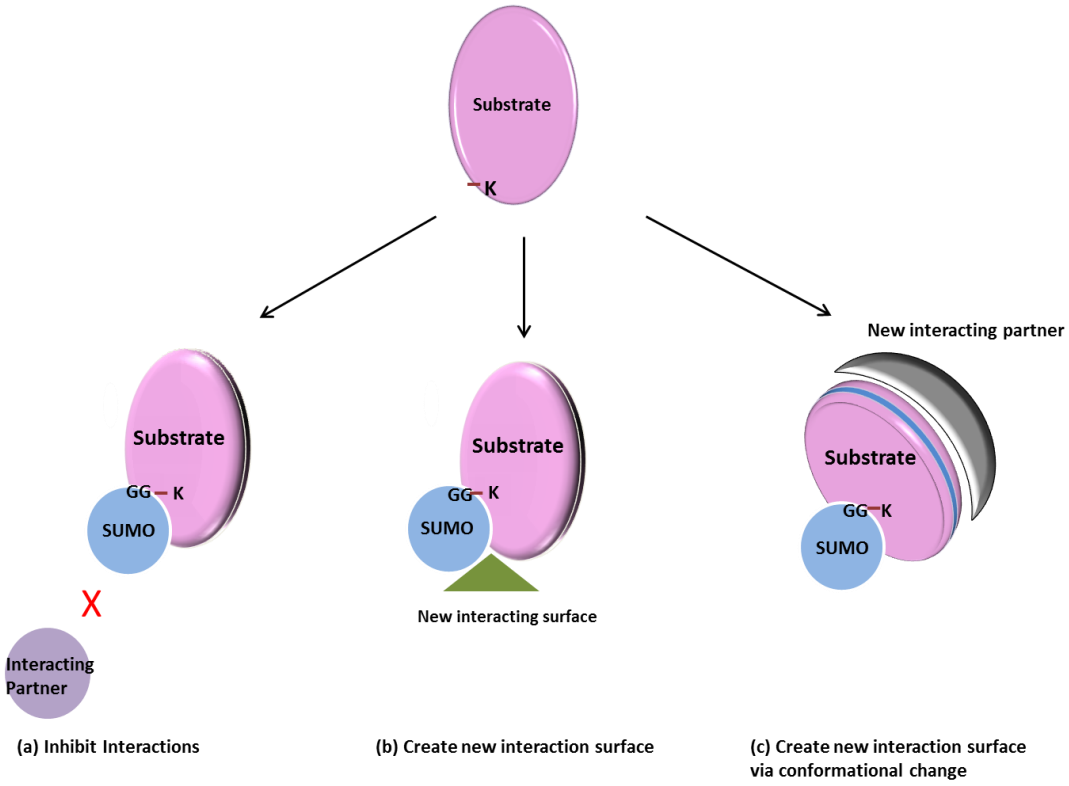


Figure 1.3 Schematic representation of possible molecular consequences of protein SUMOylation
(a) Inhibit interactions: Stearic hindrance caused as a result of covalent attachment of nearly 8kDa protein on to the target will result in abolishing protein interaction either covalent or ionic. (b) New interaction surfaces involving SUMO protein: SUMO moiety on the protein may create new surface(s) which facilitate new interactions. (c) Induce a conformational change: A possible conformational change as a result of SUMOylation may aid result in new interactions.

Protein SUMOylation is a dynamic, complex and reversible process. SUMO moieties covalently attached to the substrates, are recycled by the action of deSUMOylation enzymes. A single gene family encoding six SUMO specific Cysteine proteases has been identified in humans known as Sentrin specific proteases (SENP) (Hay, 2013) . The isoforms SENP1-3 and SENP5-7 are the human homologues. Along with the isopeptidase function, they are characterised to have the C terminal hydrolase activity which is required for the maturation of pro SUMO proteins. There are reports of SNEPs themselves undergoing post translational modification. For example, SENP3 is rapidly

turned over under normal cellular conditions by ubiquitination. Extracellular stress stimulus causes stabilisation of SNEP3 relocating it from cytoplasm to nucleus. SNEP3 rapidly deSUMOylates p300 which regulates transcription of Hif1a, a key regulator of stress response (Girdwood et al., 2003).

1.4.2. Protein SUMOylation in CNS

Protein SUMOylation has proven to be an essential part in the regulation of many neuronal cellular pathways. Although, the SUMOylation pathway was linked to diverse

neurological functions and disorders, underlying mechanistic details of how SUMO conjugation of substrates contributes to health and disease remain largely unknown. This section is a short review to evaluate the role protein SUMOylation in neurobiological processes.

The highest levels of Ubc9 expression (SUMO E2 enzyme) and SUMO1 conjugation has been detected during early brain development in rodents. High expression of Ubc9 reported in dentate gyrus of hippocampus and pyramidal neurons in cerebral cortex indicates a possible role of Ubc9 and protein SUMOylation in synaptic signalling and plasticity. Spatio-temporal regulation of SUMOylated substrates and components of SUMO cascade machinery during rat brain development implies its crucial role during the neurogenesis (Watanabe et al., 2008). Localisation of SUMO-1 and Ubc9 in Cajal bodies (CBs) is another example of SUMO mediated regulation of cellular processes in neurons. CBs are small nuclear organelles, which regulate ribonucleo proteins involved in mRNA processing (spliceosome machinery). Neuronal proteins coilin and survival of motor neuron protein (SMN), marker proteins of CBs, were described as putative substrates of SUMO conjugation (Carvalho et al., 1999). NR2E3 is a key transcription factor, which activates rod specific genes or represses cone specific genes. This is a key step in controlling rod photoreceptor differentiation in the retina. NR2E3 is SUMOylated in a PIAS3 dependent manner. High expression of PIAS3 is observed in mouse retina during retinal development (Blackshaw et al., 2004, Onishi et al., 2009). MEF2A (Myocyte Enhancer Factor 2A) is another example of a transcription factor implicated in synaptic

activity whose activity is strictly regulated by acetylation and phosphorylation dependent SUMOylation (Shalizi et al., 2006). It was speculated that SUMOylation may act as switch between transcriptional activator and repressor roles of MEF2A (Beg and Scheiffele, 2006). Leak K^+ channels (example: leak K2P1 channel), crucial for neuronal excitability are also SUMO modified. It has been proposed that SUMOylation regulates K2P1 channel activity by directly obstructing the channel pore or altering its charge distribution (Plant et al., 2005). GSK3β, a key enzyme in Wnt signalling pathway and a DISC1 binding partner, is also a substrate for SUMOylation (Wilkinson et al., 2010). It is also one of the few substrates to get SUMOylated on a lysine residue with in a non-consensus region. GSK3β SUMO mutants (non SUMOylable) are unstable and excluded from the nucleus, which implies that this modification may be crucial in regulating the enzyme activity (Eun Jeoung et al., 2008). SUMOylation has also been implicated in the pathogenesis of Alzheimer's characterised by progressive dementia. Neurological hallmarks of Alzheimer's include neuronal plaques of β amyloid and fibrillar plaques composed of hyper phosphorylated tau protein, both of which are SUMO substrates (Wilkinson et al., 2010). A direct genetic link between the SUMO system and AD has also been hypothesised as an SNP (intron 7) on Ubc9 gene has been shown to be associated with AD in certain ethnic populations (Ahn et al., 2009). Likewise, α-synuclein, implicated in Parkinson's disease interacts with SUMO 1 (Pountney et al., 2005). SUMO protein levels in cells also alter under conditions of cellular stress. Although, SUMO2/3 levels are low compared to that of SUMO1, under conditions of oxidative and osmotic stress, SUMO2/3 conjugation of proteins increases, suggesting that they may act as reserve pools (Martin et al., 2007). SUMO2/3 conjugation is also known to increase in cases of transient global cerebral ischemia in mice. Hence, it was hypothesised that SUMO conjugation may be a protective mechanism employed by the cell to down regulate cell surface markers and receptors and thereby prevent cell death (Martin et al., 2007). In addition, SUMO proteins are known to be actively transported across the synapse upon neuronal stimulation (Paul, 2008). SUMO E1 and E3 enzymes were found to be in low levels in post mortem brain tissue of SCZ patients (Paul, 2008).

Protein aggregation is the hallmark of several psychiatric illnesses such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and Huntington's disease. The proteins contributing to these diseases Tau, α-synuclein, amyloid precursor protein, SOD1,

Huntington and atrophin-1 are SUMO substrates (Krumova and Weishaupt, 2012). Although, its yet unknown how protein aggregation contributes to the disease mechanism, it is speculated that it is possibly mediated by SUMOylation as it regulates protein solubility and stability (Krumova and Weishaupt, 2012). To summarize, elucidation of SUMOylation mediated regulation of synaptic transmission and neuronal differentiation has just started to emerge. Possible suggested roles include regulation of resting membrane potential and synaptic protein-protein interactions. SUMOylation adds complexity to these processes, as they are already known to be modulated by other PTMs such as phosphorylation, ubiquitination, and acetylation.

1.5. Ubiquitination/Ubiquitylation

1.5.1. Introduction

A majority of cellular proteins are tagged by ubiquitin (ubiquitin) via an enzyme cascade known as Ubiquitination. This process is famously known as a prerequisite for targeted protein degradation by the proteasome. Hershko and collegues, using various biochemical fractionation and enzymology techniques, discovered that certain proteins when added to reticulocyte extract, became covalently tagged to a protein known as ubiquitin, and that these Ubiquitinated proteins were gradually destroyed by an ATP-dependent protease present in the extract (Hershko et al., 1980, Hershko et al., 1979, Ciechanover et al., 2012). This team further identified and characterised the enzymes in the ubiquitin cascade and named them as E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ligase). The other major component of this pathway is the ATP dependent protease, which gradually degrades the ubiquitin conjugated protein and is now named as the 26S proteasome (Adams, 2003, Baumeister et al., 1998, Braun et al., 1999, De Mot et al., 1998, Tanaka, 1998). The ubiquitin moiety, E1, E2, E3 ligases, DUB (deubiquitinating enzymes) enzymes and the 26S proteasome collectively constitute the UPS pathway.

The UPS (Ubiquitin Proteasome System) is involved in controlling many cellular processes including protein localisation, transport across membranes, antigen processing and maintenance of the stoichiometry in protein-protein complexes. The UPS pathway provides one of the many means through which the cell discards defective, mis-folded, non-functional and aggregated protein (Hershko and Ciechanover, 1998). Ubiquitin, which is a major component of this tightly regulated cascade, acts as signalling messenger to regulating the most complex cellular functions. There has been emerging evidence of its involvement in other cellular pathways like gene expression regulation, subcellular targeting of proteins, regulation of protein confirmation, and DNA repair (Di Fiore et al., 2003, Schwartz et al., 1988). Proteins tagged with a single ubiquitin or ubiquitin chain(s) are processed by a multi subunit protease complex, the 26S proteasome. However, ubiquitin itself is spared from the proteasomal action by it release from the substrate by the action of specific peptidases known as deubiquitinating enzymes (DUBs), making this post translational modification reversible. Discovery of deubiquitinating enzymes supports the theory that free ubiquitin moieties can be recycled by the cell (Matsui et al., 1982). They belong to an enzyme class of hydrolases that hydrolyze the isopeptide bond between the C-terminal glycine and either the substrate lysine or ubiquitin lysine (if in a chain). Ubiquitin can bind to proteins either covalently or *via* ionic interactions. Ubiquitin may also be conjugated to target substrate proteins and modulate their function without affecting their stability and other non-destructive roles can also be accomplished by non-covalent interaction with substrate proteins (Finley et al., 1989).

1.5.2. UPS (Ubiquitin Proteasome System): Components and pathway

Components of the UPS system are well conserved from lower to higher eukaryotes and a homologous system is absent in prokaryotes. Ubiquitin is a 76 amino acid protein, 8.3kDa in size. Upon conjugation to the target substrate, ubiquitin may affect their stability, localisation, conformation and activity. The complex surface design of ubiquitin enables it to form straight or branched chains of different lengths, which confer great versatility to this protein modification. The name ‘ubiquitin’ can be justified by its omnipresence and diverse cellular functions. Ubiquitin was discovered as a thymus hormone by Goldstein et al in 1975 (Goldstein et al., 1975). However, the exact identity and its extended role in

proteolysis was elucidated by Avram hershko and colleagues in 1978 (Hershko et al., 1980). It is one of the most conserved proteins known so far and the difference from insects to humans is only one amino acid.

Ubiquitin conjugation is an active process, which involves the transfer of an activated ubiquitin moiety to the substrate (Etlinger and Goldberg, 1977) as represented in the schematic of ubiquitin conjugation cycle (Figure 1.4). In an ATP dependent step, ubiquitin is transferred to an ubiquitin activating enzyme (also known as UAE or E1), to form a thiol ester bond between the C terminal glycine (G76) of ubiquitin and the side chain of a cystine residue within a highly conserved region with the E1. Activated ubiquitin is then transferred from E1 to the cysteine residue on one of the several ubiquitin conjugating enzymes (also known as Ubc or E2). Ubiquitin is then transferred on to the substrate directly or with the help of certain accessory proteins called E3 ligases, depending on the class of E2 enzyme involved.

The ubiquitin conjugation enzyme cascade is hierarchical, with the presence of one E1, very few characterised E2s and significantly larger number E3s with much more to be identified. Two isoforms of the E1 enzyme (E1a and E1b) have been characterised so far. To date, just over 30 genes have identified in E2 class of enzymes. E2 enzymes were discovered to have a conserved Ubc domain (ubiquitin conjugating) of about 150 amino acids. E3 ligases primarily act as substrate recognition machines that act to bring E2s and substrates together in the correct conformation to facilitate ubiquitin transfer (Hershko et al., 2000). E3 ligases will be discussed in detail in section 1.5.3.

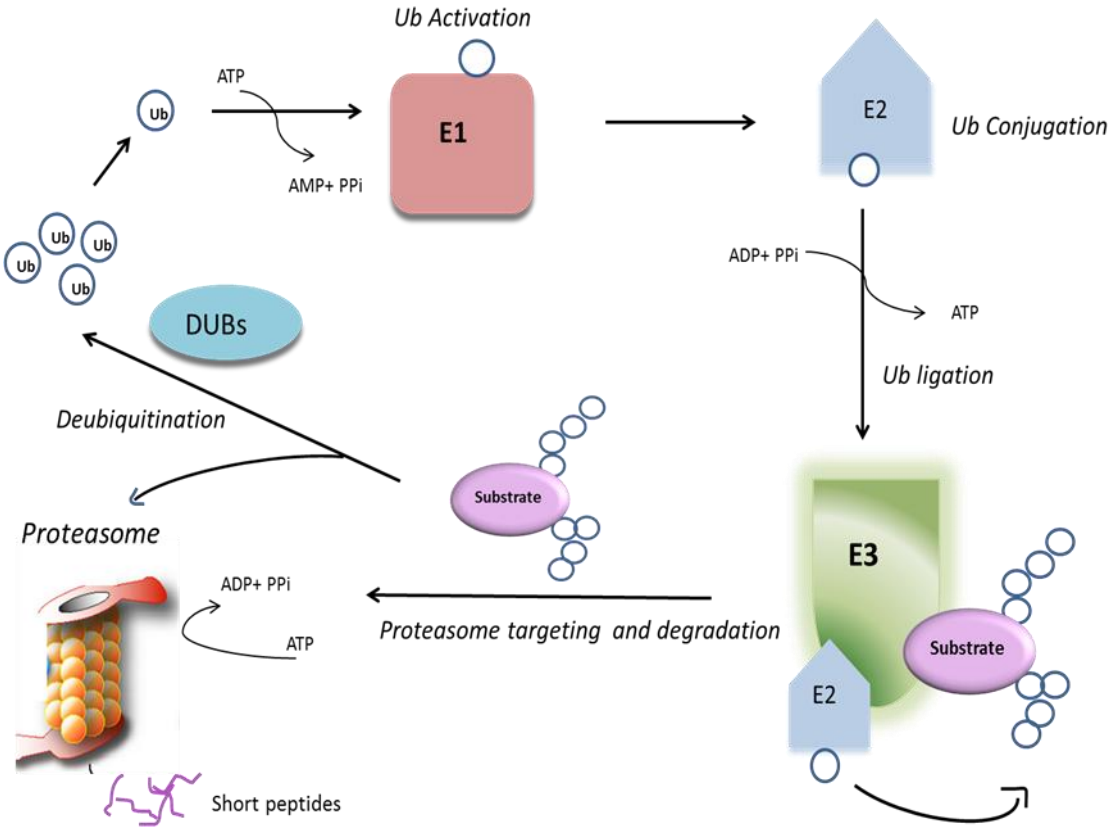


Figure 1.4 Pictorial representation of the ubiquitin cascade
Ubiquitin is activated by ATP mediated covalent binding to ubiquitin-activating enzyme (E1) which is then transferred to ubiquitin-conjugating enzyme (E2). Depending on the class of E3 ligase that regulates the substrate (section 1.5.3), Ubiquitin is then transferred helps transfer ubiquitin to the target substrate. This cascade results in the attachment of multiple ubiquitin moieties in form of chains which are subsequently recognised by the 26S proteasome for degradation and ubiquitin is recycled which is mediated by deubiquitinating enzymes (DUBs).

The ubiquitination process relies on formation of an isopeptide bond between the C-terminal glycine of the ubiquitin and specific lysine(s) on the substrate protein. Ubiquitin itself has 7 lysine residues. Once a mono ubiquitinated substrate is formed, ubiquitin can get self ubiquitinated on one or more of these lysine residues to form poly ubiquitin chains either straight or branched in form (Figure 1.5). Substrates can also be multi, mono or poly ubiquitinated at different sites. Polyubiquitin chains can either be formed on the substrate or built on E2 and E3 ligase complexes and transferred to the substrate together (Ravid and Hochstrasser, 2007). The type ubiquitination of a substrate can be classified as (1) Mono ubiquitination at a single site (2) multi-ubiquitination at multiple sites on the substrate (3) Poly-ubiquitintion with variable length of ubiquitin chains

attached at one or more sites on the substrates. There have been reports of substrates carrying nearly over a dozen ubiquitin molecules (Hochstrasser, 2006). Using peptide mapping and site directed mutagenesis studies, K48 and K63 have been identified as the sites on Ubiquitin involved in chain formation (Chau et al., 1989). However, formation of chains having both types of the above linkages concomitantly has been reported. Various models have been hypothesised for poly-ubiquitin chain assembly, reviewed in Mark Hochstrasser (2006). (Hochstrasser, 2006)). The fate of the ubiquitinated protein depends on the type of the ubiquitin chains conjugated to it (Figure 1.5). In general, proteins tagged with K48 chains are targeted to proteasome and K63 linkage chain are degraded by lysosome (Kirkin et al., 2009). More recent investigations reveal formation of other type of linkages, like K3 whose functional outcome still needs to be studied. Multi ubiquitin chain assembly, in some cases also requires an E4 class of enzymes, also known as E4 chain elongation factors (Hoppe, 2005). There has also been increasing evidence of ubiquitin tagging to the N terminus of proteins (Ciechanover and Ben-Saadon, 2004), and the side chains of amino acids like threonine, serine and cysteine (Cadwell and Coscoy, 2005, Ishikura et al., 2010).

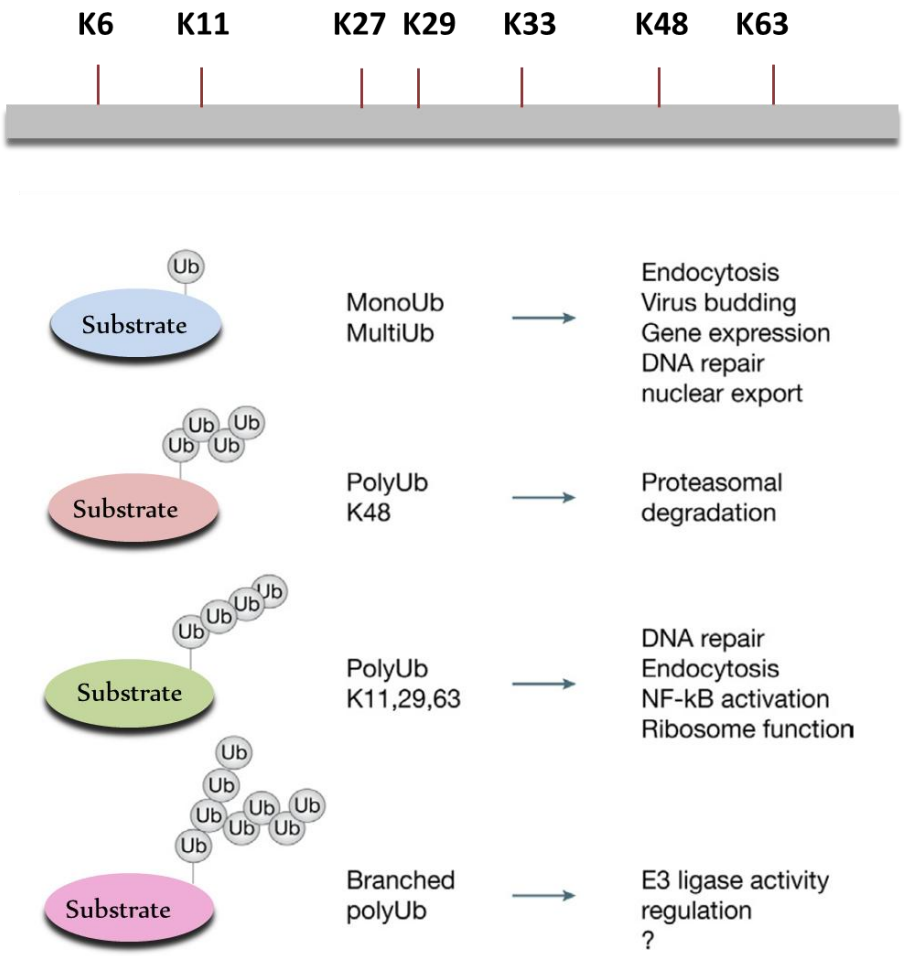


Figure 1.5 Various effects of different types of ubiquitin chain conjugation of substrates Type of ubiquitin linkage is designated by the identity of the lysine side chain amino group amino group involved in the linkage i.e, Lys6-, Lys11-, Lys27-, Lys29-, Lys33-, Lys48-, Lys63-linked, which in turn dictates the fate of the substrate.

Poly-ubiquitinated proteins are targeted to 26S proteasome, a multi enzyme complex localised ubiquititiously in the cell (Baumeister et al., 1998, De Mot et al., 1998). The 26S proteasome comprises of a cylindrical 20S subunit (core particle) and a 19S regulatory unit (regulatory particle). This multi-subunit enzyme complex has nearly 64 subunits encoded by 32 genes. To briefly describe the structure of the proteasome, the cylindrical core 20S subunit is flanked by the 19S subunit on either side. The 19S particle in turn has a lid which recognises ubiquitinated proteins and the base has nearly 6 ATPases and caps the 20S subunit core. The 19S unit disassembles the ubiquitin chains, unfolds the proteins and channels them in to the 20S subunit (Glickman et al., 1998, Braun et al., 1999). The 20S core particle has number of polypeptides, which form 4 rings stacked to

form the cylindrical core. The outer two rings comprise of 7 α subunits, complexed with the 19S particle and allow unfolded proteins to pass through. The inner 2 β rings have 3 active sites, which are categorised based on their substrate specificity as chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolase-like (PGPH) (Adams, 2003). The 20S core particle progressively degrades the substrate protein generating short peptides of about 3-25 amino acids long (Adams, 2003). Though the proteins targeted to the proteasome are processed in to short peptides, NFκb is one of few examples where proteins are only partially processed as a part of signal transduction regulation (Maniatis, 1999).

UPS functions can be broadly categorised as follows

1. Regulation of protein half-Life: Enzymes, cell surface receptors, ligands which serve regulatory function in various signalling pathways require to be turned on and off to meet the cellular needs. UPS degrades unwanted proteins and maintains the physiological levels of the substrates. Other than phosphorylation, ubiquitination is another PTM that is also involved in regulating enzyme activity by labelling them for destruction, for example, PDE4 enzymes degrade and maintain intracellular cAMP pools. These enzymes are in turn regulated by ubiquitin conjugation.

2. Degradation of misfolded proteins: Misfolded proteins are toxic to the cells and are degraded by UPS. Nearly ~30% of newly synthesised polypeptides are degraded via UPS (Wittke et al., 2002). Misfolded proteins forming toxic aggregates form a hallmark of neurodegenerative disease such as Alzheimer's and Parkinson's disease. Though the exact reason is not yet known, the common cause of protein mis-folding was thought to be SNPs and truncations (Selkoe, 2004).

3. Non proteolytic functions: While there is still a debate lingering around physiological consequence of each type of ubiquitin chain formation on the substrate, a number of studies are still uncovering new roles of ubiquitin pathway. Involvement of ubiquitin modification in various types of DNA repair, antigen processing and endocytosis during an immune response are just a few of many examples illustrating non proteolytic function of

ubiquitin in cell signalling (Chen and Sun, 2009). Ubiquitination and subsequent proteasomal degradation of IκB leading to activation of NF-κB signalling pathway implicated in DNA repair.

The following section is a short review on ubiquitin E3 ligases, a crucial component of UPS which confers specificity towards its substrates.

1.5.3. Ubiquitin E3 ligases

Ubiquitination is temporally and spatially regulated and the complexity is increased by the presence of diverse number of E3 ligases. To date, 600 E3s have been estimated to be encoded by the human genome (Bernassola et al., 2008). The two major classes of E3 ligases are the RING finger proteins and HECT domain proteins. The HECT domain forms a thioester with ubiquitin before the ubiquitin is transferred to the substrate, while the RING domain present in the RING class proteins, provides a docking surface for E2 enzymes and the target protein facilitating the active transfer of ubiquitin from the former to later, as represented in the schematic Figure 1.4 (Pickart, 2001). Accumulating evidence suggests the association of E3 genes with neurogenetic disorders (including UBR1 (Johanson–Blizzard syndrome), NHLRC1 (Lafora's disease)) (Tai and Schuman, 2008).

1.5.3.1 HECT

Homologous to E6-AP carboxyl terminus (HECT) class of E3 ligases role has been emphasised by the study of tumours caused by HPV virus. With the help of the viral proteins, E6-AP induces the ubiquitination of the tumour suppressor gene p53 (Scheffner et al., 1995). The important characteristic feature of this family of E3's is a conserved stretch of 350 amino acids in their C- terminus called HECT domain, with a cysteine residue at the catalytic site. The HECT class of E3 ligases differ from the RING E3s by possessing an intrinsic catalytic site. HECT E 3s form complexes with E2 enzymes and transfer the active ubiquitin to themselves via the cysteine residue within their active site forming a ubiquitin-thiolester intermediate. This class of E3 ligases actively catalyse the transfer and ligation of ubiquitin to the target substrate (Bernassola et al., 2008).

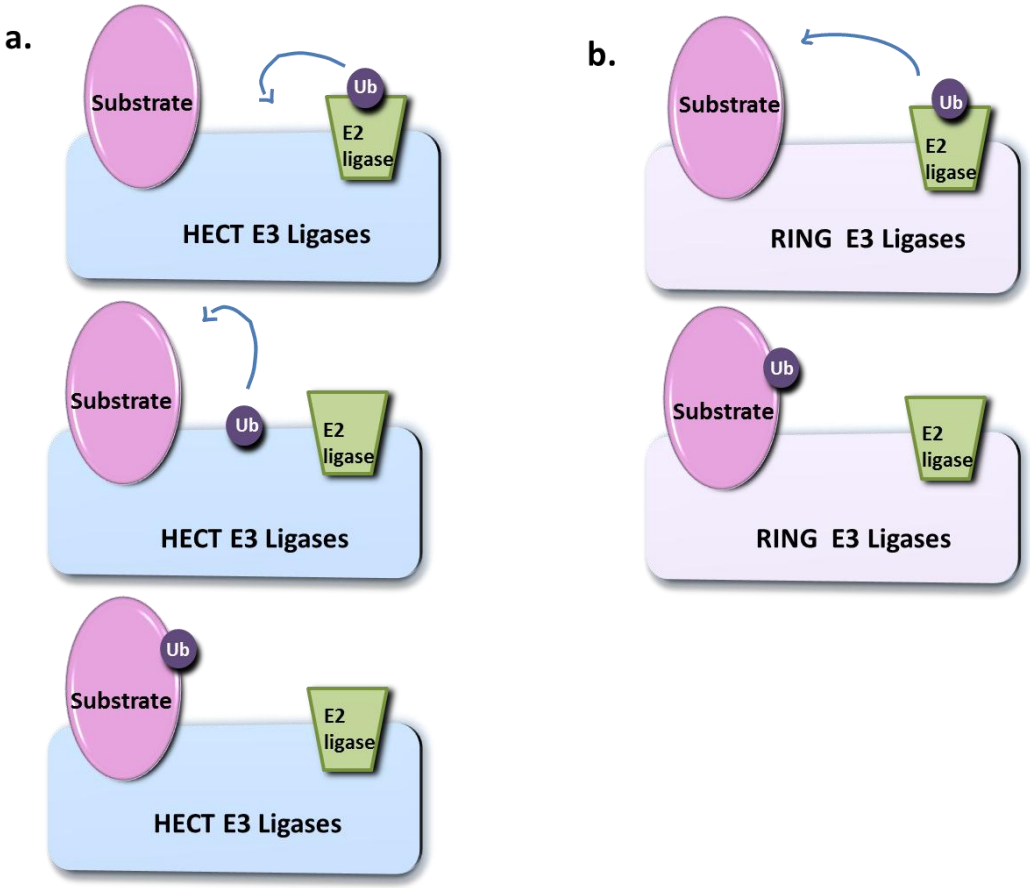


Figure 1.6 Schematic representation of mechanism of ubiquitin conjugation by HECT and RING class of E3 ligses
E3 ligases responsible for transferring the ubiquitin from ubiquitin-conjugated E2 to the targeted substrates are primarily classified in to two classes. a. The HECT-domain E3 ligases transfer the ubiquitin by forming a thioester bond between the E3 ligases and the ubiquitin. Ubiquitin is then transferred again to the substrates. b. The RING-domain E3 ligases interact with the Ubiquitin-conjugated E2 which transfers the ubiquitin directly to the substrates.

1.5.3.2 RING (Really Interesting Gene) E3 ligases

This is the largest family of E3 ligases, containing hundreds of genes and more being uncovered. The main characteristic feature of this class is the presence of the so called RING domain. The RING domain is a cysteine and histidine rich region forming a cross-brace accommodating 2 zinc ions (Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X2-Cys-$X_{(4-48)}$-Cys-X_2-Cys, where X is any amino acid)) (Borden and Freemont, 1996, Joazeiro and Weissman, 2000). Initially RING class proteins were believed to be involved in DNA

repair, however, the breakthrough in the delineation of their real function came via the discovery of a subunit of the SCF E3 ligase Rbx11 (Kamura et al., 1999, Seol et al., 1999). These studies revealed the catalytic role of members of a RING family. It has been suggested that most of the RING class E3 enzymes form a docking platform for the E2 ligase and the substrate protein and catalyse the transfer of the active Ubiquitin from the former to the later without forming an E3 Ubiquitin intermediate with a covalent bond. Zheng et al solved the structure of the Cbl-Ubch7 complex, which uncovered a number of features of this domain (Zheng et al., 2000).

RING E3 ligases are also regulated by ubiquitination, often by autocatalytic function, consequently regulating its enzymatic activity. For example, Mono ubiquitination of Mdm2 (E3 ligase which regulates the activity of p53) by autocatalysis is speculated to be critical in inhibiting its activity. An example, where auto ubiquitination switches on the ligase activity of an E3 enzyme is that of Bard1-Brca1 heterodimer (Simons et al., 2006). Diverse mutations in Parkin gene, a RING E3 ligase, were reported in a familial form of Parkinson's disease known as autosomal recessive juvenile Parkinson's disease (AR-JP) (Yi and Ehlers, 2007). Brca1, a tumour suppressor gene mutated in nearly 90% breast and ovarian cancers is an E3 ligase for substrates primarily involved in cell cycle and DNA repair. It forms a heterodimer with another E3 ligase, Bard1 and it has been established that this complex formation enhances auto ubiquitination of Brca1, potentially increasing the E3 ligase activity of Brca1 (Simons et al., 2006).

RING E3 ligases are known to function as oligomers frequently. As mentioned above, while Brca1-Bard1 is one example another popular example in which these E3 ligases function as dimer is that of the formation of heterodimer complex of Mdm2 and MDMX. They play a crucial role in constantly regulating the turnover of p53, a tumour suppressor, by Ubiquitination. Wang et al, reported that though Mdm2 alone is able to regulate p53 levels, it functions more effectively in complex with MDMX forming a heterodimer (Wang et al., 2011). As observed in Traf, c-Cbl and Siah RING E3 ligases may also form homo dimers (Deshaies and Joazeiro, 2009). However, the functional consequence of RING E3 ligase oligomerisation is not well understood yet.

Members of the RING family functioning as multi subunit complexes are discussed in detail in the following section.

1.5.3.3 SCF (Skp1 Cullin Fbox)

These are the well characterised, largest family of RING finger type E3 ligases that form a protein complex to bridge the E2 and the substrate. SCF complexes and their components were first discovered during cell cycle studies in yeast. Sic1 is an inhibitor of Cdk, which is required for the G1 to S phase transition. At the cell cycle checkpoint, Sic1 degradation is initiated by its phosphorylation by G1 cyclin (Cln)-Cdk kinases and targeted by Ubiquitination via the action of a SCF complex (Schwob et al., 1994). One well-characterised mammalian E3 ligase belonging to multi subunit RING E3 ligase family is CDL (Cullin dependent ligase).

A member of CDL family, SCF^{Skp2} quaternary structure has been elucidated which suggests that Cullin1 functions as a scaffold protein interacting with all the components of the protein complex. Its N-terminal helical region that adopts a long stalk-like structure and interacts with Skp1 and $Fbox^{Skp2}$ while its C terminal domain assumes a globular α/β domain which interacts with Rbx1 (Zheng et al., 2002). Cullin proteins are modified by NeDDylation, on their C terminus which induces a conformational change in Cullin proteins, which inturn leads to activation of the E3 ligase enzyme (NeDDylation is covalent attachment of ubiquitin like NEDD8 protein(s) catalysed by an enzyme cascade analogous to ubiquitination) (Duncan et al., 2012). To date, 7 members of Cullin family have been identified and characterised (Marín, 2009). These are Cullin 1, 2, 3, 4A, 4B, 5, and 7. Anomalies in their proteins levels, structure and SNPs have been reported in several tumours. In a comprehensively characterised example of E3 ligase complex Von Hippel-Lindau (VHL), Cul2 serves as a scaffold. Hif1a, a transcription factor regulated by cellular oxygen levels, serves as a substrate for the above mentioned complex (Maxwell et al., 1999). Several mutations in the sequence of VHL have been reported in Von Hippel-Lindau disease, primarily characterized by tumours of the eye, brain, spinal cord,

kidney, pancreas, and adrenal glands (Lee and Zhou, 2010). The role of Cul4B in neural development has also been reported recently (Liu et al., 2012, Chen et al., 2012). A study by Liu et al in NT2 cells, revealed the regulatory role of cul4B in neural progenitor growth and differentiation.

The other component of SCF complex is Rbx protein. Two homologues identified in humans are Rbx1/ROC1/Hrt1 and Rbx2/SAG/ROC2/Hrt2. In SCF^{Skp2} , Rbx1 interacts with Cullin protein via its N terminus (of Rbx1) and E2 enzyme via the RING domain on its C terminus (Zheng et al., 2002). Rbx1 interacts with all the known Cullin family proteins (Ohta et al., 1999). The function of Rbx1 is to bind and bring the E2 enzyme into close proximity with the E3 specific substrate. Apart from recruiting E2 enzyme, other precise physiological functions of Rbx1 in ubiquitination remain largely unknown. However, it is known to function as an E3 ligase for Cullin NEDDylation (a PTM essential for the E3 activity of SCF) (Megumi et al., 2005).

The other component of SCF complex is Skp1. Although, there are a number of homologues reported in lower eukaryotes, humans have only one functional Skp1 gene. Immunoprecipitation studies and yeast 2 hybrid analysis suggest that Skp1 functions as an anchor for Cullin and Fbox proteins (Patton et al., 1998). The human Skp1 was initially characterised as a component of a protein complex containing Cyclin A –Cdk2 and Fbox Skp2. This complex Ubiquitinates p27Kip, an inhibitor of CDK, which is a regulatory kinase of the cell cycle check point. Cell cycle is arrested in either G1 or G2 phase in yeast Skp1 mutants suggesting its role in cell cycle (Piva et al., 2002).

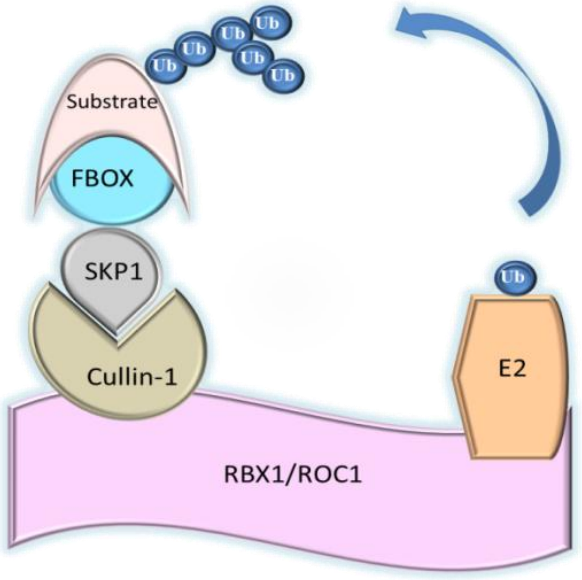


Figure 1.7 Schematic of the SCF complex
SCF (Skp1–Cullin–F-box) complex: Components of SCF complexes include the scaffold protein Cullin-1, which interacts with Skp-1 and the F-box proteins at the amino-terminus. The C temrinus of Cullin-1 associates with the RING-domain interacting molecule Roc1, which in turn interacts with ubiquitin-conjugated E2 enzymes. Substrates are recognized through the C-terminus of F-box proteins and Ubiquitin is transferred to the substrates from E2. The interaction between the F-box proteins and the substrates may require the phosphorylation of substrates.

1.5.3.4 FBP: Fbox protein

Fbox was originally identified as a region of homology in proteins such as Cdc4, β-TrCP, Met30 which have Trp-Asp repeats (WD repeats) (Bai et al., 1996). Name Fbox, was first given by Bai et al, to a motif they identified in Cyclin F (Bai et al., 1996). As a part of the SCF E3 ligase complex (Figure 1.7), the primary function of the Fbox protein is substrate recognition and hence the Fbox often confers specificity to the SCF complex with respect to substrate selection.

The structure of Fbox proteins has been elucidated and explains their functional role as adaptor proteins bringing the substrate in to proximity of the SCF catalytic core. Usually, they have an N terminal Fbox motif and the C terminal substrate recognition domain. The F-box motif consists of approximately 50 amino acids and is crucial for its interaction with Skp1. Depending on their composition and structural properties, Fbox proteins are classified into the following categories, Fbxl (LRRs, leucine rich repeats, example: Skp2),

Fbxw (WD 40 rich, example: Fbxw7 and β-Trcp) and Fbxo (a different or no Fbox, example: Cyclin F, Fbxo7) (Cenciarelli et al., 1999). However, mere presence of Fbox motif does not assure of its E3 ligase activity. For example, Ctf13 forms a part of the kinetochore complex along with Skp1 as a component of CBF3 (Centromere DNA-binding protein complex) while other components of the SCF complex are absent and there has been no reported ubiquitin ligase activity in this complex (Stemmann and Lechner, 1996). Certain Fbox proteins are also known to possess other intrinsic enzyme activity such as DNA helicase activity of hFbh1 which regulates homologous recombination (Nelson et al., 2013).

As this family constitutes a structurally diversified group of proteins, their role in wide range of cellular processes such as cell division, differentiation, cell survival and apoptosis has been proposed. Dysregulation of Fbox protein-mediated ubiquitination has been implicated in many disease pathologies including mood disorders, sleep disorders and Parkinson's disease. Table 1.1, presents few examples of Fbox proteins, their specific substrates and the pathways they regulate.

Table 1.1 Examples of Fbox proteins, their substrates and physiological role

Fbox protein	**Substrate**	**Function of Substrate**	**Reference**
βTrCP	β-catenin	Wnt signalling	(Petroski, 2008, Maniatis, 1999)
βTrCP	REST (repressor element 1-silencing transcription factor)	Neurogenesis	(Stegmüller and Bonni, 2010)
Fbxw7	Cyclin E	Cell cycle	(Zhang and Koepp, 2006)
Fbxw7	Notch	Neuronal stem cell differentiation	(Grim et al., 2006)
FBX2	BACE1 (β-secretase 1)	amyloid precursor protein (APP) processing; implied in Alzheimer's disease pathology	(Gong et al., 2010)
Skp2	Myb	Gene transcription	(Schulman et al., 2000)
Fbxl3	CRY1 and CRY2	Circadian rhythm	(Siepka et al., 2007)
Fbxo7	p27	Inhibitor of Cyclin dependent kinases; Cell cycle regulation	(Nelson et al., 2013)

Mammalian Fbox protein family is still expanding with the discovery of Fbox function in many proteins from time to time. The following section is a short review on Fbxw7 protein which has been implicated in control neural stem cell differentiation and brain development.

1.5.3.5 Fbxw7

Fbxw7, also known as hCdc4 and SEL10, is a member of the Fbox family, which functions as a subunit of SCF complex. Fbxw7, was identified during a genetic screen for cell cycle mutants in yeast (Hartwell et al., 1973). Cdc4, a yeast homologue of Fbxw7, regulates cell cycle by targeting Sic1 for ubiquitination. Sic1 is an inhibitor of Cdk that is required for the G1 to S phase transition. At the cell cycle checkpoint, Sic1 degradation is initiated by

its phosphorylation by G1 cyclin (Cln)-Cdk kinases. Sic1 is then targeted for ubiquitination via the SCF^{Cdc4} complex (Schwob et al., 1994).

Human Fbxw7 spans 200kb on chromosome 4, encoding 3 mRNA transcripts – α, β and γ generated as a result of intron splicing. Each isoform has a unique first exon, followed by ten shared exons. Unique transcriptional control is achieved by separate promoters for each isoform. At the protein level, each isoform shares a unique N terminal region followed by common regions (Figure 1.7). The unique N terminal region has been reported to regulate the sub cellular localisation of individual isoforms. The C terminal region is responsible for substrate recognition and promotion of ubiquitination (Bai et al., 1996). Isoform specific mutations have been reported in several tumours, implying their different roles as tumour suppressors. One of the well characterised Fbxw7 interactions is with Cyclin E. Three different groups independently identified Fbxw7 as the Fbox E3 ligase required for Cyclin E proteolysis (Welcker et al., 2003, Orlicky et al., 2003, Hao et al., 2007a). X ray crystallographic studies later followed, elucidating the three dimensional structure of SCF^{Fbxw7}- Cyclin E complex (Hao et al., 2007a).

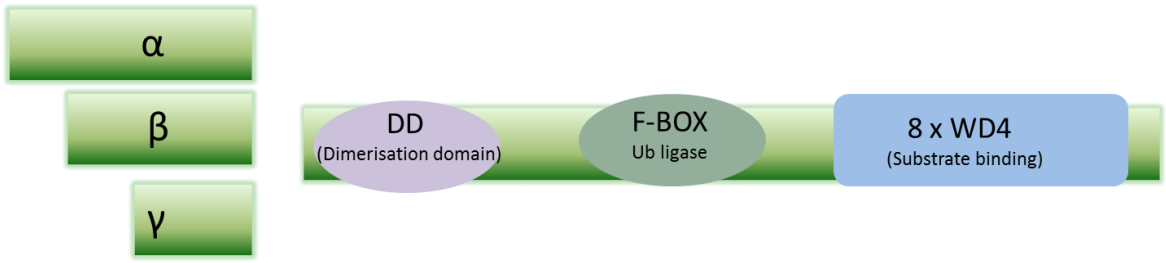


Figure 1.8 Schematic of Fbxw7 isoforms
Three functional domains identified in Fbxw7 and the variable N terminus in the three isoforms of Fbxw7.

The proposed functions of Fbxw7 are achieved by a 40 amino acid long Fbox domain which associates with Skp1, a D domain (dimerization domain) through which the isoforms dimerize and 8 repeats of WD40, which is the substrate binding domain (Zhang and Koepp, 2006). Tertiary structure of Fbxw7 (Figure 1.9) reveals that the F box domain and WD40 domain (Trp-Asp) are linked via an α helical linker. The Fbox domain

comprises of five α helices, designated as α0 to α4. There are eight copies of the WD40 motif in Cdc4, altogether. They form an eight blade β-propeller structure. However, F box proteins such as Met30 and β-TrCP have only 7 blade β- propeller structure in contrast to Cdc4, which has 8 α helical extension that link the F box domain of Cdc4 to its WD40 domain. This helical linker comprises of two α helices denoted as α-5 and α-6. These 2 helices along with the α3 and α4 of the F box domain, form a stalk positioning the WD40 domain at a distance (Figure 1.8) (Orlicky et al., 2003).

The elucidation of the tertiary structure of Fbxw7 facilitated development of small molecule inhibitors targeting its interaction with relevant substrates (Aghajan et al., 2010). Many research groups demonstrated dimerization of Fbxw7 and its homologues is mediated by the D domain (Figure 1.8). Although the significance of this dimerization yet remains to be elucidated, it was hypothesised that each SCF^{Fbxw7} complex within the dimer recruits its own ubiquitin conjugating enzyme E2, resulting in proper spatial orientation between the substrate binding domains of each Fbxw7 monomer and increasing the efficiency of ubiquitin conjugation (Welcker and Clurman, 2008).

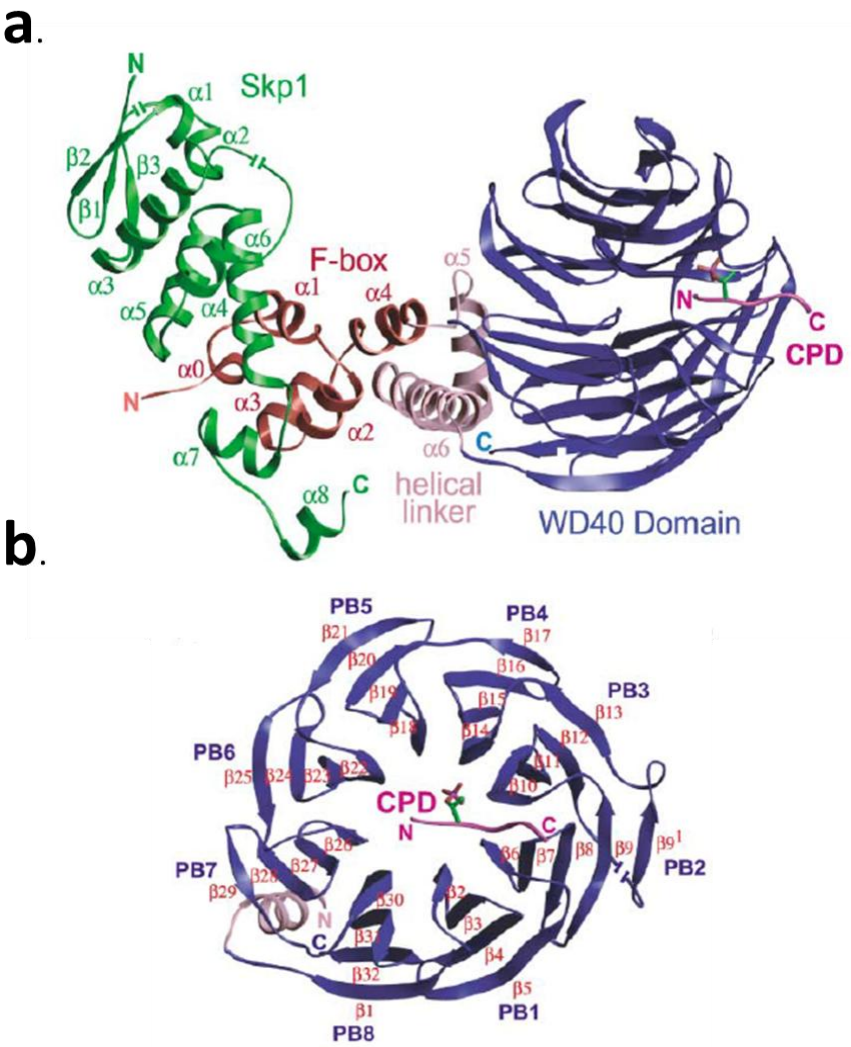


Figure 1.9 The Skp1-Cdc4 CPD complex
Adapted from Orlicky et al., (2003).
Ribbons representation of Skp1 (green), the F-box domain (residues 274-319 in red), the helical linker region (residues 331-366 in pink) and the WD40 domain of Cdc4 (residues 367-744 in blue). The bound Cyclin-E derived CPD peptide is in purple with the phosphor threonine moiety shown in ball and stick representation. Positions of disordered loop regions are shown as ribbon breaks. (b) The WD40 domain of Cdc4. β-propeller blades are denoted PB1 to PB8. Ribbons and CPD peptide are colored as in (a).

Analysis of Fbxw7 substrate sequences revealed that they might need to be phosphorylated within a conserved sequence for the Fbxw7 to bind. These phospho-epitopes are described as Cdc4 phosphodegrons (CPD). Knockout studies identified Fbxw7 as key regulating factor of neuronal stem cell viability and neuronal progenitor differentiation (Hoeck et al., 2010b). It is known to regulate these biological processes by regulating the turnover of its substrates such as AP-1, c-Jun and Notch, all known to be involved in neuronal population maintenance and self-renewal (Wang et al., 2012a).

As one of the characterised substrate of Fbxw7, Notch plays a crucial role in neuron fate and differentiation. Notch is a transcription factor, which, upon proper extra cellular stimulus, is processed and translocated in to the nucleus to regulate the expression of genes that induce cell proliferation and prevent differentiation. Studies on Fbxw7 conditional knockout mice showed that Fbxw7 regulated the abundance of Notch proteins by ubiquitin mediated proteolysis and thus played a pivotal role in regulating the ratio of neurons and glial cells, a process critical in brain development (Matsumoto et al., 2011).

1.5.4. UPS impairment in neurological diseases

In highly specialised cellular networks like that of neurons, their connectivity at the synapse is critical in rapid information transfer in the brain. With millions of neurons forming trillions of synapse, it is challenging to understand how each of these synapses is individually modulated to fine-tune neural circuitry. It is understood that synaptic stability and plasticity are maintained through finely co-ordinated intra neuronal transport and post translational modification of synaptic proteins. Ubiquitination is one such vital modifications that control synaptic protein homeostasis. Protein homeostasis in post mitotic neurons is susceptible to functional disturbances while its abnormalities result in the accumulation of insoluble and aggregated proteins in the cell. Studies evaluating the incorporation of radio labelled amino acids into brain proteins suggest that proteins are constantly replenished or up regulated in brain at different developmental stages (Cajigas et al., 2010). Studies implicated the significance of tight and controlled regulation of protein turn over in brain to enable normal brain processes (Lin and Man, 2013).

A few examples which highlight the significance of UPS mediated regulation of proteins involved in neuronal signalling are as follows: β-catenin is one of the well-studied substrates of the SCF E3 ligase beta βTrcp. This transcription factor has been shown to regulate neural progenitor proliferation (Wang et al., 2004). Neural secretory proteins like Bone morphogenic proteins (BMPs) and Sonic Hedgehog (Shh) are involved in

neurogenesis and development signalling pathways. The downstream effector proteins of these regulatory proteins are tightly regulated by the UPS system. PSD95 (Postsynaptic Density protein), an important scaffolding protein involved in localisation of receptors at the post synaptic membrane (such as NMDARs : N-methyl-D-aspartate receptor and AMPARs: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) are regulated by UPS (Mabb and Ehlers, 2010). Another notable example to demonstrate the importance of UPS regulation of signal transduction is that of Notch. Notch is a membrane receptor protein that plays a significant role in neurogenesis in response to extra cellular signals (Lathia et al., 2008). Recent studies discovered that Notch degradation is mediated by Fbxw7, thus Fbxw7 action can serve to regulate neuronal progenitor differentiation (Matsumoto et al., 2011).

The role UPS has in the cell cycle has been documented in actively dividing cells, however not much is known about its role in non-dividing cells like neurons. Only a few CNS disorders involving the UPS, directly or indirectly, are discussed here. Angelman syndrome characterised by mental retardation, epilepsy, ataxia and speech problems was the first human mental illness related to a genetic defect (defective UBE3A gene encoding E6-AP, a Ubiquitin E3 ligase (Kishino et al., 1997)) in the UPS. A frame shift mutant form of ubiquitin has been reported in patients with Alzheimer's disease and Downs syndrome (van Leeuwen et al., 1998). The inability of this mutant ubiquitin moiety to undergo proteasomal degradation can be one of the possible disease mechanisms in at least one of the above disease (Fischer et al., 2003). Huntington's chorea (HD) is one of the nine PolyQ repeat neurodegenerative disorders characterised by the presence of increased tri nucleotide repeats of CAG which are translated into long stretches of glutamine residues increasing their tendency to aggregate. A mutant of the Htt protein (encoded by Huntigton/Htt gene) is deposited in the form large microscopic inclusion bodies (IB) which is the hallmark of HD. IBs interfere with the proteasome and impair the UPS function. Though the exact disease mechanism is not yet elucidated, it is hypothesised the impairment of UPS function may disturb the cellular protein dynamics resulting in cytotoxicity leading to subsequent cell death (Finkbeiner and Mitra, 2008). Parkin (PRKN2), a ubiquitin E3 ligase and UCHL1, a human DUB enzyme, have been implicated in the pathology of autosomal recessive juvenile Parkinson's disease (Segref and Hoppe, 2009). Spinocerebellar ataxia is caused by a mutant form of ataxin-1, which is resistant to

proteasomal degradation (Klimaschewski, 2003). Micro array analysis of transcripts from peripheral blood revealed the UPS as one of the canonical pathway disrupted in SCZ and bipolar disorder (Bousman et al., 2010). Finally, a more recent study performed on post mortem brain samples of SCZ patients, uncovered abnormal levels of UPS enzymes and protein ubiquitination (Rubio et al., 2013). The above-mentioned findings not only emphasise the role of dysregulated protein degradation pathways in mental illnesses, but also provide scope for new therapeutic drug targets.

1.5.5. UPS as drug target

UPS became a therapeutic attraction for the first time in 2003 following the approval of Bortezomib (Velcade®), a proteasome inhibitor approved by the FDA for the treatment of multiple myeloma. This was followed by the Nobel prize in chemistry awarded to Aaron Ciechanover, Avram Hershko and Lewin Rose for their discovery of ubiquitin mediated protein degradation in 2004. Since then, UPS components have become an attractive drug targets and number of inhibitors targeting this signalling pathway are being developed. As of 2013 quite a few of them have entered clinical trials. Examples include Proteasome inhibitors - CEP-18770 and MLN-9708, E1 enzyme inhibitor – MLN4924, E3 enzyme inhibitor – RO5045337 (a Nutlin which antagonises Mdm2-p53 interaction). Till date there is no report of a DUB inhibitor in clinical trials, although there are several under preclinical development, reviewed in (Mattern et al., 2012).

The E1 enzyme has been described as a potential therapeutic target for treatment of hematologic malignancies (Xu et al., 2010). Tertiary structure of the E2 has been solved and provides insight in to three structural orientations of the catalytic site and the interactions with other proteins. Structure activity relationship studies would help design molecules targeting the active site of E2 enzyme. E2 and E3 enzymes meet the criteria for an ideal drug target as they are enzymes, which can be screened for small molecule inhibitors or specific antibodies. Since they are either expressed in low levels or not expressed in normal cells, their inhibition may have minimal effect on normal cell growth and function, thus minimizing toxic side effects as well as achieving maximal therapeutic index. Different strategies of targeting the E3 ligases would include small molecule

inhibitors or peptides that can target E3-E2 interfaces, E3-substrate interfaces or E3 ligase catalytic sites.

Proteasome inhibitors, though proven effective as a therapeutic strategy, have associated toxicity and side effects due to the non-selective nature of their action (many proteins get degraded by the 26S proteasome). As mentioned earlier, Bortezomib (velcade), was one of the first drugs targeting proteasome for treatment of multiple myeloma, however, excessive toxicity and drug resistance after prolonged use defined the need for the development of new inhibitor classes, which are more specific to the target (Bedford et al., 2011).

Ubiquitin Ligases (E3s) provide an alternative and attractive therapeutic target as their actions are limited to a handful of protein substrates. The following scenario serves as a best example of E3 ligase as a drug target. p53 is a tumour suppressor gene and transcription factor which regulates cell cycle progression and induces apoptosis depending on the stimuli, thus preventing malignant transformation. In nearly 50% tumours p53 is inactivated and altering its activity has been considered as a strategy for therapeutic intervention for cancer. p53 protein levels and activity are maintained under strict control by its cognate E3 ligase Mdm2 (murine double minute 2). Mdm2 binds to p53 at its transactivation domains and blocks its activation. Hence by inhibiting p53-Mdm2 interaction, stabilizes p53 and activated p53 (Shinohara and Uesugi, 2007) (Ding et al., 2013). X ray crystallographic structure of p53 (15-29) bound to Mdm2 (17-125) reveals an interaction between hydrophobic N terminal stretch on Mdm2 and amphipathic α helix of p53 peptide (Verkhivker, 2012, Vassilev et al., 2004). This information led to the development of benzodiazephine analogues and polyaromatic compounds, which disrupt the above complex. Several other compounds were later identified which disrupt the p53-Mdm2 complex. These up regulated p53 protein levels, activated the p53 pathway and induced cancer cell apoptosis (Wong et al., 2003). Other examples of irreversible inhibition of E3 ligases are (1) Tetrazole compound (Ro106-9920) which blocks ubiquitination of IκBα, (2) Ro106-9920 which reduces the serum levels of TNFα and prostaglandins in lung (Wong et al., 2003). Allosteric inhibitors can also be used to block the conformational changes or other post translational modification required for the E3 ligase activation. Compounds with imidazole and benzosulfonamide nuclei interfere with Mdm2 ubiquitination of p53 in this manner (Lai et al., 2002). Virtual ligand

screening (VLS) has also been used to screen for compounds with the ability to interfere with E3 ligase activity. *In silico* based approaches have identified small pockets in the E3 ligase that are suitable for docking with small molecules that could act as inhibitors. This approach has been used to inhibit Skp2 directed degradation of p27 (Wu et al., 2012).

High-throughput screens are another popular approach in drug discovery. Multiple steps in the ubiquitination cascade can be targeted using this approach. Inhibitors can interfere with kinase activity of the "priming kinase" (required for E3 ligase binding to the substrate) or attenuate SCF complex assembly either by interfering with the interaction directly or allosterically. An Inhibitor of the SKp1-Met30 interaction is an example for this approach and is selective to the F box domain of Met30 and hence the inhibitor doesn't interfere with the binding of other Skp1 binding F Box proteins (Aghajan et al., 2010).

1.6. DISC1 as a possible therapeutic target for neurological disorders

Various genetic studies have implicated the DISC1 locus to be a predisposing genetic factor in major mental illnesses including, SCZ, schizoaffective disorders, bipolar and depression (Chubb et al., 2008, Song et al., 2010, Ishizuka et al., 2006). However, no specific allele, mutation or SNPs has been proved to be a risk factor across various studies. As discussed earlier, DISC1 has a conserved nuclear localisation signals (NLS), which regulate its cytoplasmic/nuclear distribution. The nuclear form of DISC1 is increased in patients with sporadic SCZ, major depression, and substance alcohol abuse (Sawamura et al., 2005b). This study suggests the importance of the NLS domains on DISC1 and proposes it as a putative drug target for the aforementioned diseases. Protein aggregates are a hallmark of neurological diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) and DISC1 also forms aggregates both in vivo and in vitro. These aggregates could possibly interfere with the physiological functions of DISC1 and disrupting these aggregates can be considered as a potential therapeutic strategy.

cAMP specific PDE4 (phosphodiesterase) enzymes are potential interacting partners of DISC1 which were identified in the yeast 2 hybrid screen (Camargo et al., 2007) PDE are

the enzymes, which degrade cAMP, a crucial secondary signalling molecule. Raised cellular levels of cAMP caused the dissociation of DSIC1-PDE4 complex (Murdoch et al., 2007a). Of various isoforms of PDE4, PDE4B locus has been identified as a possible risk factor for SCZ (Millar et al., 2005). While genetic variants of PDE4B and PDE4D were shown to be directly associated with SCZ (Pickard et al., 2007, Hennah and Porteous, 2009, Fatemi et al., 2008), DISC1 has been shown to specifically interact with certain isoforms of PDE4B and PDE4D and this interaction is modulated by cellular cAMP levels (Murdoch et al., 2007). Hence, the DISC1-PDE4 interaction has gained much attention in the studies of pathophysiology of SCZ.

Studies show that shRNA knock- down of DISC1 in adult mice resulted in aberrant dendritic morphology and mis-positioning of adult born dentate granule cells (Duan et al., 2007), abnormalities in axonal targeting (Faulkner et al., 2008), abnormal development of dentate gyrus neurons and dendritic abnormalities and induced mTOR signalling (Zhou et al., 2013a). RNAi mediated knock down of DISC1 in differentiating PC12 cells caused significant inhibition of neurite outgrowth and this could be rescued by the co transfection of WT DISC1 (Kamiya et al., 2005a). *In utero* gene transfer of RNAi in mouse embryos resulted in reduced neuronal migration and reduced dendritic arborisation of cortical neurons (Kamiya et al., 2005b). In the absence of knockout DISC1 mice, the above studies as well as transgenic DISC1 mouse models (reviewed in (Jaaro-Peled, 2009)) emphasize the importance of DISC1 in the mechanism behind neurological disease.

As proposed by the neurodevelopmental hypothesis of SCZ, the symptoms of SCZ generally tend to appear in late adolescence and it has long been believed that the biological events causing the disease may have begun at a much earlier stage (Ayhan et al., 2011). Though other genes have been implicated in SCZ and neurogenesis, such as neuregulin-1 and dysbindin, most of the studies were focussed on DISC1 due to its increased association in linkage and association studies. The importance of the Scottish translocation has long been a debate for several reasons. One such reason is that, since the translocation is in the middle of an open reading frame, the protein even though translated, may be unstable and eventually degraded leading to haploinsufficiency (both alleles need to be functional for a wild type functional genotype). However, to date, there is no proof of DISC1 truncated protein being translated. Truncated DISC1 protein

might interfere with function of the wild type protein product from the other allele, resulting in total loss of function. This is known as dominant negative effect.

As discussed earlier, the phosphorylated form of DISC1 interacts with Bardet-Biedl Syndrome (BBS) proteins at the centrosome, while non-phosphorylated forms up regulate Wnt signalling via interaction with GSK3β which acts as a developmental switch between progenitor proliferation to post mitotic neuronal migration (Ishizuka et al., 2011b). This study emphasises the role of DISC1 as a scaffold protein function depends on several interacting partners. One source kinase of the phosphorylation of DISC1 at Ser713 is Cdk5 (Ishizuka et al., 2011b). Cdk5 inhibitors CP-668863 and CP-681301, developed by Pfizer have been considered as potential drugs to provide neuro protection in Alzheimer's disease (Hikida et al., 2012).

1.7. Aims of my Ph.D.

As detailed in my previous sections, genetic studies implicated DISC1, already associated with SCZ and other mood disorders, but also in autism spectrum disorders, attention deficit and hyperactivity disorder (ADHD) and Asperger syndrome. While lymphoblasts cell lines of the Scottish family members carrying the translocation were shown to have reduced expression of mRNA transcripts, no changes were detected in SCZ patients outside this family which suggests the complexity of regulation of DISC1 expression and protein processing. In addition, atypical antipsychotic drugs such as olanzapine and risperidone were shown to increase DISC1 transcript levels in mouse frontal cortex and hippocampus suggesting that they may exert their effect by modulating DISC1 expression (Olincy et al., 2011, Chiba et al., 2006). Abnormal brain development and function has been reported with RNAi mediated knockdown of DISC1 indicating that loss of DISC1 function is not desirable for appropriate brain development (Maher and LoTurco, 2012, Hikida et al., 2012).

To date, little is known about the post translational modifications on DISC1. Phosphorylation of DISC1 at Ser713 was shown to function as a molecular switch between progenitor proliferation and post mitotic neuronal migration in the developing cortex.

From the above evidence it can be proposed that understanding how DISC1 protein levels are modulated may hold the key to understand DISC1 associated pathology and uncover key targets for therapeutic intervention. With recent studies implicating the dysregulation of ubiquitin and Ubl signalling pathways in schizophrenia, it would be interesting to investigate how DISC1 expression is regulated by these signalling pathways and how it may contribute to disease development (Altar et al., 2005, Rubio et al., 2013).

Hence through my work I set out to address the following key questions:

1. Is DISC1 a potential SUMO substrate in cells? What are the key SUMO acceptor sites on DISC1? What is physiological significance of DISC1 SUMOylation? As a scaffold protein with many interacting partners, does SUMOylation effect its protein interactions such as DIXDC1 and Ndel1 which have been implicated in neuronal precursor proliferation, differentiation and migration. I addressed these aspects using in vitro peptide array technology, UFDS (Ubc9 fusion directed SUMOylation) and Immunoprecipitation in chapter 3.

2. In chapter 4, I investigated the ubiquitin modification of DISC1 protein using proteasome inhibitor MG132. Immunoprecipitation, Western blotting and Mass spectroscopy studies were used to identify potential lysine moieties which are modified by ubiquitin conjugation. Human Fbox siRNA library screen was performed to identify putative Fbox that regulates ubiquitination of DISC1 i.e., Fbxw7.

3. In chapter 5, utilising peptide array approach, I had mapped the crucial interaction sites between DISC1 and the Skp1-Fbxw7 followed by a series of optimisation screens via which I developed certain disruptor peptides to target DISC1- [Skp1-Fbxw7] complex. Lead peptides were shortlisted following their ability to stabilise DISC1 protein levels in HEK293 cells. Neuronal progenitors generated from iPS cells were also explored for their potential as cell line models for psychiatric diseases.

4. In chapter 6, I discussed my work which involved developing a sensitive in vitro FP assay to monitor DISC1- [Skp1-Fbxw7] interaction. This assay was adapted to perform a high throughput chemical library screen to identify small molecules which disrupt this complex.

2.

Materials and Methods

2.1 Materials

All chemicals used in this study were of analytical grade supplied by Thermo Fisher scientific, Invitrogen or SIGMA Aldrich unless otherwise specified. All peptides used in the present chapter were custom made by ProImmune, UK at >85% purity, assessed by Mass spectroscopy and HPLC. Labelled peptides used as tracers in FP assays were tagged with FITC (Fluorescein isothiocyanate) on the amino terminal end of the peptides. All the drugs and disruptor peptides were dissolved in dimethly sulfoxide (DMSO) and used at a final DMSO concentration of 0.001%.

2.2 Molecular Biology Methods

2.2.1 Transformation of competent cells

DH5α competent cells (Invitrogen) stored at -80°C were carefully thawed on ice just before use. 1-10 ng of DNA was added to 30 µl of competent cells in a sterile eppendorff and incubated on ice for 10 min. The cells were then heat shocked at 42°C for 45 sec then placed on ice for 2 min. The cells were then added to 300 µl Luria Broth (LB) media (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 170 mM NaCl) and incubated at 37°C for 1 hr with shaking. 50-100 µl of transformation mix was then spread on 100 mm petri dishes containing pre poured LB agar plates (L broth with 1.5% (w/v) agar) containing appropriate antibiotic (ampicillin, 50 µg/ml, kanamycin 30 µg/ml) using a

sterile disposable spreader. These plates were incubated upside down overnight at 37°C. The growth of bacterial colonies indicated successful cell transformation.

2.2.2 Isolation of plasmid DNA from E coli

Single colonies were selected from the overnight incubated agar plates and grown overnight in 5 ml LB media containing appropriate antibiotic in an orbital shaker at 37^{o}C. QIAprep Miniprep Kit (Qiagen) was used to isolate smaller amounts of plasmid DNA. Alternatively, for a larger volume of bacterial culture, 500 ml LB media supplemented with appropriate antibiotic was inoculated with 5ml starter culture grown for about 6-8hr and incubated overnight at 37^{o}C. For large scale plasmid DNA extraction the QIAprep Maxiprep kit (Qiagen) was used according to the manufacturer's instructions. The purified DNA was then eluted with sterile double distilled H20 and stored at 4^{o}C for short term storage or -20^{o}C for longer term.

2.2.3 Storage of plasmid DNA

Overnight incubated bacterial culture was mixed with 30% sterile glycerol solution at 1:1 ratio to give a final glycerol concentration of 15% in cryovials and snap frozen using dry ice and stored at -80°C.

2.2.4 Quantification of DNA concentration

The concentration of purified DNA was determined using Nanodrop 3300 spectrophotometer (Thermo Scientific). Absorbance wavelength was set at 260 nm and 280 nm and the A260:A280 ratio determines the purity of the nucleic acid extract where a value of 1.8 and 2.0 is considered pure for DNA and RNA, respectively. The ratio of A260:A230 ideally should be between 2-2.3 and a lower reading may indicate organic solvent contamination in the preparation.

2.2.5 Total RNA extraction

The protocols described in this section are described, according to Standard Operating Procedure of Pfizer, for the work performed as part of a 10 week placement at Pfizer Neuroscience research Unit, Cambridge, MA, USA).

Homogenisation: Culture medium was aspirated off culture dishes and 1ml TRIzol (per each well of a 6 well culture plate) (Ambion 15596-026) was added.

Phase Separation: The homogenised samples were incubated for 5 min at RT to allow complete dissociation of nucleoprotein complexes. 0.2 volumes of chloroform per 1ml TRIzol reagent was then added and vigorously mixed followed by 2-3 min incubation. The samples were centrifuged at no more than 1,000 g for 15 min at 4˚C. Following centrifugation the mixture separates into a lower red phenol chloroform phase (which constitutes protein), an interphase and a colourless aqueous phase. The aqueous phase, which exclusively contains the RNA, was carefully transferred to a fresh tube without disturbing the other phases.

RNA Precipitation: RNA in the separated aqueous phase is precipitated by adding 0.5 volumes of isopropanol per 1 ml of TRIzol reagent used. The samples are thoroughly mixed and incubated for 10 min at RT.

RNA Wash: The RNA was pelleted by spinning at no more than 10,000 g. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. Samples were then centrifuged at no more than 7,500 g for 5 min at 4˚C.

Redissolving the RNA: The ethanol was carefully aspirated off, the RNA pellet was air dried and re-suspended initially in 100 µl followed by quantification.

DNase treatment of purified RNA: Purified RNA was further subjected to DNase treatment to remove any DNA contamination. TURBO DNA free kit (Ambion AM1907) was used and the DNase treatment was done according to the protocol mentioned in the instruction manual.

RNA precipitations clean up: Re-suspended RNA was re-precipitated to remove any salts or organic solvents that may have co-precipitated. NaCl was added to a final concentration of 0.1M followed by the addition of 2.35 volumes of ethanol. The samples were then thoroughly mixed and incubated at -20^{o}C for >30min or overnight. The RNA was then precipitated at 4˚C by spinning at 10,000 g. The supernatant was carefully aspirated and the RNA pellet was air dried. The RNA was re-suspended in water and stored at -80˚C.

2.2.6 cDNA synthesis

High Capacity RNA to cDNA Kit (Applied Biosystems 4387406) was used according to the manufacturer's instruction manual.
Reaction was set up as following:

Table 2.1 Standard PCR reaction conditions

Components	Volume
2x RT buffer	10 µL
20x Enzyme mix	1 µL
RNA (2µg)	X µL
Water	20 µL final reaction volume

Cycling conditions	Time (minutes)
37°C	60
95°C	5
4°C	Forever

The cDNA samples were stored at -20C° for subsequent RT-PCR analysis.

2.2.7 TaqMan real-time PCR

Gene specific pairs of primers and TaqMan probes were designed by Applied Biosystems (inventoried TaqMan® gene expression assays). All assays were purchased from Applied Biosystems and assay IDs are given in Table 2.3. The relative expression of DISC1, Pan FBXW7 and alpha FBXW7 isoform was assessed in 2 separate cDNA samples from the neuronal progenitors generated from all 6 different iPS cell lines. Real-time PCR was performed from reverse transcribed cDNA samples using the Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen) following the manufacturer's instructions. Briefly, 100 ng of cDNA were added to a 96-well MicroAmp® Fast Optical Reaction Plate (applied Biosystems) with 12.5 µl TaqMan probe mix, 1 µl each of the 10 µM primer pair and 0.25 µl of 10 µM fluorogenic probe (either gene-specific inventoried assays or endogenous reference assays). Thermal cycling and fluorescent monitoring were performed using the

ViiA 7 Real Time PCR system. Each PCR amplification was run in quadruplicate using the following conditions: initial denaturation at 95°C for 15 sec, followed by a total of 40 cycles (3 sec at 95°C for denaturation, 30 sec at 60°C for annealing and extension). Fluorescence data were collected during the extension step of each cycle. Negative controls using RNA as template were also included in all runs to test for the presence of genomic DNA contamination.

Table 2.2 Inventoried primer probe sets purchased from Invitrogen

Gene	Assay ID
TBP	Hs99999910_m1
PSMB2	Hs00267650_m1
RPL13A	Hs03043884_g1
RPN1	Hs00161446_m1
RPLP0	Hs02992885_s1
DISC1 (pan)	Hs00257791_s1
FBXW7 (pan)	Hs00217794_m1
FBXW7 (α isoform)	Hs01023824_m1

Table 2.3: Standard PCR reaction conditions

Component	Per Reaction
Master Mix	5 µl
Taqman Assay	0.5 µl
Water	1.5 µl
cDNA (5 ng/µl)	3 µl
Total volume	10 µl

2.2.8 Transient transfection of plasmid DNA

Plasmid DNA used in the present study was transiently transfected into Human embryonic kidney 293 (HEK 293) cells using Polyfect transfection (Qiagen) according to the manufacturer's instructions. To describe briefly, cells were plated at a density of 4x 10^5 cells per 100 mm dish. At 50-60% confluency, transfections were performed with 8µg of circular plasmid DNA using Polyfect transfection reagent according to the manufacturer's instructions. The assay was scaled down for smaller culture dishes following the instruction manual. Cells were incubated for at least 48 hr for sufficient protein expression.

2.2.9 si RNA transfection – Fbox siRNA library screen

ON Target PLUS siRNA library for Fbox (G-105625-05) was purchased from Dharmacon, Thermofisher Scientific. HEK293 cells were plated at a density of 3 x 10^3 cells/ well in 6-well plates. 24 hr after incubation, the cells were transfected with control nonspecific siRNA or the test siRNA from the above library at a final concentration of 25 nM/each well using DharmaFECT (Dharmacon - Thermo Scientific) according to the manufacturer's protocol. The cells were incubated for 64 hr at 37°C in a CO_2 incubator. Gene expression knockdown was analysed by performing Western blotting.

2.3 Cell culture

All cell culture procedures were carried out in a Class II hoods using standard aseptic techniques and sterile instruments. All culture reagents were supplied by Sigma. The tissue culture flasks, dishes, and pipettes were supplied by Corning. All cultures were examined regularly under a phase contrast inverted microscope (Leitz Diavert, Germany).

2.3.1 HEK293 cells

The HEK293 cells were used for the present study. These cells were maintained in growth media containing DMEM (SIGMA) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/µg penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were passaged at about 80% confluency. For cell passage, growth medium was aspirated and the cells were washed in sterile phosphate buffer saline (PBS) to remove traces of media. The PBS was then aspirated off and the cells were treated with 2mL trypsin-EDTA solution per 75 cm^2 culture flask of cells to dissociate cell monolayer. Growth medium was added to quench the trypsin-EDTA solution and cells were collected by centrifugation at 10,000 g for 2 min at room temperature. The supernatant was removed without disturbing the cell pellet which was then re-suspended in appropriate fresh growth media and re-plated according to the requirement. For cryopreserving the cells, the cell pellet was re-suspended in 10% dimethyl sulfoxide (DMSO) containing growth medium and stored at -80˚C.

2.3.2 iPS cell culture and neuronal progenitor differentiation

All the protocols described in this section were according to the SOP (standard operating procedure) followed at Pfizer Neuroscience Research Unit, Cambridge, USA.

2.3.2.1 iPSc generation and culture

All the iPS cells (except Detroit 551 cell line) used in the present study were generated by Chiang et al (Chiang et al., 2011). Detroit 551 iPS cell line was generated by Dr.Sandra Engle, Pfizer Inc. Detroit 551 fibroblasts were obtained from ATCC (CCL-110) and maintained in Knockout DMEM (Life Technologies 10829-018) with 15% FBS (Life Technologies), 1x Nonessential amino acids (Life Technologies 11140-050), 2 mM L-glutamine (Life Technologies 25030-081), 1x Gentamicin (Life Technologies 15710-072), 0.1 mM β-mercaptoethanol (Sigma M-7522). The cells were reprogrammed with individual lentiviruses expressing c-Myc, KLF4, SOX2, Oct3/4, Nanog, and LIN28 using standard techniques (Takahashi, Okita et al. 2007). One colony, Detroit 551-3, with ES-like morphology, high expression of pluripotency markers (e.g. Oct3/4, TRA-1-60, TRA-1-81, SSEA4), a normal karyotype, a normal karyotype and the ability to form all 3 germ layers in a teratoma assay was used in all subsequent experiments. The cells were maintained on mitomycin C (Sigma M0503)-treated mouse embryonic fibroblasts (MEF) and grown in hPSC media [DMEM/F12 (Life Technologies 11330-057), 20% Knockout serum replacement (Life Technologies, 10828-028), 2 mM L-glutamine (Life Technologies 25030-081), 1x Gentamicin (Life Technologies 15710-072), 0.1 mM β-mercaptoethanol (Sigma M-7522), FGF2 (Life Technologies PHG0263)]. Cells were enzymatically passaged using 1 mg/ml Dispase (Life Technologies 17105-0410) to every 4-6 days to fresh MEF.

2.3.2.2 Neuronal Progenitor differentiation

Neuronal differentiation protocol was adapted from the protocol of (Pankratz et al., 2007). Embryoid body formation was induced on the first day, assigned as day 0. Culture media was removed from the appropriate culture dishes with iPS cell colonies and washed with warm (37°C) DMEM-F12 media. 1ml Dispase (Invitrogen- 17105-041) was added per p100 culture plate and incubated for 37°C for 2-5 min until the edges of the cell colony start curling up. Disapase was aspirated off and washed with DMEM-F12

media. Using Stempro EZPassage (Invitrogen 23181-010) the colonies were gently broken until they were dissociated from the plate. The iPS cell colonies were transferred from half of the p100 dish to 100 mm non coated, non-treated, non-adhesive cell culture dish with hPSC media [DMEM/F12 (Life Technologies 11330-057), 20% Knockout serum replacement (Life Technologies, 10828-028), 2 mM L-glutamine (Life Technologies 25030-081), 1x Gentamicin (Life Technologies 15710-072), 0.1 mM β-mercaptoethanol (Sigma M-7522), FGF2 (Life Technologies PHG0263)], to promote preferential adhesion between cells over adhesion to the culture dish.

On day 2, the embryoid bodies were collected by centrifugation in appropriate centrifuge tubes at 500 g for 5 min at RT. Supernatant was discarded while extreme care is taken not to disturb the pellet which was re-suspended in 10 ml of GABA differentiation media [50% Neurobasal Media (Life Technologies 21103-0490), 50% DMEM/F12 (Life Technologies 10565-018), 0.5x N2, 0.5x B27 (Life Technologies 17504-044), 1 mM L-glutamine (Life Technologies 25030-081), 1x Gentamicin (Life Technologies 15710-072), 10µM SB431542, 0.2µM LDN-193189 (StemGent 04-0074), 20ng/ml FGF8b (R&D Systems 423-F8-025), Shh (R&D Systems 1845-SH-025/CF), 10 ng/ml FGF2 (Invitrogen PHG0263)], supplemented with ROCK inhibitor (10 µM) and transferred to cell culture plates pre-coated with matrigel. Cell culture dishes were coated with matrigel according to manufacturer's instructions (BD Matrigel matrix- 354277). Fresh GABA Media change was done from day 4 to day 10.

Cells were passaged on day 10 by aspirating the media and washing 3 times with PBS. 1 ml of accutase (Invitrogen A1110501) was used to gently lift the cell off the dish. Equal volume of DMEM-F12 media was added to neutralise the enzyme activity and the cells were collected by gently pipetting the suspension and centrifuged at 1000 g for 5 min at RT. Supernatant was disposed and the cell pellet was re-suspended in GABA media supplemented with 10µM ROCK inhibitor and plated in 1:1 ratio on pre coated POL coated plates (20 µg/ml poly-L-ornithine hydrobromide (Sigma P3655)/ 5 µg/ml natural mouse laminin (Life Technologies 23017-015) (POL)-coated plates).

Fresh media change was performed every day with GABA media. Confluency and morphology of the cells was microscopically observed on a daily basis to detect unwanted

differentiation. Cells can be passaged using the protocol described above depending on the confluency after day 15. From this point onwards neuronal progenitors can be maintained and expanded as required in NP media [50% Neurobasal Media (Life Technologies 21103-0490), 50% DMEM/F12 (Life Technologies 10565-018), 0.5x N2, 0.5x B27 (Life Technologies 17504-044), 1 mM L-glutamine (Life Technologies 25030-081), 1x Gentamicin (Life Technologies 15710-072), 10ng/ml EGF (R&D Systems 236-EG), 10ng/ml FGF2 (Invitrogen PHG0263)]. Cells were maintained in appropriate dishes or multi well plates as required at 1.62 x 10^5 per cm^2 area of the culture vessel.

2.3.3 Preparation of Cell Lysates

Cells were briefly washed with PBS and then lysed in to ice cold cell lysis buffer 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 10mM DTT with Roche protease inhibitor, phosSTOP (Roche, 04906837001), 2mM NEM (N-ethylmaleimide). The cell suspension was then incubated on an end-over-end rotor at 4°C to ensure proper cell lysis and then centrifuged at 13,000 g for 10 min at 4°C. The protein concentration in the supernatant was analysed by Bradford method (Bradford, 1976).

2.4 Protein techniques

2.4.1 Co-Immunoprecipitation

Cell lysates were adjusted to equal protein amount (1 µg/µl) using cell lysis buffer. Cell lysates normalised for the protein concentration were incubated with mouse IgG (Millipore, 12487) Protein A agarose beads (life technologies, 15918-014) for 2 hr at 4°C. Agarose beads were collected after a brief centrifugation and the collected supernatant was added with HA tagged agarose (SIGMA; A2095) washed 3 times in cell lysis buffer and the final volume is adjusted to 750 µl and incubated over night at 4°C. The beads were precipitated and washed three times to remove non-specifically bound proteins. Immune complexes were eluted from the beads by boiling them in SDS loading buffer. The complexes were analysed on SDS PAGE. Tagged agarose beads used in the present study include: Flag (Sigma), HA (Sigma), V5 (Abcam), IgG (Sigma).

2.4.2 Denaturing Co-Immunoprecipitation

Cells were scraped in to cell lysis buffer with 1% SDS (Sodium Dodecyl Sulphate, an anionic detergent) and boiled for 5 min at 95°C. Bovine pancreatic DNase 1 (EMD_BIO-260913) was added to degrade the DNA and prevent the lysate from becoming viscous. The lysates were then spun down at 13000 g for 10 min to precipitate cell debris. Lysates normalized for protein concentration were then diluted with the cell lysis buffer to a make final SDS concentration to 0.1%. Immunoprecipitation was carried out as explained in previous section.

2.4.3 SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) was carried out to separate proteins based on their molecular weight. SDS (Sodium Dodecyl Sulphate) is an anionic detergent which imparts uniform negative charge to the polypeptide backbone. The cell lysates were boiled in 5x SDS loading buffer (10% SDS, 300 mM Tris-HCl, pH 7.2, 0.05% bromophenol blue, 50% glycerol, 10% β-mercaptoethanol) at 95°C for 5 min and were then loaded on to NuPAGE precast 4-12% gels in MOPS buffer along with pre-stained protein marker (Bio-Rad). Protein samples were resolved on the gel according to the manufacturer's instruction manual.

2.4.4 Western blotting

The resolved proteins on NuPAGE gel were electrotansfered onto nitrocellulose membranes (Whatman GmbH) in 1X transfer buffer with 20% methanol for 2 hr at 27 V or overnight at 11 V. The membrane was then blocked in 5% (w/v) non-fat dry milk (Marvel) in TBST (25 mM Tris-HCl; pH 7.6, 100 mM NaCl, 0.5% Tween 20) for 1 hr at room temperature or 4°C over night. Membranes were then probed with specific primary antibodies at appropriate dilutions (Table 2.4) in 1% (w/v) marvel in TBST solution and incubated for 2 hr at room temperature or overnight at 4˚C. The membranes were washed thrice for 15 min each in TBST. Membranes are then probed with appropriate horseradish peroxide (HRP) conjugated anti-immunoglobulin G (IgG) secondary antibody (Table 2.5) diluted 1:5000 in 1% milk/TBST solution for 1 hr at room temperature.

Membranes were then washed 3 times for 10 min. Chemiluminescence (ECL) Western blotting Substrate (Thermo Scientific) was used for detecting the HRP conjugates on the membrane. Chemiluminescent images of immune detected bands were recorded on blue-light sensitive autoradiography X-ray films which were then developed using the Kodak® X-Omat Model 2000 processor. Band intensities were quantified using image J (Girish and Vijayalakshmi, 2004).

Table 2.4 List of primary and secondary antibodies, their source and working dilutions.

Primary Antibody	**Supplier**	**Part number**	**Host animal**	**Dilution**
HA	Santa Cruz	sc-7392	Mouse	1:2000
Penta His HRP	Qiagen	34460	Mouse	1:2000
Ubiquitin Lys 48	Millipore	05-1307	Rabbit	1:2000
Ubiquitin Lys 63	eBioscience	14-6077-82	Mouse	1:1000
FK2 (Pan mono and poly ubiquitin chains)	Enzo life sciences	BML-PW8810-0500	Mouse	1:3000
His HRP conjugated (Kit)	Qiagen	34460	Mouse	1:2000
Fbxw7	Abcam	ab12292	Rabbit	1:1000
c-Myc	Santa Cruz	Sc-764	Rabbit	1:2000
Myc tag	Cell Signalling		Mouse	1:1000
α-Tubulin	Sigma Aldrich	T5168	Mouse	1:10,000
GAPDH	Merck Millipore	AB2302	Mouse	1:20,000
Alpha hDISC1	Gift from Prof. Takayama, University of Tokyo		Rabbit	1:1000 (over night incubation)
GFP	Santa Cruz	Sc-9996	Mouse	1:3000
Flag HRP conjugated	Sigma		Mouse	1:5000
V5-HRP	Invitrogen	R961-25	Mouse	1:5000
Ubc9	Santa cruz	Sc-1-759	Rabbit	1:2000
Cyclin E	Cell signaling	4129	Mouse	1:2000
Notch1	Cell signaling	3608	Rabbit	1:2000
c-Jun	Cell signaling	9165	Rabbit	1:2000

Table 2.5 List of secondary antibodies, their source and working dilutions.

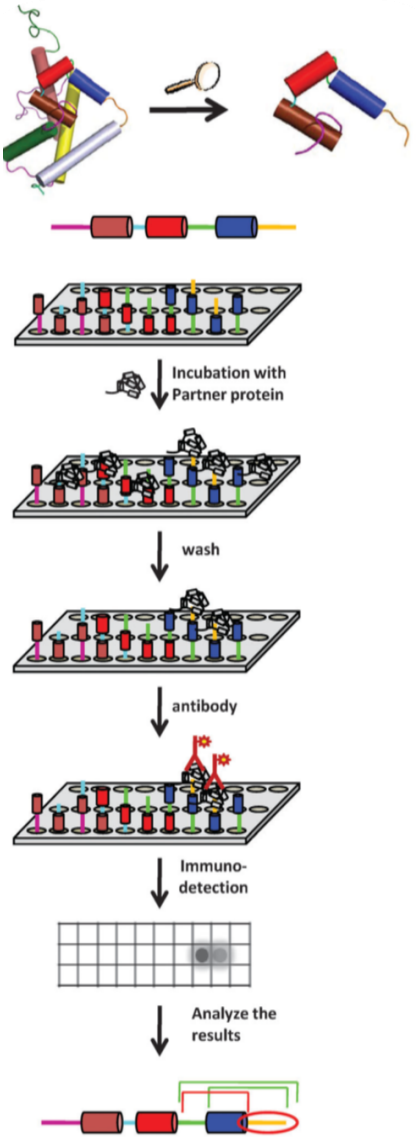
Secondary Antibodies	Supplier	Part number	Dilution
Mouse IgG - HRP	GE healthcare	NXA931	1:5000
Rabbit IgG-HRP	Sigma Aldrich	A6154	1:5000
IRDye 800CW Donkey anti-Mouse IgG	Licor	926-32212	1:10,000
IRDye 800CW Donkey anti-Rabbit IgG	Licor	926-32213	1:10,000

2.4.5 SPOT synthesis of peptides – Mapping DISC1-FBXW7 binding sites

Peptide arrays were generated in- house by SPOT synthesis on the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments). Principle behind this technique is the spotting of 25-mer peptides on a cellulose membrane. These peptide arrays can be used to identify protein interaction motifs by overlaying with purified proteins. It can also be used to perform *in vitro* assays like phosphorylation, SUMOylation etc. Either domain within a protein or the full length protein can be spotted as overlapping 25-mer (for *in vitro* SUMOylation assay – section 2.4.6) or shorter peptides, each shifted by five amino acids to increase the reliability of the screen.

To elucidate DISC1-Fbxw7 interaction, peptide library was generated constituting DISC1 phosphodegron motif C^{191}-G-P-E-V-P-P-T-P-P-G-S-H-S-A-F-T-S-S-F-S-F-I-R-L^{215} , full length, N terminal truncations, C terminal truncation, with phospho Thr and phospho Ser at 198 and 202 positions respectively. This library was spotted (linked covalently) on to microscope glass slides. Arrays were stored dry at -20°C prior to use. The slides were washed in 1xTBST for 1 minute followed by blocking with 5% PhosphoBLOCKER (AKR-103, CELL BIOLABS INC.) in TBST (w/v) (150 mM NaCl, 0.1% Tween20, 20 mM Tris-HCl and pH 7.5) for 1hr at room temperature to block non-specific binding. The array was then incubated with 1µg/ml of purified, recombinant SCF^{Fbw7} complex (Millipore, 23-030) in 1% PhosphoBLOCKER solution in TBST at 4°C overnight. Follwonig day, the array was washed 3 times for 10 minutes in TBST and incubated with His-HRP antibody at 1 in 2000 dilution

in 1% blocking solution for 2 hours at room temperature. After a further 3 washes the array was exposed to ECL and film (as in section 2.4.4).



A peptide library is designed with multiple partly overlapping peptides no longer then 25mers, while maintaining the secondary structures (helices and loops) intact. For full length protein arrays, each secondary element should overlap with the next secondary element, and also with the previous element. (for disruptor peptide development, peptides need not overlap since, the precise binding sites has been mapped).

Incubation with the interacting protein

Wash steps to remove non specific interaction

Detection of the binding protein is done using specific antibodies followed by ECL detection

Each dark spot represents binding of the protein to a specific peptide

Figure 2.1 Schematic of peptide array workflow
Adapted from (Katz et al., 2011).

2.4.6 *In vitro* SUMOylation of hDISC1 peptide array

Human DISC1 (hDISC1) peptide array was pre-blocked in 5% (w/v) BSA (bovine serum albumin, SIGMA) in TBST for 1hr at room temperature with gentle rocking. This was followed by a brief washing in TBST for 5 min. SUMO kit (Enzo life sciences, UW8955) was used to SUMOylate hDISC1 peptide array. This kit contains purified recombinant His tagged human SUMO1, SUMO2, SUMO3 proteins, active E1, E2 enzymes, ATP (required co factor for SUMO conjugation). A reaction mixture was prepared with all the above kit

components and made up to 2ml with sterile PBS (phosphate buffered saline). The array was incubated at 30°C for 60 min and then washed in TBST, 3 times for 5 min each. This was followed by probing with His HRP conjugated antibody at appropriate dilution mentioned for 2hr at room temperature followed by 3 times, 5 min washes with TBST. The array was ECL detected as explained in section 2.4.4.

2.4.7 Immunocytochemistry

All immunocytochemistry studies were performed at Pfizer NRU, Cambridge, MA, USA, as a part of 10 week placement (discussed in chapter 5). iPS cell colonies and neuronal progenitors (NPs) were stained for relevant markers for characterisation. NPs were plated at 100 cells per cm^2 area of 35mm MatTek dishes (Invitrogen). Cells were fixed with 4% paraformaldehyde followed by three washes with TBS (Tris-buffered saline; 150mM NaCl, 20 mM Tris, pH 7.4). Cells were permeabilised by incubating with 0.2% Triton dissolved in TBS. Cells were blocked for 1 hour with blocking solution (10% donkey serum, 4% BSA in TBS). The primary antibody was diluted in antibody diluent (Dako, S0809) to required concentration (dilutions were represented in the Table 2.6 and 2.7). 50 µl of diluted primary antibody was added to the coverslip for 2 hr or overnight incubation at 4°C. The dishes were washed thrice with TBS. The fluorescent antibody (Alexa 594-conjugated antibody or Alexa 488-conjugated antibody, depending on the primary antibody choice, was diluted at 1:200 using diluent and added to the dish for 1 hour (this procedure was repeated if a second fluorescent probe was needed). The coverslips were washed 5 times with TBS before mounting a cover slip with Immunomount (sandon). Cells were visualized using the Zeiss LSM 710 confocal microscope (Zeiss, Oberkochken, Germany).

Table 2.6 List of Stem cell marker antibodies used to characterise the iPS cell lines

Marker	**Localisation**	**Host animal**	**Dilution**	**Source**
Oct4A	Nuclear	Anti rabbit	1 : 800	Cell signalling, 2890
Tra-1-81	Surface	Anti mouse	1:1000	Cell signalling, 4745S
Nanog	Nuclear	Anti goat	1:100	Millipore, MABD24
SSEA4	Surface	Anti mouse	1:100	Millipore, MABD43034
DAPI	Nuclear			Invitrogen

Table 2.7 List of Neuronal Progenitor cell marker antibodies used to characterise the NPs generated from the iPS cell lines

Marker	Localisation	Host animal	Dilution	Source
Nestin	Filament	Anti Mouse	1:200	Millipore, MAB5326
SOX-2	TF (nuclear)	Anti goat	1:100	Santa cruz, SC-17319
Pax6	Nuclear	Anti Rabbit	1:300	Covance, PRB-278P

2.4.8 Mass Spectroscopy sample preparation

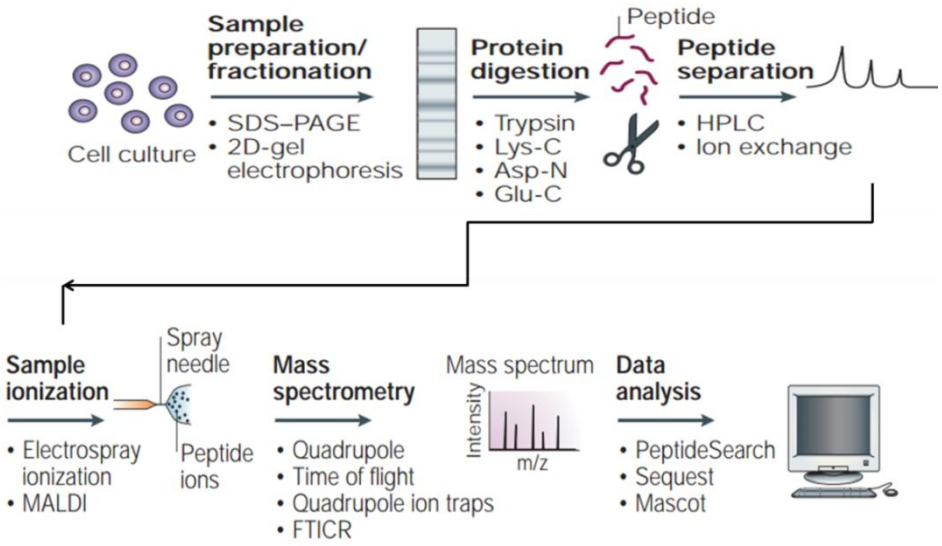


Figure 2.2 Schematic of Mass Spectrometry (MS) technique.
Figure adapted from (Steen and Mann, 2004)

Mass spectrometry is a powerful technique used to quantify unknown compounds, identify sample composition and characterise structure of various molecules such as proteins. In the present study, MS studies were performed to detect the following: (1) DISC1-SUMO conjugates and the corresponding acceptor lysine residues (2) DISC1-ubiquitin conjugates, type of ubiquitin chains and the acceptor lysine residues. HEK293 cells were transiently transfected with relevant constructs using Polyfect transfection reagent. 48hr post transfection, cell cultures were treated with MG132 at 10 µM final concentration (where ever applicable) before proceeding to cell lysis and subsequent co-Immunoprecipitation (described in chapter 4 and 5 for DISC1 SUMOylation and DISC1 ubiquitination respectively). Flag tagged agarose beads or 14F2 (DISC1 antibody 1) were used as bait to detect DISC1-ubiquitin and DISC1 –SUMO conjugates respectively.

Immunoprecipitate complexes were separated using SDS PAGE on bis tris acrylamide gel. The gel was then stained with Colloidal blue stain, Invitrogen (part number: LC6025) according to manufacturer's instructions. This was followed by gel de-staining in ultra-pure double distilled water over night. Relevant bands of interest (as indicated in the respective results chapter 3 and 4) were then excised under sterile conditions. The Mass spectroscopy analysis of the samples was out sourced to M.W. Keck Facility-Mass Spectrometry and Protein Chemistry Services Yale University, Connecticut, USA. In brief, the proteins in the excised gel bands were subjected to double digestion with Glu-C (a serine protease which cleaves at the C-terminus of glutamic acid and aspartic acid residues) and trypsin (a serine protease which primarily cleaves proteins and peptides at the C terminal side of lysine and arginine). Protein digests were analysed using Thermo Scientific LTQ-Orbitrap XL mass spectrometer.

The MS spectra were searched using the Mascot algorithm for MS spectra data (Hirosawa et al., 1993). MASCOT software search engine identifies proteins by comparing the MS data to peptide sequence databases. This program cleaves the protein *in silico* depending on the cleavage enzyme used in MS and determines the theoretical mass of each peptide. Each peptide is then scored based on the probability of a match between the sample peptides and the protein database. The score is a measure of the reliability of identification i.e., the number of peptides identified for each protein/peptide is proportional to the MASCOT score for that protein /peptide. For details more details on MASCOT score, refer to http://www.matrixscience.com/home.html.

2.4.9 Fluorescence polarization (FP) assay

Purified 'His6-tagged Fbw7 (residues 263-707)-Skp1' protein complex was a kind gift from Bing Hao, Structural Biology Program, Memorial Sloan-Kettering Cancer Centre, New York, USA. All the peptides used in the present study were custom made by Proimmune, UK. FP assays were performed in 384-well black, low-binding, round-bottomed plate (Sigma Aldrich) and read in Mithras LB 940 Multimode microplate Reader. Saturation experiments were performed to determine the K_d values of fluorescently labelled traces (peptides) with Skp1-Fbxw7 protein complex using 0.1 µM each of the peptides and serial dilutions of Skp1-Fbxw7 (example: 0.1 µM – 50 µM) in assay buffer PBS to produce a total

reaction volume of 10 µL. Each assay plate was covered with adhesive aluminium foil before reading polarization values using 485 nm and 535 nm excitation and emission wavelengths with Mithras plate reader.

Competitive FP binding assays were performed with 5 µL total volume of the fluorescent tracer (final concentration in the well - 0.1 µM) and Skp1-Fbxw7 complex (final concentration in the well 0.4 µM) was pipetted in each well in quadruplicates. Disruptor peptide or compound was titrated in a dose dependent manner using no fewer than 6 concentrations. Compounds and the disruptor peptides were serially diluted to minimise pipetting errors. For every assay negative controls were performed with the Skp1-Fbxw7 complex. The output reading was obtained as mP units (milli polarisation units). mP values were plotted against log concentration of the protein complex (for binding assays) and compound/disruptor peptides (competition curves). The principle of this assay and the Z'-factor for the competitive FP assay is further elucidated in chapter 6.

3.

DISC1 is a potential SUMOylation target

3.1 Introduction

While the complex pathophysiology of schizophrenia remains unclear, a number of recent molecular studies have uncovered interesting new insights, which are crucial to the understanding of the disease mechanism. Since its discovery, the DISC1 gene has been found to be associated with a range of psychiatric illnesses such as SCZ, bipolar disorder, major depression and autism (Hashimoto et al., 2006, Song et al., 2010). Several groups have independently reported that genetic variations in DISC1 can be associated with abnormal brain function and structure, along with impaired cognitive functions (Carless et al., 2011, Singh et al., 2011, Nakata et al., 2009, Zhang et al., 2006). Further to this, considerable effort has been put into understanding how DISC1 exerts its physiological function by studying its localisation and interacting partners. DISC1 has been shown to be localised in various sub cellular compartments such as mitochondria (Park et al., 2010) and the centrosome (Miyoshi et al., 2004) and altered localisation was reported to be associated with disease phenotypes.

Independent studies have reported associations between SNPs (single Nucleotide polymorphisms) within the DISC1 locus and various clinical and morphological brain phenotypes in patients with SCZ (Lipska et al., 2006b, Hashimoto et al., 2006). One such SNP, S704C, was identified in individuals with reduced hippocampal grey matter and was also identified in SCZ patients with positive symptoms (Leliveld et al., 2009, Singh et al., 2011). These studies suggest a role for the S704C allele in regulating human brain

morphology and function. Other DISC1 variants such as L607F and R264Q have been reported in both healthy individuals and in SCZ patients, and are known to affect cortical morphology, though the exact mechanism by which these SNPs affect the brain morphology is as yet unclear. It is interesting to note that no DISC1 mutations were observed in certain cases of chronic mental disorders (Leliveld et al., 2008). Hence, in this scenario it's unclear how a healthy DISC1 gene contributes to the disease aetiology. It may be hypothesised that post-translational modifications of DISC1 could be a reason for the wild type and the mutant DISC1 proteins becoming dysfunctional in these respective scenarios contributing to the pathophysiology of the disease.

3.1.1 Evidence of DISC1 aggregation contributing to impaired neuronal function

Carsten Korth and colleagues have conducted extensive studies of DISC1 aggregates in various cell lines (Figure 3.1). They reported the presence of DISC1 in detergent insoluble fraction prepared from post mortem brain tissue of individuals with chronic psychiatric illness including SCZ (Korth, 2012). Studies in neuroblastoma cells demonstrate that the 100kDa DISC1 isoform can aggregate and that the resulting insoluble fraction fails to interact with Ndel1, a well characterised DISC1 interacting partner (Leliveld et al., 2008). DISC1 aggregation also influences the selection of the scaffold's interacting partners. For example, DISC1 aggregates have a reduced ability to interact with Ndel1 (Leliveld et al., 2008) while they co-recruit soluble fractions of Dysbindin (Ottis et al., 2011). DISC1 aggregation was also found to be influenced by cellular stress (such as hydrogen peroxide) and disease polymorphisms. DISC1 Ser704Cys variant was shown to have an increased propensity to form aggregates (Korth, 2012).

DISC1 aggregates are also cell-invasive, a scenario mirrored in other psychiatric aggregation illnesses with proteins such as α-synuclein and polyglutamine (Bader et al., 2012). Independent studies by Kittler et al, reported the recruitment of GFP-DISC1 aggregates to aggresomes and subsequent degradation by the autophagy pathway. Microscopic studies show GFP-DISC1 co-localised with autophagy markers such as LC3

(microtubule-associated protein light chain 3) in COS-7 cells and primary neuronal cultures (Atkin et al., 2012). In COS7 cells, over-expressed GFP DISC1 co-recruits endogenous DISC1 from soluble fractions under physiological stress. Mitochondrial transport and neuronal function is also be impaired due to increased DISC1 aggregation (Atkin et al., 2012). This may be attributed to yet unknown conformational changes in the protein, which in turn may be influenced by post-translational modification events.

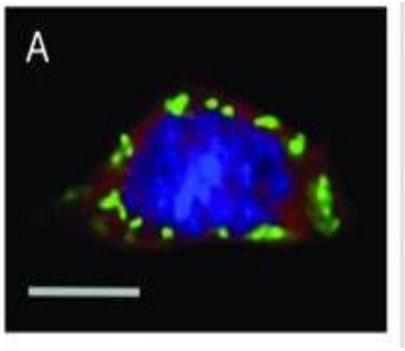


Figure 3.1 Laser scanning confocal microscopy of DISC1 aggregates in neuroblastoma cells.
Figure taken from Korth et al. (2012). Mouse neuroblastoma cells (CAD cells) permanently transfected with monomeric red fluorescent protein (red) and transiently transfected with untagged, full length DISC1, stained with α-DISC1 mAb 14F2 and a secondary FITC-labelled antibody (green) and DAPI stained nucleus (blue). Scale : 10 µm

Furthermore characterisation of these DISC1 aggregates led to the identification of a distinct oligomerization domain which lies towards the C-terminus (amino acids 640-854). Genetic variants within this region such as S704 and C704 were reported to disrupt this oligomerization of the DISC1 (Bader et al., 2012, Leliveld et al., 2009). It is also interesting to note that this C terminal oligomerization domain on DISC1 corresponds to the fragment lost in the Scottish translocation, and therefore it may have an underlying and crucial role in the disease mechanism.

3.1.2 SUMOylation dependent regulation of protein aggregation in chronic mental disease

As discussed in chapter 1, post translational modifications such as phosphorylation and SUMOylation have long been implicated in a number of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (Bence et al., 2001). SUMOylation is a post-translational modification characterised by the covalent conjugation of a small ubiquitin like modifier (SUMO) to the target protein. The SUMO pathway is detailed in section 1.4. The functional role of the SUMO pathway was emphasised by Ubc9 (SUMO E2 enzyme) knockdown studies, which show that this pathway is crucial for eukaryotic cell viability (Anderson et al., 2009). Studies on the role of SUMOylation in the central and peripheral nervous system reveal a wide array of mechanisms by which this PTM can contribute to the neuropathology of various diseases. Members of the SUMO cascade regulate the activity of several signalling molecules contributing to cerebellar development. One such example is MEF2A, whose activity has been shown to be repressed by SUMOylation at K403 leading to post synaptic granule neuron differentiation (Martin et al., 2007). Diseases such as NIID (Neuronal intranuclear inclusion disease) are characterised by neuronal intra-nuclear inclusions (NIIs) carrying SUMO-1 and SUMO substrates such as PML (promyelocytic leukaemia protein), RanGAP1 and HDAC4 (histone deacetylase 4) (Takahashi-Fujigasaki et al., 2006).

Emerging evidence describes SUMOylation as a powerful regulator of many signal transduction pathways and gene expression cascades, with a majority of SUMOylated substrates localised in the nucleus. As previously discussed (in section 1.4.1), the functional consequences of SUMOylation of a substrate may include altered sub-cellular and sub-nuclear localization, altered gene expression, as well as cross-regulation with other post-translational modifications, such as ubiquitination. Such consequences are often attained at the molecular levels via the alternation of protein - protein interactions of the SUMO conjugated protein, by altered protein conformation, inhibition of an existing binding site or the creation of a new binding site for the interacting proteins.

Pathological protein aggregates formed as a result of altered protein folding, stability or targeting have been identified as a hallmark of many neurodegenerative disorders such as

Parkinson's disease (aggregates of α-Synuclein constituting Lewy bodies) and Huntington Disease (aggregates of Htt protein). However, recent studies have shown that deficiency of a SUMO acceptor lysine in α-synuclein renders the Lewy bodies more toxic than the wild type sequence (Krumova et al., 2011). Although, in some instances the converse was true, where it was observed that SUMOylation reduced aggregation of mutant Huntington protein and ataxin-7. There are also reports of SUMOylation dependent enhancement of aggregate formation, as seen in super oxide dismutase 1 (SOD1), a causative factor of ALS (Amyotrophic lateral sclerosis, a motor neuron disease) (Krumova and Weishaupt, 2012). Progressive ataxia and dementia have also been demonstrated to be associated with insoluble protein aggregates, showing strong immune reactivity for SUMO1 (Martin et al., 2007).

3.2 Experimental aims

DISC1 has repeatedly been implicated as a candidate for susceptibility to SCZ and other chronic mental disorders, and such studies have also shed light on the role of DISC1 in neuronal development. Formation of DISC1 aggregates and reports of cell invasion by these aggregates have provided proof of DISC1 mediated pathophysiology in mental illnesses. However, the exact mechanistic relation of DISC1 to these diseases remains unclear.

As several other aggregation prone proteins were known to be SUMO conjugated and SUMOylation has been strongly implicated in several so called 'aggregopathies' such as Parkinson's disease, Huntington's disease, it was hypothesised that SUMOylation may play a role in regulating the formation of DISC1 aggregates.

My aims in the present section were:

1. To determine whether DISC1 is a substrate for SUMO conjugation.

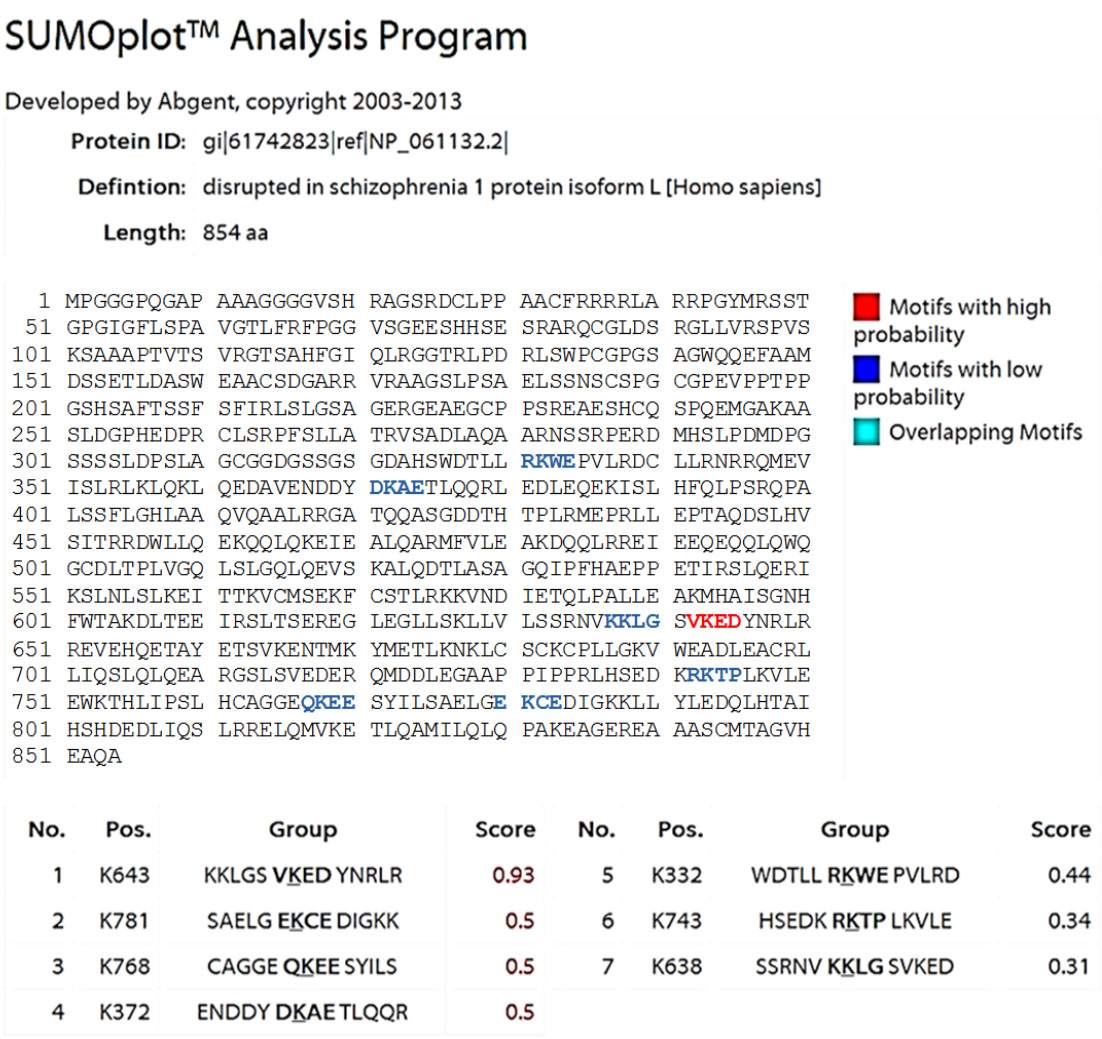
2. To identify SUMO acceptor lysines (K) on DISC1.

3. To analyse the significance of my findings in light of what has already been published and to explore the future perspectives.

3.3 Results

3.3.1 DISC1 is a putative SUMOylation target

SUMOylation usually occurs on a lysine residue entrenched in a core consensus motif ψKxE/D (where ψ is a branched hydrophobic and x is any amino acid). Ubc9 (the SUMO E2 enzyme) directly recognises this consensus motif and covalently bonds the SUMO residue to the lysine residue within this motif (Bernier-Villamor et al., 2002). To add to the complexity of this system, certain SUMO substrates also contain SUMO Interacting Motifs (SIMs) which allow substrates to interact non-covalently with SUMO moieties (Meulmeester and Melchior, 2008). The SUMOplot™ Analysis Program designed by Abgent to predict putative SUMO consensus sequences was used to determine if DISC1 possessed SUMO consensus sequences. The SUMOplot Analysis Program predicts and assigns probability scores to sites where SUMOylation may occur in a given protein. The SUMOplot™ score system is based on the following criteria: direct amino acid match to SUMO consensus sequence, and substitution of the consensus amino acid residues with amino acid residues exhibiting similar hydrophobicity. The results of the analysis suggested putative SUMO consensus sequences present within DISC1 (Figure 3.2).



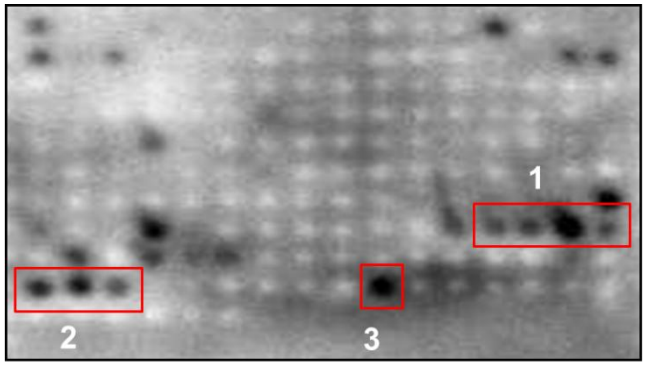
No.	Pos.	Group	Score	No.	Pos.	Group	Score
1	K643	KKLGS VKED YNRLR	0.93	5	K332	WDTLL RKWE PVLRD	0.44
2	K781	SAELG EKCE DIGKK	0.5	6	K743	HSEDK RKTP LKVLE	0.34
3	K768	CAGGE QKEE SYILS	0.5	7	K638	SSRNV KKLG SVKED	0.31
4	K372	ENDDY DKAE TLQQR	0.5				

Figure 3.2 SUMOPlot analysis of DISC1 protein sequence
Human DISC1 Protein sequence (NCBI accession number NP_061132.2) was analysed using the free software SUMOplot™ developed by Abgent. Link to SUMOPlot algorithm: (http://www.abgent.com/SUMOplot). Lysine residues (K) with a higher score have a higher probability of undergoing SUMO conjugation.

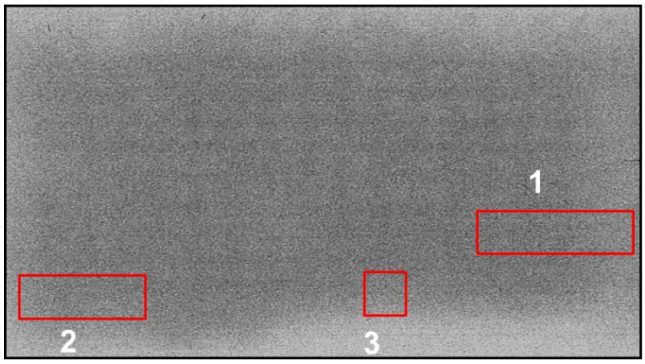
SUMOplot software predicted 7 potential SUMO acceptor lysine residues on DISC1 protein with varying probabilities (Figure 3.2). K643 was predicted with high confidence score as it falls within a strict consensus ψKxE/D. Other sites were sub-optimal in this regard and received lower scores.

3.3.2 DISC1 peptide array is selectively SUMOylated *in vitro*

Although search engine software such as SUMOplot is useful in identifying the SUMO consensus sites on substrates, follow-up experiments are necessary to validate the sites. Novel peptide array technology has been employed in number of studies to characterise post-translational modifications such as Ubiquitination and SUMOylation. This approach has been used to detect SUMO sites in substrates such as PDE4D5 (Li et al., 2010). A study by K. Schwamborn and group describes a method by which peptide array technology can be employed to identify SUMO target (Schwamborn et al., 2008). Peptide arrays of overlapping 25-mer peptides sequentially shifted by five amino acids and spanning the entire indicated DISC1 protein sequence (in Figure 3.1) were incubated with SUMOylation assay mixture and this was followed by the detection of conjugated SUMO with anti His antibody (to detect His-tagged SUMO moieties). Dark spots represent positive areas of SUMO conjugation, while clear spots are negative for the modification by SUMO (Figure 3.3). While no spots were detected on a control array that contained the SUMOylation assay without the E2 ligase Ubc9, there were putative SUMO conjugation sites identified on the test array (Figure 3.3).



Test:
SUMOylation assay mixture



Control:
SUMOylation assay mixture with out Ubc9 (SUMO E2 enzyme)

1.

Spot 125	L-E-G-L-L-S-K-L-L-V-L-S-S-R-N-V-K-K-L-G-S-V-K-E-D
Spot 126	S-K-L-L-V-L-S-S-R-N-V-K-K-L-G-S-V-K-E-D-Y-N-R-L-R
Spot 127	L-S-S-R-N-V-K-K-L-G-S-V-K-E-D-Y-N-R-L-R-R-E-V-E-H
Spot 128	V-K-K-L-G-S-V-K-E-D-Y-N-R-L-R-R-E-V-E-H-Q-E-T-A-Y

2.

Spot 145	Q-M-D-D-L-E-G-A-A-P-P-I-P-P-R-L-H-S-E-D-K-R-K-T-P
Spot 146	E-G-A-A-P-P-I-P-P-R-L-H-S-E-D-K-R-K-T-P-L-K-V-L-E
Spot 147	P-I-P-P-R-L-H-S-E-D-K-R-K-T-P-L-K-V-L-E-E-W-K-T-H

3.

Spot 154	E-Q-K-E-E-S-Y-I-L-S-A-E-L-G-E-K-C-E-D-I-G-K-K-L-L

Figure 3.3 *In vitro* SUMOylation of DISC1 peptide array
Upper panel: *In vitro* SUMOylation of peptide arrays of DISC1 protein. Putative SUMOylation sites appear as dark spots, identified are highlighted in boxes numbered 1,2,3. Bottom panel: Corresponding peptide sequences of the spots. Putative SUMO consensus site within the peptides are highlighted in red (K643, K743, K768).

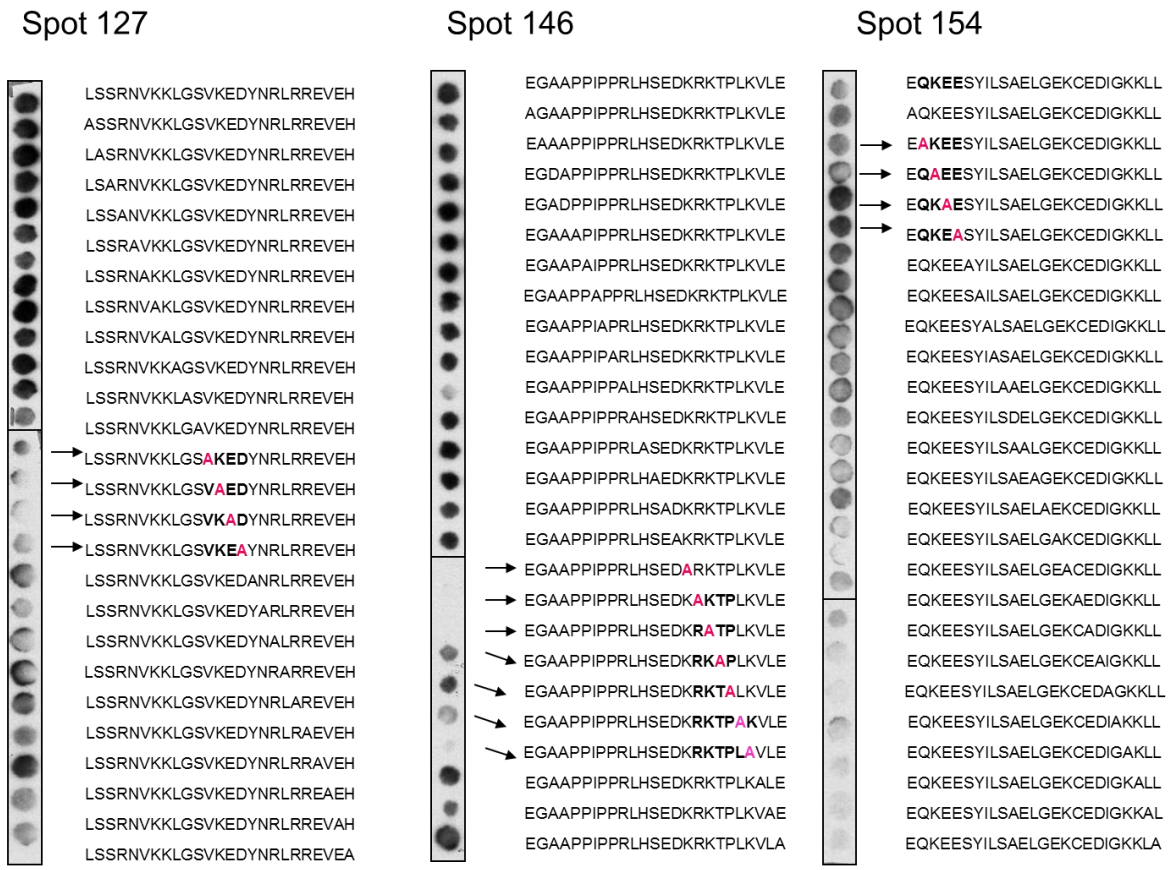


Figure 3.4 Alanine substitution peptide arrays of the positive hits from in vitro peptide array analysis
***In vitro* SUMOylation assay on alanine scanning peptide array of the peptides identified in Figure 3.3. This peptide array library consisted of peptides with SUMO consensus site sequentialy substituted with Alanine. *In vitro* SUMOylation assay was carried out on these peptide array libraries and probed with His tag antibody (to detect His tagged SUMO moeities) (described in section 2.4.6). Positive interaction appears as dark spot. SUMO consensus site moities were substituted with alanine are indicated in bold letters while each alanine substitution within this consensus is highlighted in red.**

Putative SUMO conjugation sites identified in initial DISC1 peptide array screen were further characterised by mutational analysis. Positive peptides identified were re-synthesised with sequential mutation of each amino acid to alanine and *in vitro* SUMOylation was carried out (Figure 3.4). In sequences corresponding to spot 127 and 146, replacing the residues within the consensus motif with alanine abolished the SUMO conjugation of those peptides. However, in peptides corresponding to spot 154, replacing the motif with alanine had no or very little effect on SUMO conjugation indicating that the initial observation of positive SUMO attachment may be considered as an artifact.

While SUMO prediction algorithms and *in vitro* peptide array screens are extremely useful in identifying putative SUMOylation substrates, they are essentially artificial screening tools that require validation to account for their limitations. It has been reported that this motif is found in over a third of all characterised proteins, which leads to the real possibility of identifying false positives using the SUMO prediction software (Song et al., 2005). Many other proteins with the characterised consensus motif are not SUMO targets, while many other proteins are SUMOylated at sites other than the consensus motif. Hence, a range of biochemical validations are required before any physiological importance can be attributed to the modification.

3.3.3 DISC1 is SUMOylated in HEK293 cells

3.3.3.1 Ubc9 and PIAS E3 ligases enhance DISC1 SUMOylation

SUMOylation is catalysed by Ubc9, an E2 conjugating enzyme and the E3 ligase, PIAS (PIAS1, PIAS3, PIASy). Over expression of the SUMO E2 and E3 enzymes are known to enhance SUMO conjugation of certain substrates, such as Smad4 (Lee et al., 2003). The effect of co expression of the above mentioned SUMO E2 and E3 enzymes on DISC1 SUMOylation was therefore investigated. Figure 3.5 depicts the co-expression of Flag-DISC1 with Ubc9, SUMO1/2 and various isoforms of PIAS (1/3/y). Over expression of SUMO with the E2 and E3 ligase results in the retardation of the electrophoretic migration of Flag-DISC1 compared to that co-expressed with empty vector. This suggests that DISC1 has been modified by SUMOylation.

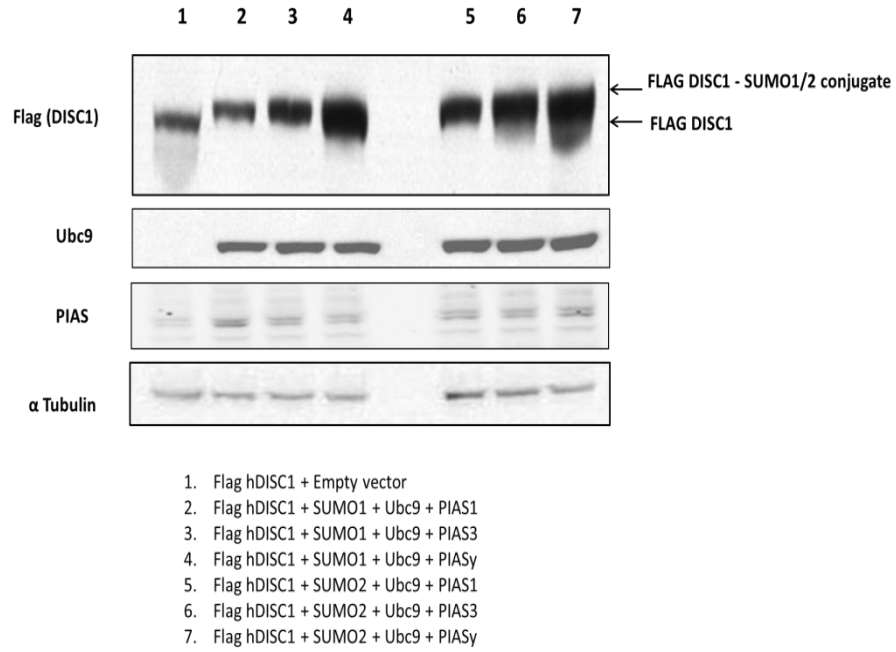


Figure 3.5 Enhancement of DISC1 SUMOylation by SUMO modification enzymes HEK293 cells were transfected with Flag-DISC1, SUMO-1, or SUMO-2 in the presence of Ubc9 and PIAS (1, 3, y isoforms). Cells were lysed in denaturing conditions and subjected to immunoblot analysis. Anti-Flag antibody was used to analyse DISC1 expression and alpha tubulin antibody was used as loading control. Slow migrating DISC1 bands were indicated as SUMO modified DISC1 species.

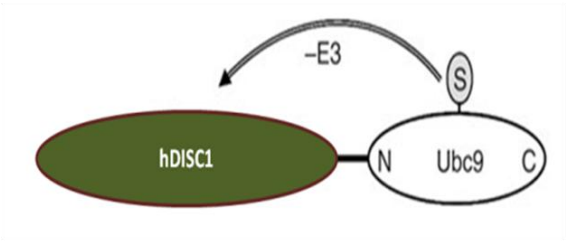
3.3.3.2 UFDS method based detection of DISC1-SUMO conjugates

Most post translational modifications, although covalent in nature, are a two-way process whereby there is an adding step (example: phosphorylation by a kinase) followed by a subtraction step (example: dephosphorylation by a phosphatase). This “on” and “off” switch allows downstream physiological changes to be transient, allowing cells to return to their quiescent state in readiness for another functional event. In this way, SUMOylation is regulated by SUMO conjugating enzymes (UbC9) and SUMO specific proteases which cleave off the SUMO moiety from the target protein i.e., DeSUMOylation enzymes. As SUMOylation requires energy and is switched on following certain cellular signals, only a small fraction of cellular proteins are SUMOylated under steady state conditions (Hay, 2013). As a result of this, the detection of SUMOylated proteins is notoriously tricky using conventional biochemical methods. Basal levels of SUMO

modified and unmodified proteins are strictly maintained under the control of SUMO E3 ligases and DeSUMOylating enzymes. One confounding factor was that specific SUMO protease inhibitors were unavailable at the time of this study to stabilise the SUMO conjugated substrates for easy biochemical analysis. In order to overcome these technical limitations, the SUMOylation of DISC1 by a novel Ubc9 fusion–directed SUMOylation (UFDS) method described in Jakobs et al. (2007) was chosen to investigate DISC1 basal SUMOylation levels. This study highlights enhanced SUMOylation of p53 and STAT1 when they were expressed as Ubc9 fusion proteins (p53-Ubc9 and STAT1-Ubc9). The principle behind the UFDS method is that substrates undergo SUMO conjugation even in the absence of SUMO E3 ligase when Ubc9 (SUMO E2 enzyme) is bought in to close proximity of a substrate by fusing it to the said substrate Figure 3.6). This study provided a novel method to study the SUMOylation of substrates in a cellular context.

To determine whether DISC1 is SUMOylated in cells, DISC1 was cloned in to plasmids that generate the fusion proteins with Ubc9 at the C-terminus (represented as WT DISC1-Ubc9, DISC1 K643A-Ubc9, DISC1 Ubc9 C93S for WT, putative SUMO dead DISC1 mutant and Ubc9 dead mutant respectively). HEK293 cells were transfected with the above vector together with GFP-SUMO1. Use of GFP tagged SUMO1 facilitates the identification of SUMOylated DISC1 species because of increased molecular weight of the SUMO conjugate that can easily be detected as a more pronounced band-shift on a Western blot. Over-expressed GFP was used as a negative control. The protein extracts were analysed by immunoblot analysis, probing with a DISC1 antibody. The gels revealed a number of slow migrating, higher molecular weight proteins (Figure 3.7) in the lysates of cells that overexpressed the DISC1-Ubc9 fusion and GFP-SUMO1. These bands representing SUMOylated forms of DISC1 were not detected in the absence of GFP-SUMO1 or where the fusions contained a catalytically dead Ubc9 (C93S) (DISC1 Ubc9 C93S serves as a dominant negative since the cysteine residue at the active site of Ubc9 is mutated to Serine which abolishes SUMO-1 conjugating ability (Jakobs et al., 2007)). Interestingly, the DISC1 alanine mutant (K643A), whereby the DISC1 SUMO consensus site identified by algorithms and peptide array (section 3.3.1 and 3.3.2) was mutated, showed diminished SUMOylation suggesting that this is a definite SUMO site, but other sites must also exist.

SUMOylation cascade under physiological conditions



SUMOylation of Ubc9 conjugated DISC1 bypassing the requirement of an E3 ligase

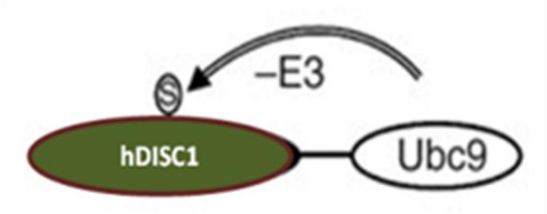


Figure 3.6 Schematic representation of UFDS (Ubc9 Fusion Directed SUMOylation) method
Adapted from (Jakobs et al., 2007). SUMO E3 ligase- dependent SUMO(s) conjugation occurs when SUMO E2 enzyme Ubc9 is brought in close proximity to the substrate by a SUMO E3 ligase. UFDS method bypasses the requirement of SUMO E3 enzyme by fusing a SUMOylation substrate (DISC1 WT/ K643A in the present study) to the SUMO conjugating enzyme Ubc9.

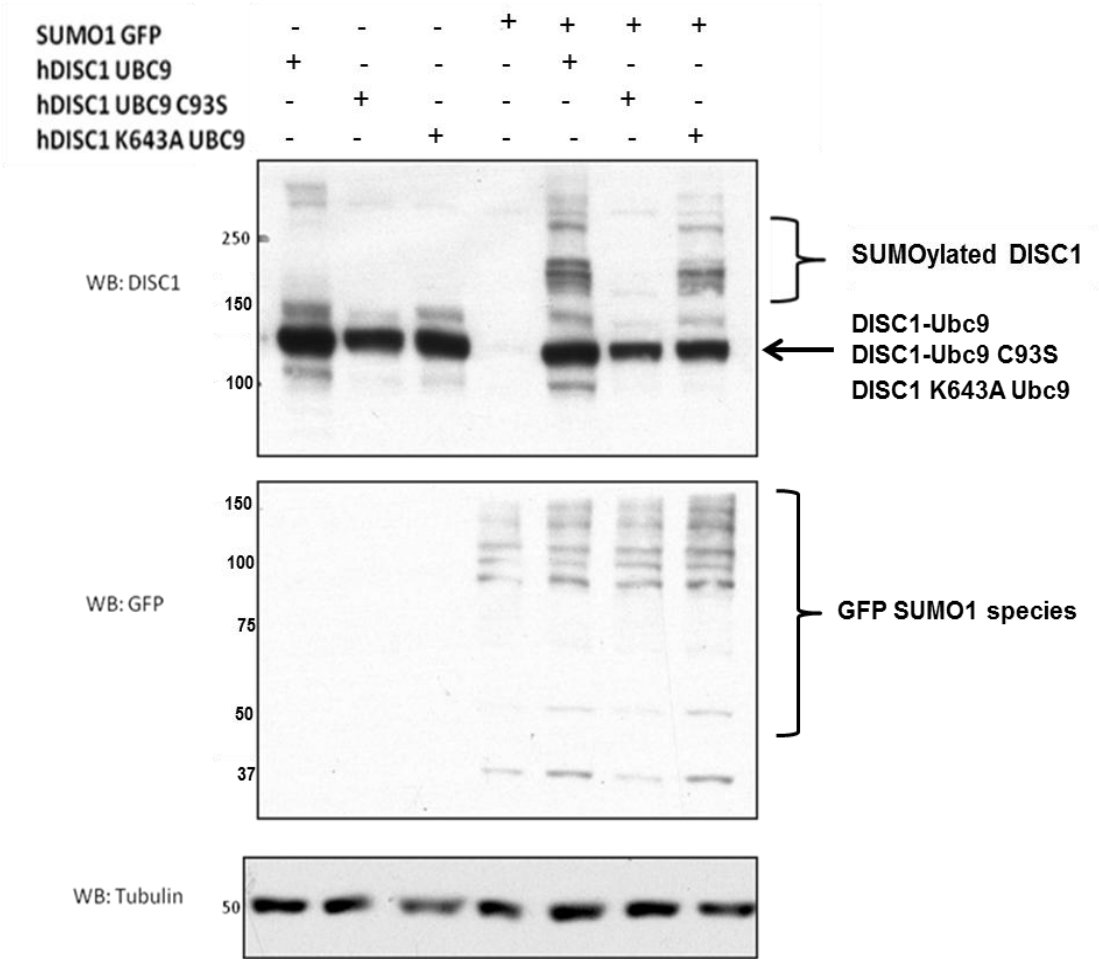


Figure 3.7 Ubc9 Fusion Directed SUMOylation (UFDS) of human DISC1 in Hek293 cell line

The expression plasmids for DISC1-Ubc9, DISC1 (K643R)-Ubc9, DISC1-Ubc9 (C93S) were transfected into HEK293 cells either alone or together with the GFP-SUMO1 expression plasmid as indicated. After 48 hr, the fusion proteins in the protein extracts were detected by Western blotting using a DISC1 antibody (Upper panel), GFP antibody (middle panel) and alpha tubulin (lower panel). DISC1-Ubc9 proteins (WT and K643A mutant) DISC1-Ubc9 C93S mutant and their corresponding GFP-SUMO1 conjugates are indicated.

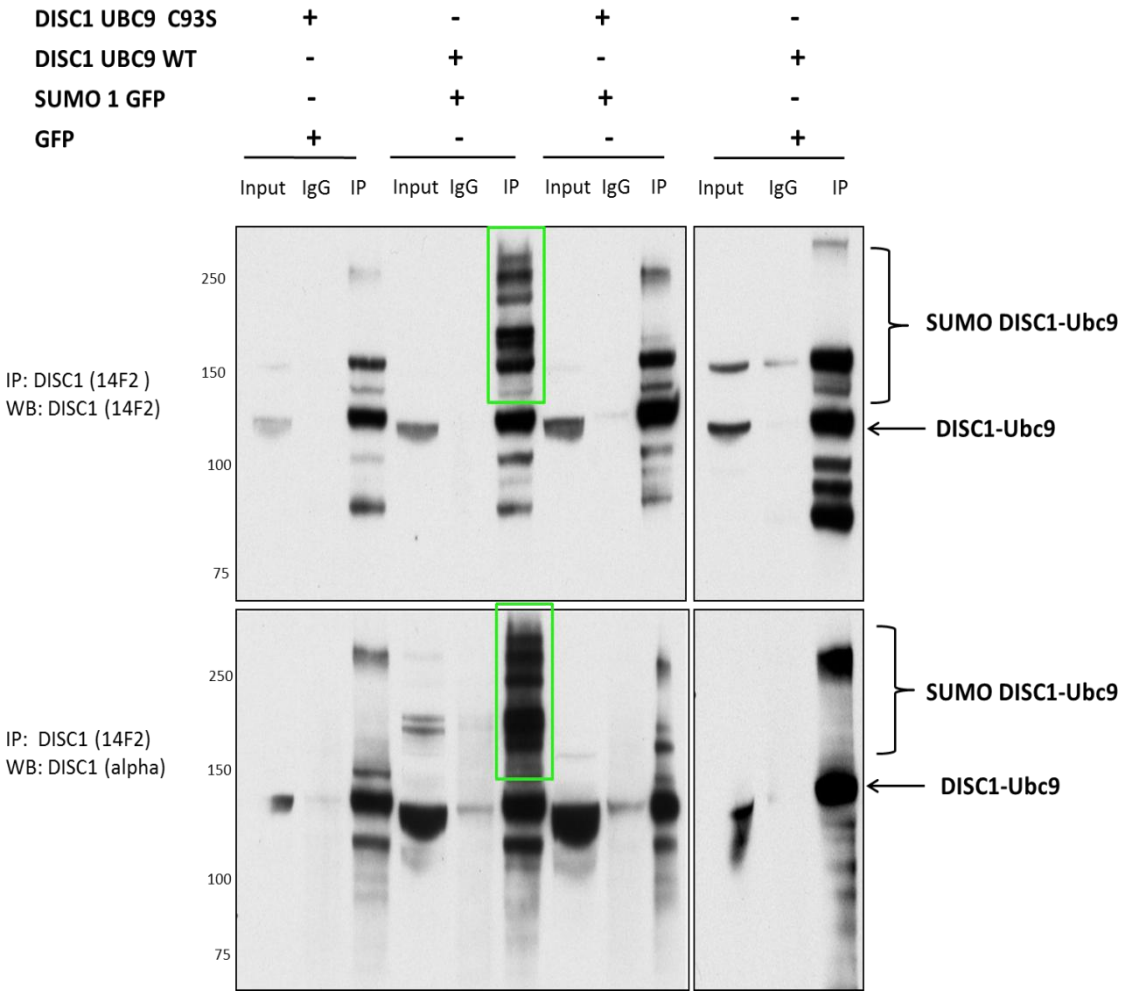


Figure 3.8 Validation of DISC1-SUMO species using two antibody approach
The expression plasmids for DISC1-Ubc9, DISC1-Ubc9 (C93S) were co-transfected into HEK293 cells either with the GFP-SUMO1 or GFP expression (negative control) plasmid as indicated. After 48 h, immunoprecipittion was performed with a DISC1 raised against a specific epitope on DISC1 and immunobloted with another DISC1 antibody raised against a different epitope to the former DISC1 antibody. Top panel: IP antibody : 14F2 , WB antibody: 14F2, Bottm panel: IP antibody : 14F2, WB antibody : alpha DISC1. IP was performed with an IgG antibody as a negative control. DISC1-Ubc9, DISC1-Ubc9 C93S mutant and their corresponding GFP-SUMO1 conjugates are indicated.

By using the UFDS method, we established, for the first time, the novel post translational modification on DISC1, SUMOylation. DISC1-Ubc9 fusion proteins were observed to be migrating at about 125kDa. Co expression of these constructs with GFP-SUMO1 resulted in the appearance of high molecular bands on the immunoblots, which correspond to the DISC1–Ubc9 SUMO1 conjugates. DISC1-Ubc9 C93S (DISC1 fusion with the catalytically inert form of UBC9) is completely devoid of these bands. This observation confirms the specificity of SUMO conjugation. A high molecular weight band was observed when DISC1-Ubc9 alone was overexpressed. This could likely be a result of endogenous SUMO conjugation of the overexpressed DISC1 Ubc9 fusion construct (Figure 3.7).

To validate the high molecular weight bands appearing in the immunoblots as SUMO DISC1 conjugates, immunoprecipitation was performed using UFDS lysates. DISC1 immunoprecipitation was performed using 14F2 (DISC1 antibody 1) and the immune complexes were probed with alpha DISC1 antibody (DISC1 antibody 2). The presence of DISC1 – SUMO conjugates in both the immunoblots rules out the possibility of non-specific DISC1 bands (Figure 3.8).

The kinetics of SUMO conjugation on DISC1K643A Ubc9 mutant were also studied. K643 site was identified as a putative SUMO acceptor site on DISC1 in *in vitro* SUMOylation studies. Ideally, alanine mutant at K643 should abolish the SUMO conjugation. However, this was not the case, DISC1 SUMO conjugate bands can still be seen when an Alanine mutant at K643 was overexpressed with GFP SUMO-1, which indicates the presence of other potential SUMO acceptor lysine residues on DISC1 as predicted by SUMOPlot software (figure 3.7). Hence, a mass spectrophotometry approach was employed to uncover potential SUMO acceptor sites on DISC1 as detailed in section 3.3.4.

3.3.4 Mass Spectroscopy studies to validate SUMO modification of DISC1 protein *in vitro*

Identification of the type and site of post translational modification on a substrate of interest is a crucial step towards understanding the physiological role of the specific modification. Consideration of the field of protein phosphorylation is a good example. Mapping of the site of phosphorylation and identification of candidate kinases has enormously contributed to our understanding of numerous signal transduction pathways. Mass spectroscopy (MS) is an advanced powerful tool, used to characterise proteins and peptides. Hence, I took a MS approach to further validate DISC1 as a SUMO substrate and identify the SUMO acceptor lysines.

To ensure maximal amounts of SUMOylated DISC1, once again the UFDS approach described in the previous section was used. DISC1-Ubc9 was co-expressed with GFP-SUMO1 in HEK293 cells. 48hr post transfection, cell lysates were prepared under denaturing conditions and DISC1 was immunoprecipitated using 14F2 (DISC1 antibody 1) antibody. DISC1 immuno complexes were subjected to SDS-PAGE separation. SDS-PAGE gels were stained with Colloidal Blue (Invitrogen) and bands were excised at indicated molecular weights in Figure 3.9, upper panel. Western blot analysis from the same samples and subsequent probing with α-DISC1 (antibody 2) - Figure 3.9 (lower panel) ensured the fidelity of the immunoprecipitations in isolation of DISC1.

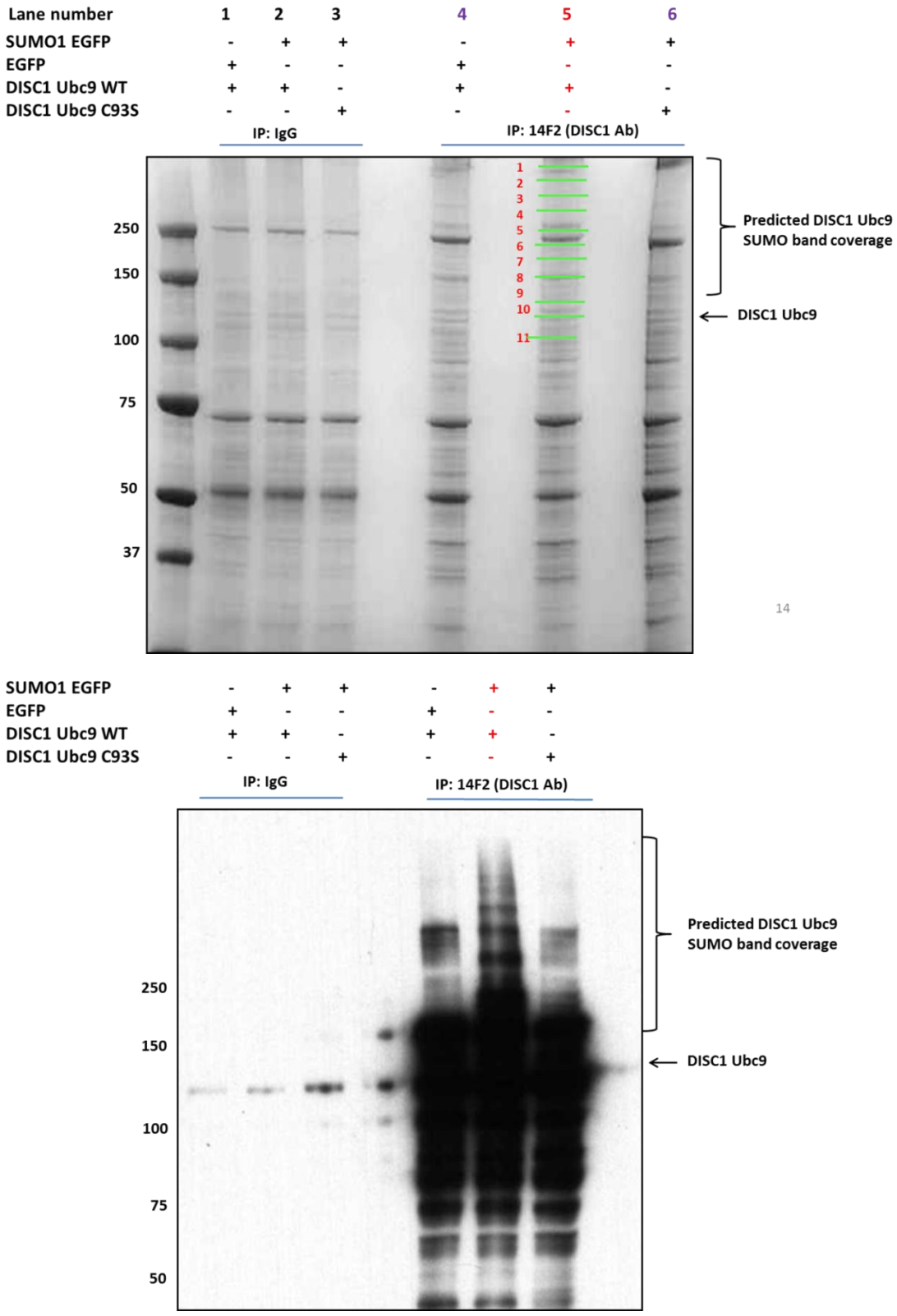


Figure 3.9 Preparation of mass spectroscopy samples for the detection of DISC1 SUMOylation

The expression plasmids for DISC1-Ubc9, DISC1(K643A)-Ubc9, DISC1-Ubc9 (C93S) were co-transfected into HEK293 cells together with the GFP-SUMO1 or GFP expression plasmid as indicated. Immunoprecipitation was performed with a DISC1 antibody 1(14F2) or IgG antibody (negative control). Top panel: Immunoprecipitate complexes

were separated by PAGE technique and coomassie stained. The number of bands indicated in the Figure (lane 5, highlighted in red) have been excised from the stained gel and stored for further analysis. Bottom Panel: 5µl of each sample have been analysed by Western blotting and probed with anti DISC1 antibody to confirm the presence of DISC1-SUMO conjugates. DISC1-Ubc9 proteins (WT and K643A mutant) DISC1-Ubc9 C93S mutant and their corresponding GFP-SUMO1 conjugates are indicated.

Table 3.1 Summary of DISC1- SUMOylation Mass Spectroscopy studies

Sample ID (figure 3.9, top panel)	Sample Collected at Molecular weight range	DISC1 peptide detected (percentage coverage)	SUMO acceptor site identified	Type of SUMO modification	Mascot Score
5.4	~300 kDa	K.**K**LLYLE.D (13.3)	K788	$SUMO_{343}$	Not significant
5.5	~250 kDa	K.**K**LLYLE.D (28.2)	K788	$SUMO_{343}$	Not significant

The above table depicts the prescence of DISC1 at two different molecular weights which correspond to post translationaly modified (PTM) DISC1 species in the respective samples Column 1 represents the lane number followed by the sample number assigned in Figure 3.9. Example: 5.4 represents lane 5 and sample 4. Column 2 is the precise molecular weight on the gel at which the band has been excised. Column 3 represents the peptide identified in the respective sample with the SUMO conjugated lysine highlighted in red. Column 4 is the position of lysine residue identified to be SUMO conjugated in the sample. Column 5 is the type of SUMO modification detected and column 6 is the MASCOT score, which was found not to be significant (MASCOT score explained in section 2.4.8).

MS studies positively detected DISC1 peptides at molecular weights higher than 125 kDa (additive molecular weight of DISC1-Ubc9) indicating potential DISC1-SUMO conjugation. $SUMO_{343}$ represents the molecular mass (~343 Da) of the peptide 'QTGG' generated from SUMO1 after GluC digestion (specific for Carboxyl side of Glutamate residue). K^{788} was identified as a putative SUMO acceptor lysine. However, the percentage peptide coverage within the samples analysed was too low indicating high contamination or poor

sample quality. Although DISC1 peptides were detected in the other samples, the confidence scoring was too low to be considered. (The principle of Mass spectroscopy techniques is explained in section 2.4.8).

3.3.5 Consequences of DISC1 SUMOylation – effect on its protein interactions

SUMO modification of substrates has many physiological consequences. Since SUMO proteins are about 10kDa size proteins, covalently conjugated to its substrate, this bulky modification may either cause stearic hindrance thereby interfering with protein – protein interactions or create additional binding surface on the substrate and promote or strengthen protein interactions (Jentsch and Psakhye, 2013). Hence, we made an attempt to understand the effect of SUMO modification on DISC1, on its protein interactions. DISC1 is known to bind many proteins involved in several critical signalling pathways reviewed in (Bradshaw and Porteous, 2010). Out of all these interacting partners we were particularly interested in two crucial interactors – nuclear distribution protein nudE-like 1 (Ndel1) and dishevelled axin domain containing 1 (DIXDC1) for the following reasons: 1. Ndel1, a component of the dynein motor protein complex interacts with DISC1 at the centrosome and mediates neurite outgrowth, migration and cortical development (Ozeki et al., 2003a, Morris et al., 2003). 2. DISC1 interaction with DIXDC1 was shown to mediate embryonic neural progenitor proliferation and neuronal migration (reviewed in section1.2.3) (Bradshaw and Porteous, 2010, Singh et al., 2010).

To investigate the effect of SUMO conjugation of DISC1 on its interaction with the above mentioned proteins, we employed the UFDS method to enhance DISC1 SUMOylation while co-expressing DISC1 binding partner of interest in HEK293 cells. Immunoprecipitation was performed using an antibody specific for the tag on the interacting partner as bait, followed by Western blot analysis of the immnuoprecipitate complexes by probing for DISC1 antibody, to examine if the binding partner precipitated only with unSUMOylated DISC1-Ubc9 or SUMOylated DISC1-Ubc9 or both.

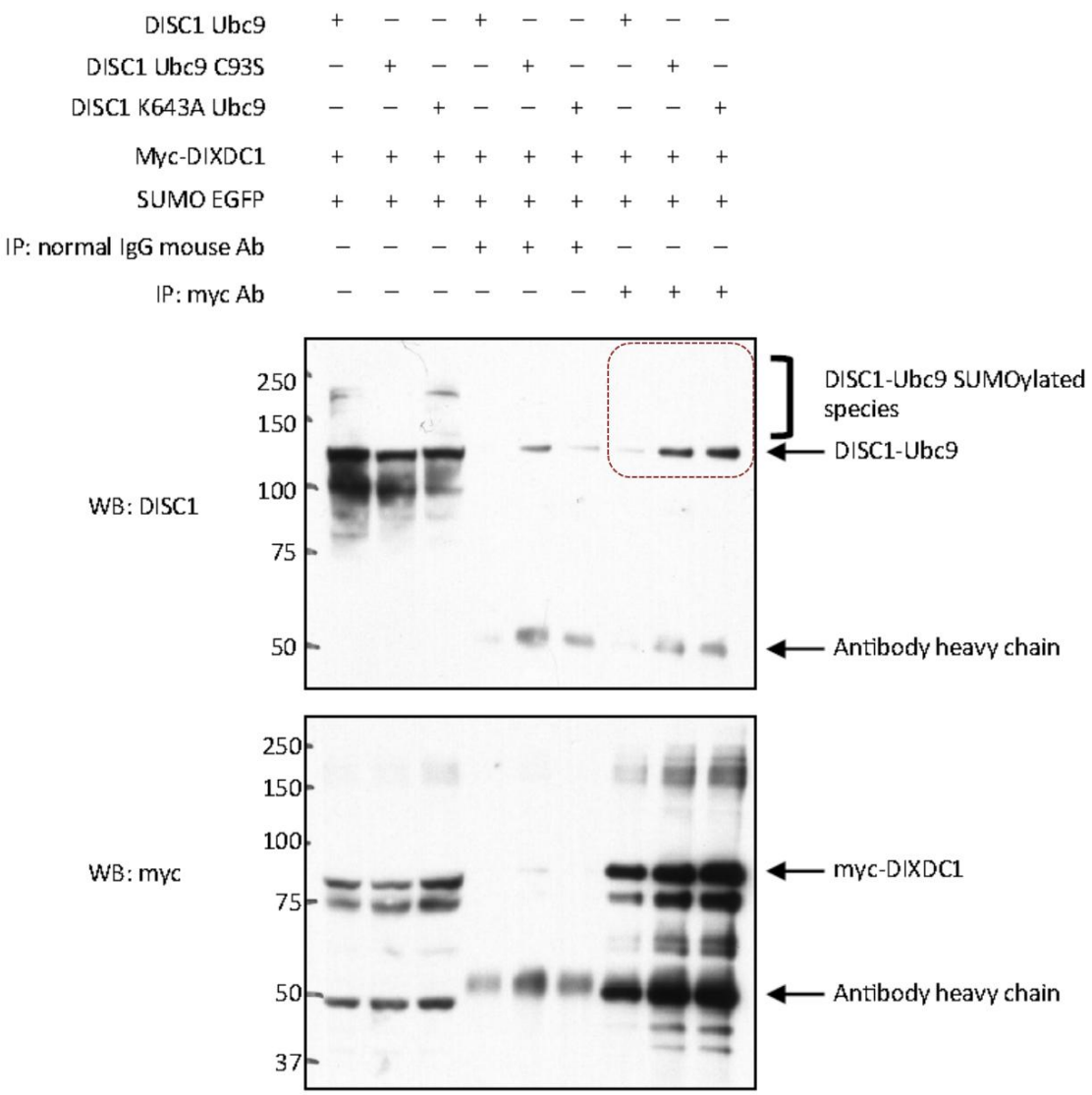


Figure 3.10a DIXDC1 may interact only with unSUMOylated DISC1
Western blot analysis of DIXDC1 and DISC1 co-immunoprecipitation (Figure legend in next page under Figure 3.10b).
This work was performed by Elizabeth M Cannell, Gardiner Laboratory, University of Glasgow.

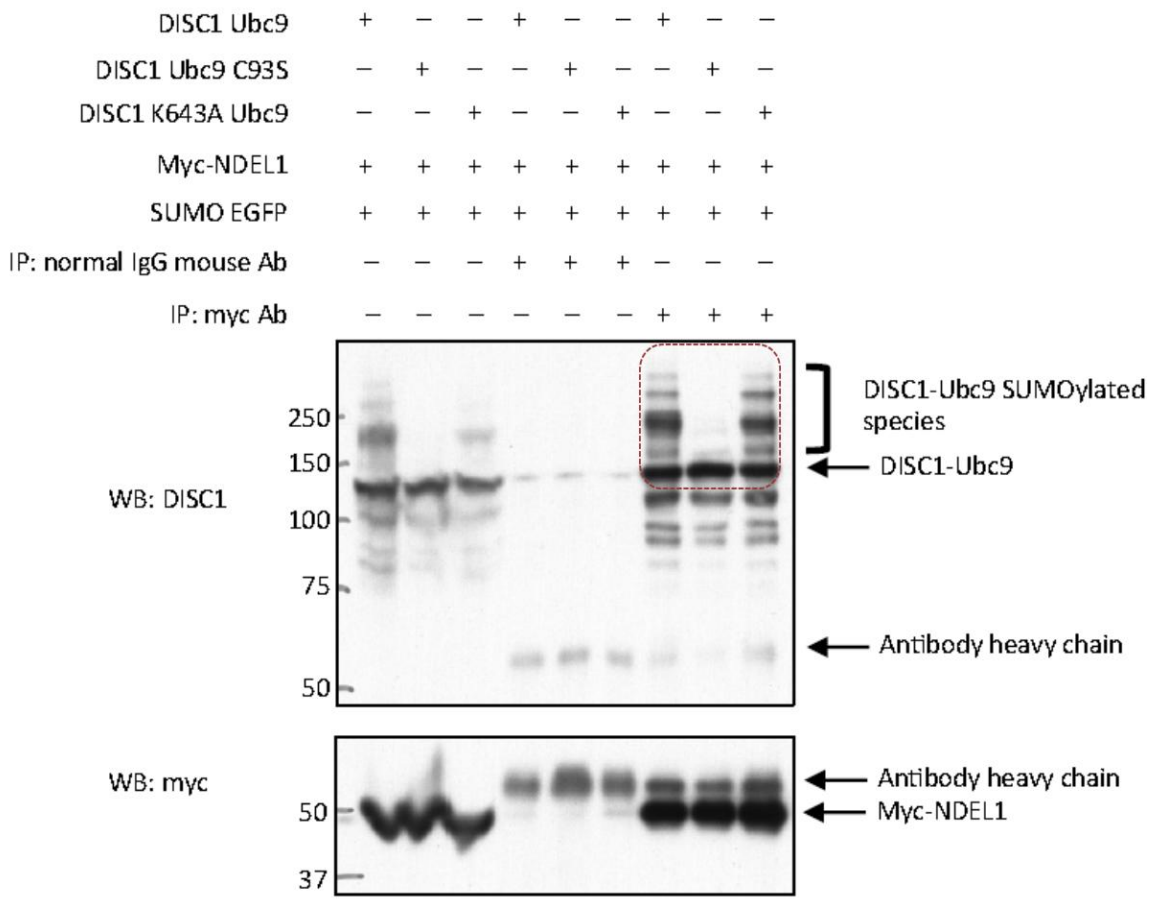


Figure 3.10b Ndel1 interacts with both SUMOylated and unSUMOylated DISC1
Western blot analysis of Ndel1 and DISC1 co-immunoprecipitation assay. Following overexpression of indicated constructs in HEK293 cells, lysate was incubated with the indicated antibody. Mouse IgG antibody was use in the negative control IP. The first three lanes of the blots are cell lysate, while the next three lanes were loaded with proteins isolated by the immunoprecipitation assay by mouse IgG and last three lanes with those isolated with Myc antibody (tag on DIXDC1 and Nudel1). Top panel: blots probed with DISC1 Ab and bottom panel: blots probed with Myc antibody.
This work was performed by Elizabeth M Cannell, Gardiner Laboratory, University of Glasgow.

Co-imunoprecipitation experiments revealed that SUMO conjugation of DISC1 may interfere and abolish its interaction with DIXDC1. As it can seen in Figure 3.10a, the high molecular weight DISC1-Ubc9 – SUMO conjugates are absent in both the test IP and negative control IP lanes (highlighted in box, Figure 3.10a). However, these results were inconclusive. Since, the SUMOylated DISC1-Ubc9 bands (bands above 100kDa) were faint

in the input lanes (first three lanes) it could be possible that the SUMO DISC1-Ubc9 levels in the immunoprecipitates were too low to be detected. More experimental repeats are needed to ascertain this, which was not within the scope of this project.

Similar experiments with Ndel1 revealed that SUMOylation of DISC1 does not alter its interaction with Ndel1 (Figure 3.10b) and the slow migrating, high molecular weight DISC1-Ubc9-SUMO bands are present in the test IP lane but not the negative control lane (highlighted with red box Figure 3.10b).

3.4 Discussion

3.4.1 DISC1 is modified by SUMO1

It has been established through the last two decades that post-translational modifications (individually or co-operatively) regulate many signal transduction pathways (Prabakaran et al., 2012). SUMOylation is one such kind of modification that is increasingly garnering interest in the field of chronic mental disease research due to its role in protein aggregation and solubility, a hallmark feature of many neurological disorders (Krumova and Weishaupt, 2013, Krumova and Weishaupt, 2012).

It has been established here that there is a novel post translational modification on DISC1 in the form SUMOylation. DISC1 could be covalently modified by SUMO1 conjugation at one or more site, as the SUMOplot algorithm identified number of putative consensus SUMOylation motifs on DISC1 (Figure 3.2). *In vitro* peptide array studies identified three of the predicted motifs K643, K768 and K743 (Figure 3.3, 3.4). However, Alanine substitution scans of the putative sites identified in the initial peptide array screen validated only two of those motifs, K643 and K743 (Figure 3.4). My preliminary studies focused on the characterisation of the K643 site as it was predicted to be more likely to be SUMO conjugated implied by highest score of 0.93 in the SUMOplot algorithm, while it is also conserved in mouse and human. However, mutational studies on DISC1 K643R-Ubc9 conjugate reduced but did not completely abolish SUMO modification. This may be

because of the presence of more than one SUMO acceptor site as predicted by SUMOplot algorithm.

Studies show that the mere presence of consensus Lys residues in the SUMO motif is often not sufficient for recognition by the SUMO conjugation machinery, there are other criteria such as the presence of downstream acidic patches or pre-existing phosphorylation (Yang et al., 2006). If the lysine residues are embedded in stable structural helices, it may not be recognised by the SUMO conjugating enzyme, Ubc9. However, it is important to note that certain substrates are SUMOylated at a non-consensus lysine residue, for example, human E2-25K (Pichler et al., 2005). If this is the case, it is difficult to predict such random sites. The UFDS method has proved to be useful in efficient SUMOylation of DISC1 in my cell line model and this represents the first reliable indication that DISC1 can be SUMOylated *in vitro*.

Mass spectroscopy, was also used in the present study to verify DISC1 as a SUMO substrate and identify the site of post translational modification. DISC1-Ubc9 migrates at about 125kDa on SDS-PAGE; however, as expected, the MS study detected DISC1 peptides in samples excised at molecular weights greater than 125kDa indicative the putative post translational modification by SUMO. Though all modifications could not be identified, $SUMO_{343}$ modification was detected at K788 ($SUMO_{343}$ refers to the molecular weight of the putative adduct, 343.1492 Da molecular weight corresponds to the peptide 'QTGG' remaining after GluC cleavage of a SUMO1-modified lysine). Only one SUMO acceptor lysine was identified in the MS studies while several bands are observed in UFDS methods based immunoblots. From the MS spectral data it was evident that the sample quality was not optimal. Absolute identification of SUMOylation sites on substrates has always been challenging due to the reversible dynamic nature of SUMOylation (achieved by the action of SUMO conjugation and deSUMOylating/SUMO protease enzymes in cells) which deteriorates the sample quality. SUMO1 forms chains less efficiently *in vitro* since it lacks the internal SUMOylation site which is present in SUMO 2 and SUMO 3 (Vertegaal, 2007). Also, successful detection of SUMO sites depends on the sensitivity of mass spectrometer which can retrieve less abundant SUMOylated peptides from the MS

samples (Knuesel et al., 2005). Unfortunately technical and biological repeats for MS studies were not feasible in this project due to time constraints and budgetary concerns.

It is also important to note that just as any other post translational modification, SUMOylation is spatio temporally regulated which confers additional levels of regulation (Loriol et al., 2012). It may be triggered by a physiological stimulus or other pre-existing post translational modification such as phosphorylation as seen in case of c-Jun and p53 SUMOylation (which require a phosphorylation event) (Muller et al., 2000). With no knowledge of the above stimuli nor the tertiary structure of DISC1, it would be difficult to induce enough SUMOylation in cells to generate high quality samples for MS, which may generate more information on this modification. However, my work provides a number of different avenues of biochemical proof that the SUMO conjugation of DISC1 occurs *in vitro*.

3.4.2 Significance of DISC1 as a SUMO substrate

Over the past few decades, there have been very few developments in psychiatric drug development and most of the newer antipsychotics and anti-depressants have been reported to cause severe side effect**s**. The increasing characterisation and appreciation of the DISC1 interactome is a factor that may provide novel opportunities to discover new classes of brain function altering drugs (Hikida et al., 2012, Soares et al., 2011).

Demonstration of the SUMO conjugation of DISC1 opens up new opportunities to explore its physiological functions. DISC1 is known as a scaffold protein with no reported enzymatic activity. Several of its interactions have been studied and known to influence various neuronal signalling pathways (Bradshaw and Porteous, 2010, Chubb et al., 2008). One of the molecular consequences of SUMO conjugation of a substrate is a conformational change leading to altered protein- protein interaction. Hence my studies may prove useful in developing further understanding how DISC1 interactions are modulated.

SUMO and other post translational modifications are known to regulate many aspects of substrate proteins. Phosphorylation of DISC1 orchestrates neuronal progenitor cell migration and proliferation in mice (Ishizuka et al., 2011c). Some substrate proteins require a pre-existing phosphorylation to induce SUMO conjugation as seen in regulation of heat shock factor HSFb and MEF2 (Hietakangas et al., 2006). SUMOylation and ubiquitination are also known to cross-talk via regulation of each other's respective enzymatic cascade. This type of regulation is quite evident in E2-25K, an E2 enzyme , which loses its ubiquitin conjugating ability upon SUMOylation (Pichler et al., 2005). The next chapter investigates another novel modification crucial in regulating DISC1 protein turn over: ubiquitination.

Post translational modification by SUMO conjugation has been shown to influence subcellular localisation of substrates such as RanGAP (a small GTPase activating protein). While SUMO-RanGAP is co-localised with the nuclear pore complex, its unmodified counterpart is predominantly cytoplasmic (Matunis et al., 1996). DISC1 is hypothesised to exert its function by interaction with specific proteins in discrete sub cellular compartments. For example, DISC1 is known to interact with NudE-like (NUDEL/NDEL1) at the centrosome and activating transcription factor 4 (ATF4)/CREB2 in the nucleus (Kamiya et al., 2005a, Morris et al., 2003). A 75kDa DISC1 isoform has been reported to be specifically enriched in the nucleus in brain autopsies of patients with sporadic schizophrenia and major depression as well as substance and alcohol abuse (Sawamura et al., 2005a) suggesting its role in the pathophysiology of these major mental illnesses. Hence, it would be interesting to investigate the role of SUMO conjugation on sub cellular targeting of DISC1.

In an attempt to develop a fly model expressing human DISC1, Akira Sawa and colleagues have shown that DISC1 appears in a punctate pattern co-localised with PML protein, a core component of PML nuclear bodies (Sawamura et al., 2008). Another study has shown that SUMO1 conjugation of PML protein is necessary for formation of PML bodies (Zhong et al., 2000). Hence, it would be interesting to investigate if DISC1 aggregation is regulated by SUMO modification.

In this project, the effect of SUMO modification of DISC1 on its interacting partner profile was explored (section 3.3.5). Of the two binding proteins chosen, DIXDC1 was likely to be interacting with non SUMOylated DISC1 although the results were inconclusive and require further characterisation. DISC1 functionally interacts with DIXDC1 and regulates neuronal progenitor proliferation during early brain development by modulating Wnt-GSK3β/β-catenin pathway (Singh et al., 2010). Their role in neuronal migration was suggested to occur through a pathway independent of Wnt/β-catenin signalling (Singh et al., 2010). If SUMOylation of DISC1 indeed abolishes its interaction with DIXDC1, it is likely that this PTM on DISC1 may be a crucial event during early brain development and is influenced by DIXDC1-DISC1 complex formation.

Another interacting partner studied in this context is Nudel1, is a Cysteine protease (endo-oligopeptidase A (EOPA)) whose activity is inhibited by DISC1 interaction in in vitro assays (Hayashi et al., 2005). Nudel also regulates neuronal differentiation of PC12 cell line (Ozeki et al., 2003a). Co-immunoprecipitation experiments show that Nudel1 interacts with both SUMOylated and non SUMOylated DISC1. However, these experiments are only qualitative and do not provide any information on the strength of binding or interaction. Presence of SUMO Interacting Motifs (SIMs) on proteins increases their binding affinity to proteins which are covalently conjugated to SUMO proteins, which is one of the possible consequence of protein SUMOylation (Jentsch and Psakhye, 2013).

Co-immunoprecipitation experiments performed in rodent brain lysates have shown that binding occurs between the C terminal region (fragment spanning from 596 to 852 residues) of DISC1 and region between the calponin homology domain and the coiled-coil domain of DIXDC1 (Singh et al., 2010). Ndel1 interacts with the C-terminal domain of DISC1 (residues 727-854) (Ozeki et al., 2003a, Soares et al., 2011). As stated earlier, the binding sites for DIXDC1 and Nudel1 on DISC1 overlap. It has been suggested that Cdk5 mediated phosphorylation on DIXDC1 results in the formation of DISC1/DIXDC11/Ndel1 complex which is indispensable in neuronal migration (Singh et al., 2010). Taking this in

to account, it is likely that SUMO conjugation of DISC1 may act as a trigger between neuronal progenitor proliferation and migration and this event mimics the phosphorylation event on DISC1 which acts as a molecular switch between proliferation of mitotic progenitor cells enabling the migration of post-mitotic neurons. Putative truncated protein in the DISC1 translocation in the Scottish pedigree (TrDISC1) lacks the binding site for both these proteins as well as the identified SUMO acceptor lysine (K643 and K788). Hence, it is likely that this PTM on DISC1 influences various physiological events possibly mediated by its interactions with various proteins.

Given the role SUMOylation in CNS related disease pathways (reviewed in section 1.4.2) it can be considered as an attractive target for therapeutic intervention. For example the increase in overall protein SUMOylation observed in neurons is a neuroprotective response (Loftus et al., 2009) while SUMO-1 expression has been shown to rapidly increase during hypoxia (Shao et al., 2004). It would be interesting to investigate if SUMO conjugation of DISC1 has such a role to play in the disease mechanism of DISC1 associated psychiatric illnesses.

To this end, future work should 1. Unequivocally identify the SUMO sites on DISC1 2. Use site directed mutagenesis to block the SUMO acceptor sites 3.analysis of the aggregation and cellular location of Wt vs mutant SUMO-null DISC1. Interesting insights into the functionality of the SUMOylation of DISC1 in animal models could be gleaned by generating transgenic mice carrying DISC1 mutations at SUMO conjugation sites.

3.5 Chapter summary

In summation, my work presented in this chapter uncovers a novel post translational modification on DISC1 in the form of SUMOylation. In vitro SUMOylation of DISC1 peptide array identified K643 as a potential acceptor lysine. SUMOylation of arginine mutant at K643 site on DISC1 was attenuated using the UFDS method suggesting that this site as a principle SUMO conjugation site on DISC1. However, this site could not be verified using MS studies due to poor sample quality. Our co-immnoprecipitation studies also suggest that SUMO modification of DISC1 may abolish its binding with DIXDC1, while its interaction with Nudel1 remained unaffected.

4.

Proteasomal regulation of human DISC1 protein

4.1 Introduction

Gene expression studies carried out on the post-mortem brain tissue of subjects with SCZ has provided insight in to possible involvement of ubiquitin proteasome system dysregulation in the SCZ disease mechanism. A number of studies used cDNA microarrays to evaluate the profile of differential gene expression in these samples vs control. Consequently, Ubiquitin carboxyl-terminal esterase L1 (UCHL1), a deubiquitinating enzyme with predominant expression in CNS (known to be associated with neurological disorders such as AD and PD) and PSMC6 (proteasome 26S subunit, ATPase, 6 encodes one of the catalytic subunits of the 20S subunit of the proteasome) are two candidate genes that were significantly down-regulated in subjects with SCZ (Altar et al., 2005, Vawter et al., 2001). Another study used a blood based micro-array approach to screen for the gene expression patterns from SCZ and BD patients belonging to two different ethnic groups and in doing so, identified a number of UPS genes to be dysregulated (Bousman et al., 2010). Various other reports of altered gene expression of UPS components in SCZ and Bipolar disorder (Middleton et al., 2002) garnered my interest.

The functional role of DISC1 in neuronal development was initially investigated by delineating its interacting partners using a yeast 2 hybrid screen (Millar et al., 2003, Ozeki et al., 2003b). Key proteins involved in neuronal development, neuron migration and synaptic signalling have been identified in these screens. These studies emphasise the functional significance of DISC1 in brain development and strengthen its role in disease

mechanisms of psychiatric illnesses such as SCZ and certain mood disorders (Hashimoto et al., 2006). The effects of DISC1 knockdown has been studied using various methods. *In utero* gene transfer was used to transiently knockdown DISC1 in mouse models to understand its role in growth and maturation of the brain (Niwa et al., 2010). This study used GFP tagged DISC1 shRNA, which was injected in to the pre frontal cortex of mice brain at E14. This resulted in morphological abnormalities in postnatal meso cortical dopaminergic maturation, in turn, resulting in several behavioural abnormalities in adulthood (Niwa et al., 2010). shRNA mediated DISC1 knockdown in cortical precursor cells in ventricular zone, abolished the migration of cells in to the cortical plate (Kamiya et al., 2005b).

Various independent studies focused on the functional consequences of the truncated form of DISC1 protein (Millar et al., 2000c)) and revealed its dominant negative role. Over expressed C-terminal truncated DISC1 (TrDISC1, amino acids 1-597) has been shown to interfere with the wild type protein product of the functional allele affecting its function, subcellular localisation and microtubular dynamics (Ozeki et al., 2003a, Kamiya et al., 2005b). However, it should be noted that TrDISC1 protein has not been detected in cells. Apart from the Scottish pedigree, several other groups independently reported many other SNPs within the DISC1 locus in various ethnic groups to be associated with chronic mental illnesses (Zhang et al., 2006, Saetre et al., 2008, Callicott et al., 2005). Exonic polymorphisms such as S704 and C704 have been implicated in SCZ and major depression respectively (Leliveld et al., 2009, Song et al., 2010). However, the effect of these SNPs or truncations on the protein confirmation or stability has not been investigated.

The hallmark of many psychiatric illnesses is pathological aggregation of defective or mutant proteins resulting in dysregulation of key signalling pathways, ultimately leading to chronic neurological disorders such as Alzheimer's disease, Creutzfeldt-Jakob's disease, Parkinson's disease and Huntington's disease (Leliveld et al., 2009). A study of DISC1 dimerisation and oligomerisation, revealed interesting leads regarding the pathological role of DISC1 (Leliveld et al., 2009). The presence of sarkosyl (detergent) insoluble DISC1 aggregates in the brains of a sub set of cases with mental disease can be

triggered by subtle conformational changes and possibly yet unknown posttranslational modifications (Leliveld et al., 2008). These putative post translational modifications in turn might abolish or create new interacting partners leading to loss or gain in function.

In spite of the increasing evidence that DISC1 is a key susceptibility factor in SCZ, not much is known about its turnover/half-life and whether its regulatory mechanisms at the protein level depend on its cycling synthesis or degradation. Very few studies investigated the levels of DISC1 at the transcript and protein level. Increased DISC1 transcript levels were reported in peripheral blood mononuclear cells of patients with schizophrenia. In an effort to identify a suitable biomarker for SCZ, the above study compared the transcript levels of various DISC1 isoforms in blood samples of patients experiencing psychosis, to those samples collected after neuroleptic doses of haloperidol was administered. A predominant decrease in DISC1 transcript levels was reported following the treatment, suggesting a strong association of DISC1 levels to the disease mechanism (Olincy et al., 2011). Another study used immunostaining techniques to investigate the effect of epileptic seizures on DISC1 levels in adult rats (Fournier et al., 2009). Electrically induced epileptic seizures significantly reduced DISC1 immunolabeling within the dentate gyrus, while the width of the granule cell layers remained the same between the control and induced (Fournier et al., 2009). This study by Fournier and collegues suggests the crucial role of DISC1 levels in cytoarchitectural abnormalities of the dentate gyrus observed in some SCZ subjects (Arnold, 1999).

Approximately 10-20% cellular proteins are short lived, with a half-life less than 3 hours. Studies using proteasomal inhibitors show that up to 90% cellular proteins are degraded by UPS (Lee and Goldberg, 1998). Ubiquitinated substrates are short lived and it is challenging to capture the signal of ubiquitinated protein of interest using conventional biochemical techniques. Proteasome inhibitors, hence, are very useful to stabilise cellular levels of modified proteins. Proteins, whose turnover is regulated by UPS, should accumulate, in a ubiquitin-conjugated form, upon proteasome inhibition. My initial experiments were concerned with investigating the consequences of inhibiting the proteasome on DISC1 and studying the dynamics of DISC1 ubiquitination (L isoform).

Commonly used proteasome inhibitors are Lactacystin, Epoxomicin and MG132. Lactacystin, a β lactone, is a natural microbial product which exerts its inhibitory action by undergoing partial hydrolysis forming clasto-lactacystin, which interacts with proteasome (Dick et al., 1996). Epoxomicin, an α1, β1 – epoxy-ketone tetrapeptide, is also a natural bacterial product which inhibits chymotrypsin-like, trypsin-like, and peptidyl glutamyl peptide-hydrolyzing (PGPH) activities of the proteasome. Epoximicin is 80 fold more potent than lactacystin (Meng et al., 1999). MG132, a peptide aldehyde, has been chosen in the present study as it is inexpensive compared to the others. Its structure and mechanism of action is explained in the following section.

4.1.1 MG132- Structure, Mechanism of action

MG132 is one of the first groups of proteasome inhibitors to be synthesised that belong to the class of peptide aldehydes. These inhibitors were developed on the basis of substrate specificity (Lee and Goldberg, 1998). MG132 (Carbobenzoxy-Leu-Leu-leucinal) is structurally a substrate analogue which inhibits the chymotrypsin like activity in the 20S subunit of the proteasome. It is a cell permeable small molecule, which exerts it inhibitory function by the formation of a transition state analogue. However, the proteolytic enzyme activity is gradually restored by the hydrolysis of the covalent bond between MG132 and the enzyme complex, hence the inhibition is reversible. Its IC_{50} is 0.1μM for chymotryptic activity of the proteasome and its K_i is 4nM. MG132 also induces neurite outgrowth in rat PC12 cell line (Obin et al., 1999).

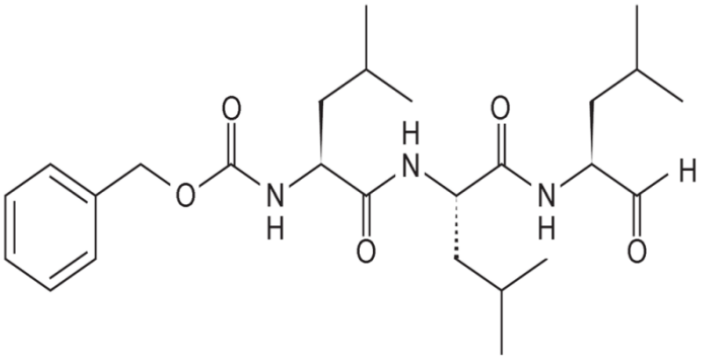


Figure 4.1 Chemical structure of Proteasome inhibitor MG132 Carbobenzoxy-Leu-Leu-leucinal.

4.2 Experimental aims

DISC1 has already been established as a susceptibility factor in SCZ and other related illnesses. Increasing evidence also suggests dysregulation of the UPS pathway in various psychiatric disorders. Reduced DISC1 protein levels observed in tissue collected from subjects with SCZ may be a part of the actual disease mechanism which is not yet understood (Lipska et al., 2006a) .

Hence I set out to investigate the following in this chapter:

1. To determine whether DISC1 protein levels are regulated by the ubiquitin proteasome system (UPS).

2. To identify E3 ligase components of the UPS system that mediates DISC1 ubiquitination.

4.3 Results

4.3.1 Proteasomal regulation of DISC1 protein in HEK293 cell line

HEK293 cells are derived from Human Embryonic Kidney cells by transformation with sheared adenovirus 5 DNA. Their ease of culture, maintenance, transfection and transduction made them a popular choice of cell line for various studies. Regardless of their origin, HEK293 cells exhibit a number of neuronal characteristics such as expressing all sub units of neurofilament proteins, neuron specific enolase and Tau (Campbell et al., 2005). They also express functional ion gated calcium potassium channels (He and Soderlund, 2010). In spite of the availability of neuroblastoma cell line SH-SY5Y, HEK293 cells were chosen for my study for the following reasons: higher detectable DISC1 protein levels and ease of transfection in HEK293 cells over SH-SY5Y cells.

My Initial experiments involved investigating the effect of proteasomal inhibition on DISC1 endogenous levels (Figure 4.2) and overexpressed HA - DISC1 (Figure 4.3) using Western blotting techniques. MG132 transiently increased DISC1 protein levels in a time dependent manner in first two hours at the indicated concentration. However, after 3hr treatment there was a significant reduction of DISC1 protein levels to below the levels seen at time = zero. MG132 time courses with cells transfected with HA DISC1 were restricted to 5hr (Figure 4.3) as an increase in cell death was observed beyond that point. This may be attributed to the possible non proteasomal enzyme inhibition effect of MG132 on lysosomal cysteine protease and calpine inhibition (Lee and Goldberg, 1998).

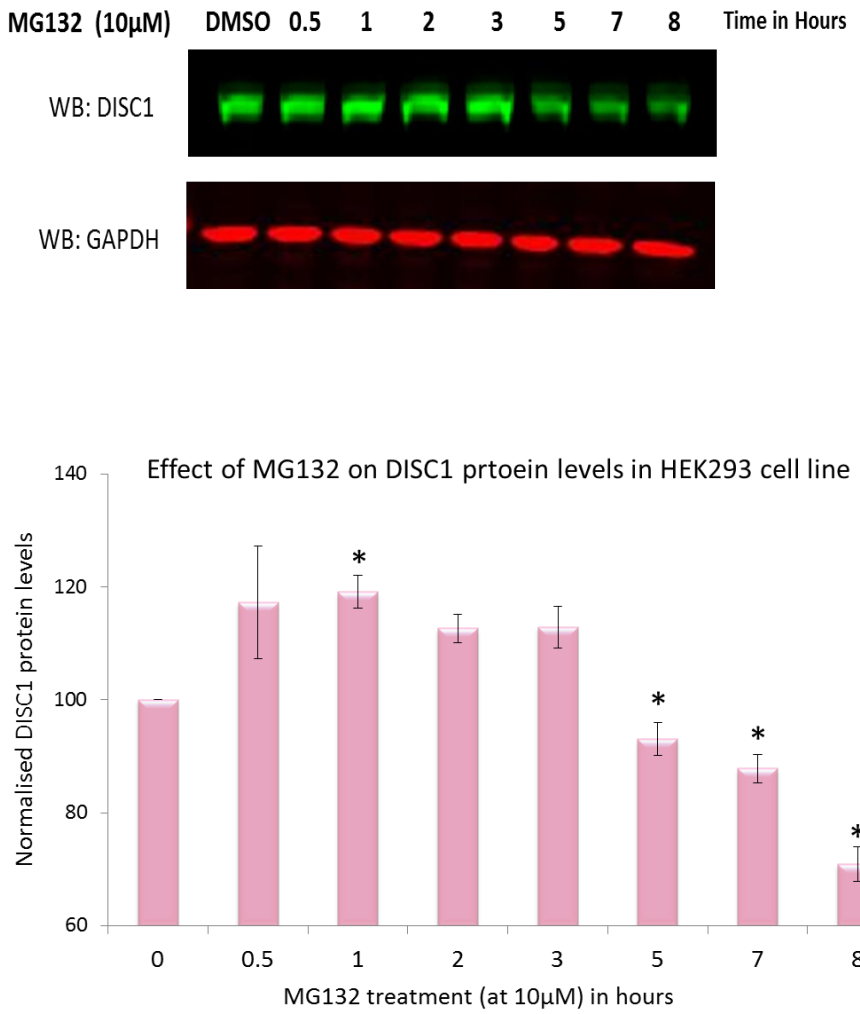


Figure 4.2 Proteasome inhibition transiently stabilises endogenous DISC1 levels in HEK293 cell line.
Top panel: HEK293 cell cultures were treated with MG132 at 10µM concentration for indicated time points and endogenous DISC1 protein levels were analysed by Western blotting technique using Anti DISC1 antibody. Anti GAPDH antibody was used as a loading control. Bottom panel: Densitometric analysis of the amount of DISC1 protein normalised to GAPDH expression levels. Data is represented as ± S.E.M. of three biological repeats. Statistical significances between control (time = 0) and a time point was calculated using one way analysis of variance (ANOVA). *P < 0.05 values were considered significant.

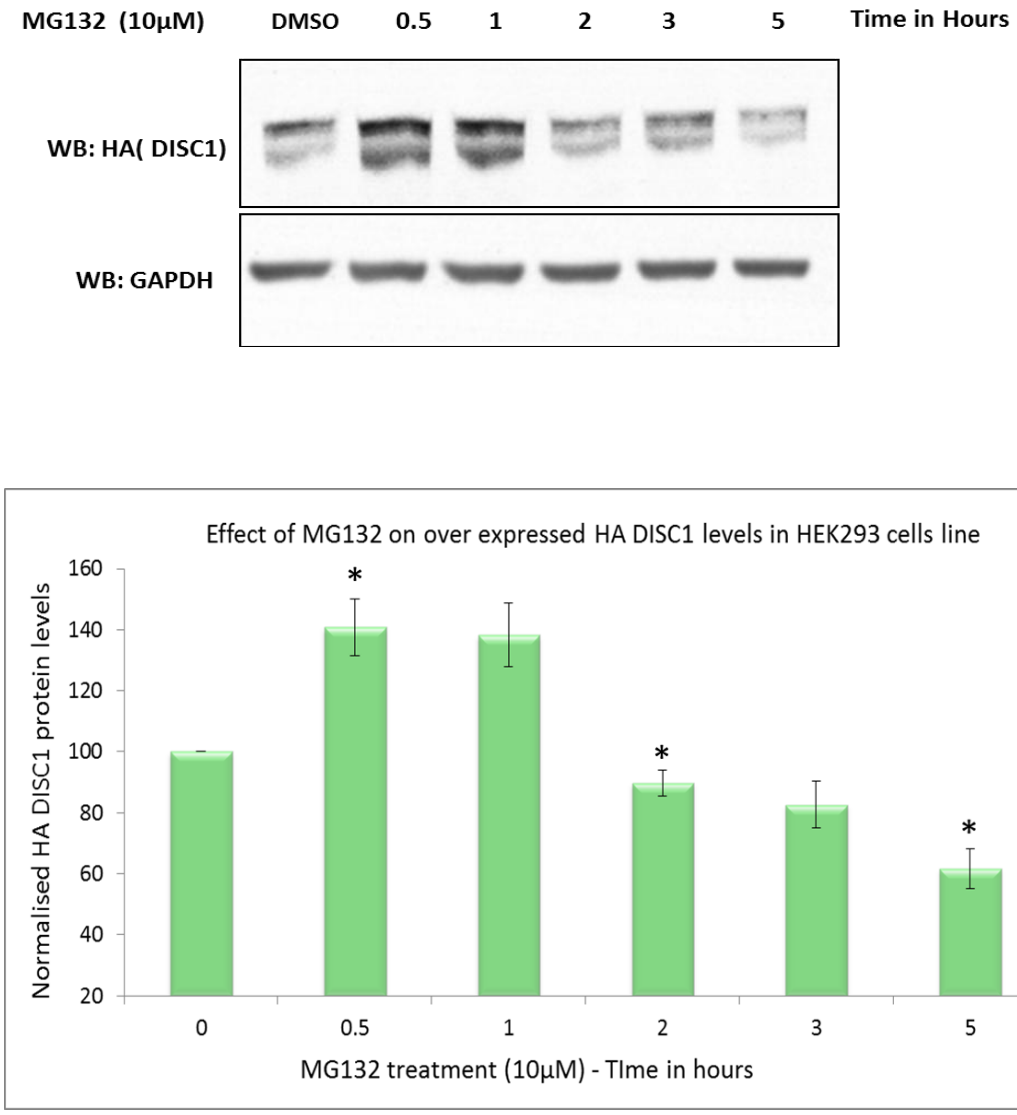


Figure 4.3 Proteasome inhibition transiently stabilises over expressed DISC1 levels in He293 cell line.
Top panel: HEK293 cell cultures were transiently transfected with HA (haemagglutinin)-DISC1. 48hr post incubation Ubiquitination cell cultures were treated with MG132 at 10 µM concentration for indicated time points and DISC1 protein levels were analysed by Western blotting technique with anti HA antibody. Anti GAPDH antibody was used as a loading control. Bottom panel: Densitometric analysis of the amount of DISC1 protein normalised to GAPDH expression levels. Data is represented as ± S.E.M. of three biological repeats. Statistical significances between control (time = 0) and a time point was calculated using one way analysis of variance (ANOVA). *P < 0.05 values were considered significant.

4.3.2 Ubiquitin chain formation on DISC1 protein – K48 and K63 linked ubiquitin chains detected

To determine whether DISC1 is a substrate for ubiquitination, HA - DISC1 was overexpressed with HA - ubiquitin or an empty vector which served as a negative control. 48 hours post transfection, cells were treated with MG132 or DMSO (vehicle) at 10µM for 3hr. The cell lysates were evaluated using a Western blotting technique. Probing with DISC1 antibody shows the presence of high molecular weight, slow migrating DISC1 species in the samples co expressing ubiquitin (Figure 4.4). Proteasome inhibition retains the high molecular weight DISC1 bands which correspond to the ubiquitinated DISC1 protein, likely to be carrying one or many type of ubiquitin chains. These high molecular weight bands are absent in the samples of HA - DISC1 co expressed with an empty vector i.e., absence of ubiquitin (Figure 4.4.).

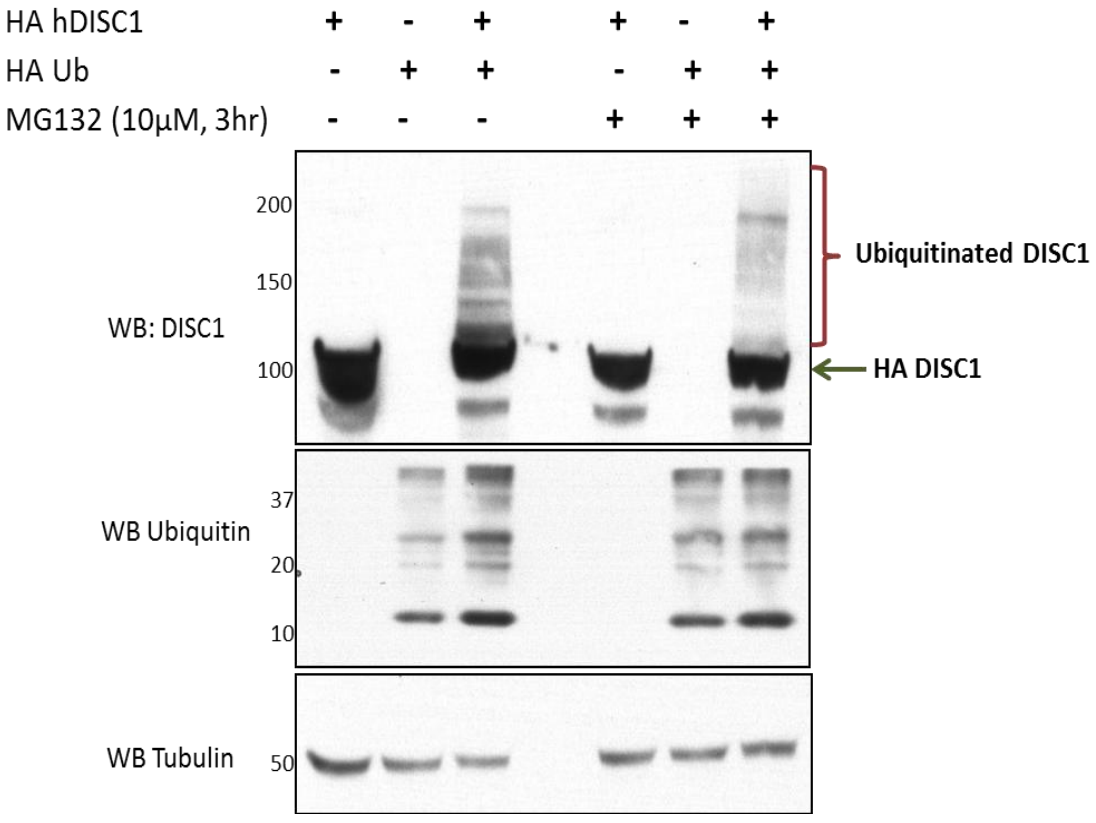


Figure 4.4 Immunoblots showing DISC1-ubiquitin conjugates from HEK293 cell lysate. HA-DISC1 was overexpressed with an empty vector or HA-ubiquitin. Samples were treated with DMSO or MG132 at a concentration of 10µM for 3hr. Cells were lysed under denaturing conditions (described in materials and methods) and analysed for the presence of DISC1-ubiquitin conjugates using Western blotting technique. Anti-DISC1

antibody was used to detect DISC1 (unmodified and ubiquitin conjugated) and anti-tubulin antibody was used as loading control.

The ubiquitination of DISC1 was further confirmed by immunoprecipitation experiments where endogenous DISC1 was purified from HEK293 treated with MG132 or DMSO (vehicle) at 10µM concentration for 3hr. Immunoprecipitation was performed using DISC1 antibody as bait and the immunoprecipitate complexes were analysed for the presence ubiquitin chains by probing with a pan ubiquitin antibody or K48, K63 type ubiquitin linkage specific antibodies (Figure 4.5). Similar experiments to detect ubiquitination of over expressed HA tagged DISC1 were also performed. Immunoprecipitation of over expressed HA tagged DISC1 from HEK293 cells was carried out using HA tag specific antibody (Figure 4.6).

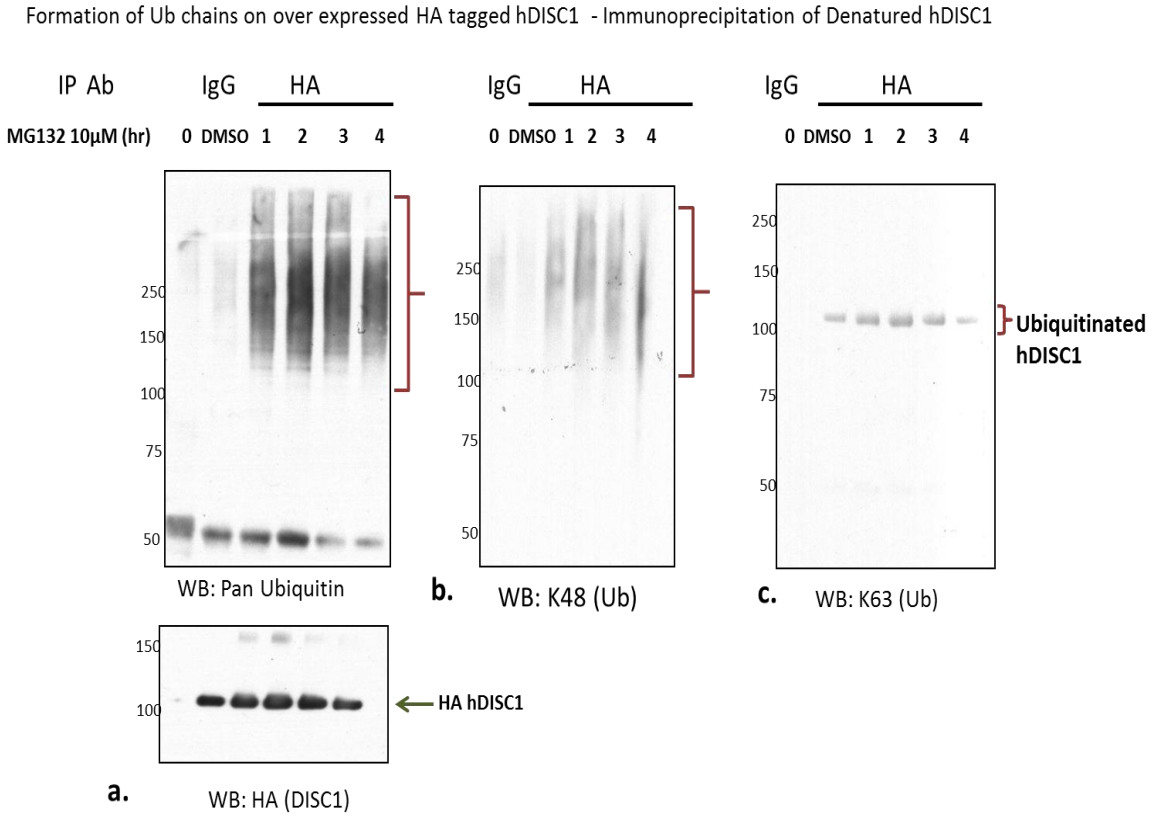


Figure 4.5 Detection of endogenous DISC1 – Ubiquitin conjugates in HEK293 cells under denaturing conditions.
Immunoblot analysis of DISC1-Ubiqutin conjugates after immunoprecipitation of endogenous DISC1 from HEK293 cell lysate. HEK293 cells cultures were treated with DMSO or MG132 at a concentration of 10µM for 3hr. Cells were lysed under denaturing conditions and immunprecipitation was performed using anti DISC1 antibody or rabbit IgG (described in materials and methods). Captured immunoprecipitate complexes were analysed by Western blotting technique. Left panel: Anti Ubiquitin and anti DISC1

antibodies were used. Immunoprecipitate complexes were also analysed with ubiquitin chain type specific antibody – middle panel: anti K48 linked Ubiquitin chain type specific antibody and right panel: anti K63 type Ubiquitin chain specific antibody.

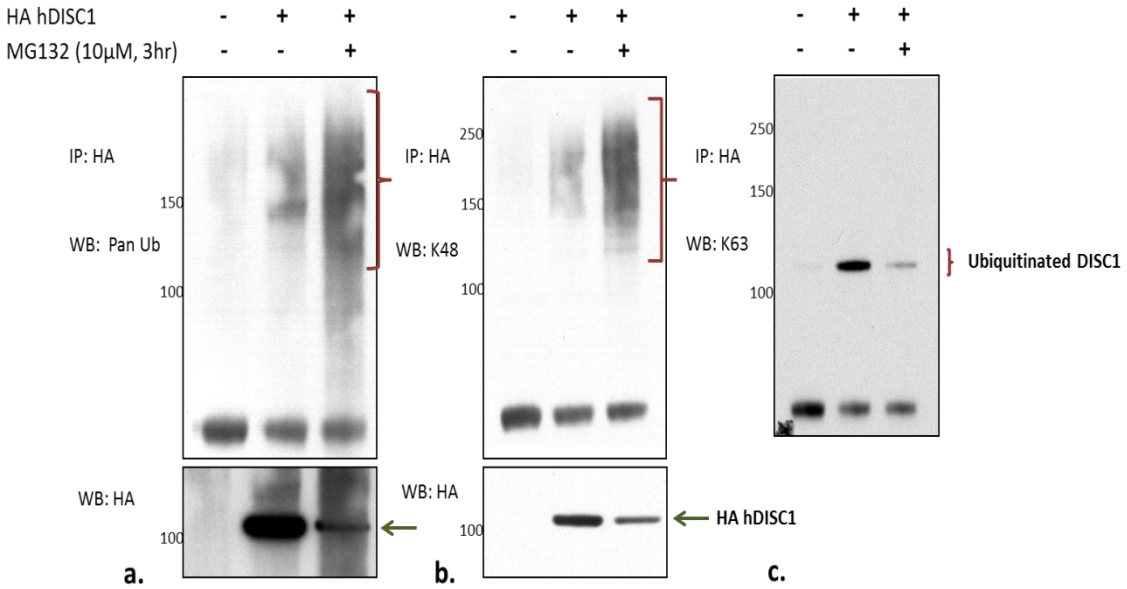


Figure 4.6 Immunoblot analysis of overexpressed human DISC1-Ubiqutin conjugates in HEK293 cell lysate under denaturing conditions:
(a) HA - DISC1 was overexpressed in HEK293 cells and treated with 10µM DMSO or MG132 for 3hr. Cells were lysed under denaturing conditions and immunprecipitation was performed using HA - agarose beads or mouse Ig G - agarose beads (negative control). Captured immunoprecipitate complexes were analysed by Western blotting technique (described in materials and methods). Left panel: Anti ubiquitin and anti DISC1 antibodies were used. Immunoprecipitate complexes were also analysed with ubiquitin chain type specific antibody; middle panel: anti K48 linked ubiquitin chain type specific antibody and right panel: anti K63 type ubiquitin chain specific antibody.

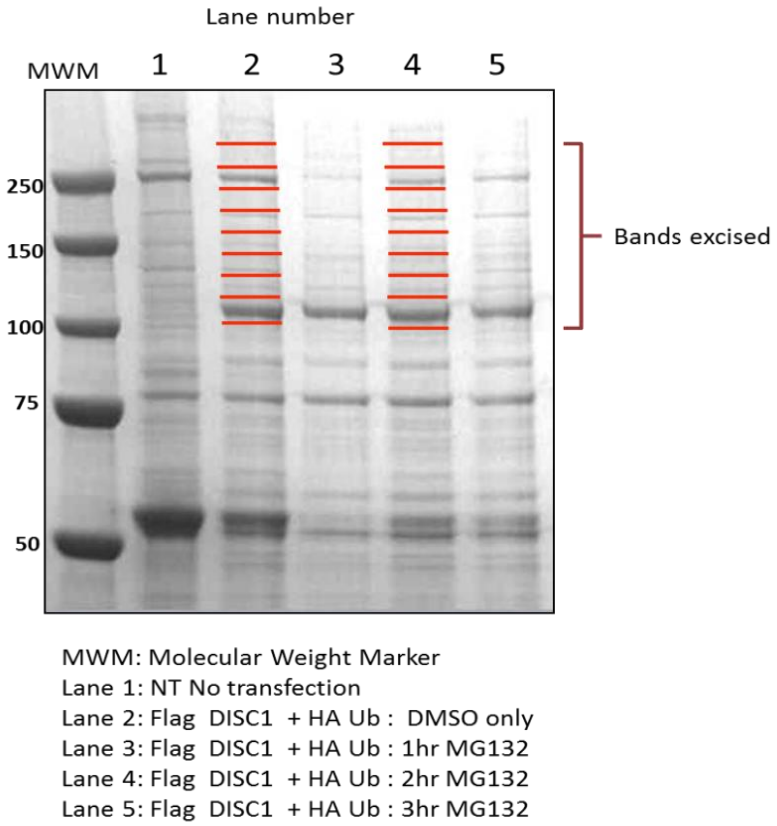
The biochemical experiments performed in this section confirm that DISC1 undergoes covalent modification with ubiquitin which can be observed as high molecular weight, slow migrating DISC1-ubiquitin conjugates observed as a smear at molecular weights above that of native DISC1 (~100kDa). Pretreatment of cells with proteasome inhibitor MG132 prior to cell lysis led to significant enrichment of ubiquitin conjugates in the sample, which can be seen as increased ubiquitin conjugate bands in the immunoblots. As discussed in Chapter 1, ubiquitin is reversible and mediated by the action of de-ubiquitylating enzymes (DUBs) which rapidly deconjugate ubiquitin from the substrates and recycle ubiquitin. The action of these enzymes hampers the detection and reliable

characterisation of protein ubiquitination. One strategy that has been efficiently employed was purifying the protein-ubiquitin conjugates under denaturing conditions i.e, use of strong detergents and heat. Protein denaturation rapidly destroys enzyme activity by disrupting protein secondary and tertiary structure by destroying hydrogen bonding, salt bridges and disulphide bonds while the backbone peptide bond and other covalent modifications remains intact (Ciechanover and Iwai, 2004). This strategy was successfully employed in the present study which allowed efficient detection of post translation medication on DISC1 and also aids further biophysical studies discussed in the next section.

4.3.3 DISC1 ubiquitination - Mass spectroscopy evidence

Identification of ubiquitin modification sites on a substrate protein is crucial to the facilitation of understanding the physiological role of ubiquitination in the context of that protein. All PTMs direct specific functional consequences for the target protein. Most often, ubiquitin receptor lysine residue(s) on the target is/are identified by site directed mutagenesis experiments which block ubiquitination. Recent advances in technology have enabled the use of Mass spectroscopy techniques to not only identify the target lysine residue which undergoes the PTM but also the type of ubiquitin chain associated with it. In this study, Flag DISC1 and HA Ubiquitin were co expressed in HEK293 cells followed by MG132 treatment and cell lysis after 48hr incubation. Immunoprecipitate complexes were collected using Flag tagged agarose beads as bait. Materials and methods section 2.4.8 describes the protocol for the immunoprecipitation and subsequent sample preparation for the Mass spectroscopy studies.

Mass spectroscopy samples were analysed at Yale School of Medicine, USA.



MWM: Molecular Weight Marker
Lane 1: NT No transfection
Lane 2: Flag DISC1 + HA Ub : DMSO only
Lane 3: Flag DISC1 + HA Ub : 1hr MG132
Lane 4: Flag DISC1 + HA Ub : 2hr MG132
Lane 5: Flag DISC1 + HA Ub : 3hr MG132

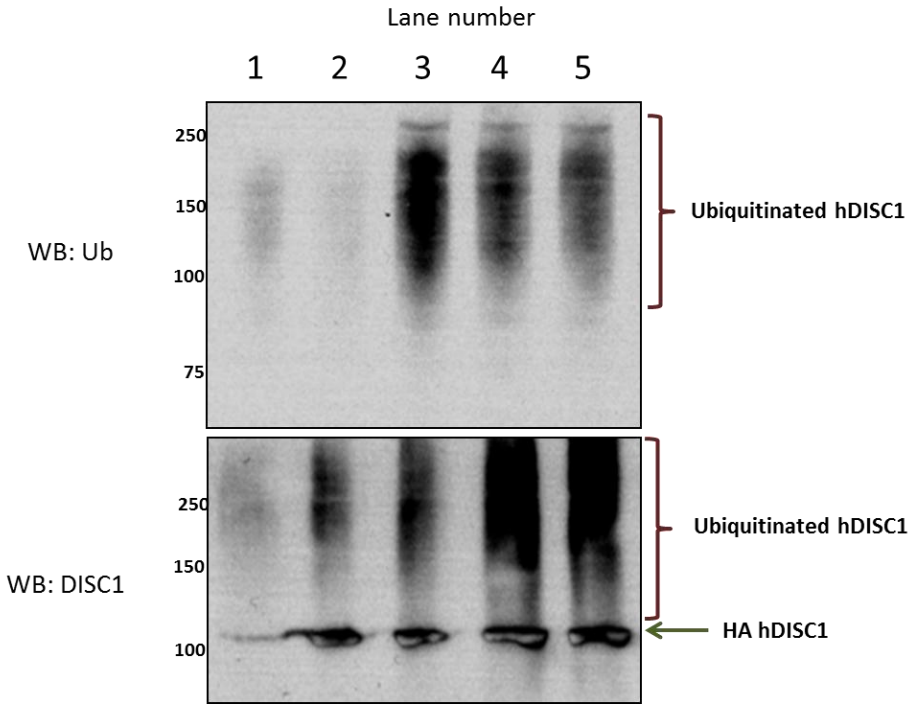


Figure 4.7 Preparation of Samples for mass spectroscopy studies to detect human DISC1 Ubiquitination in HEK293 cells.
Flag DISC1-HA Ubiquitin complexes overexpressed in HEK293 cell line were immunoprecipitated using Flag tagged agarose beads. Untransfected HEK293 cell lysate was used as a negative control. Bands were excised from a colloidal blue stained PAGE gel as indicated in this Figure (top panel) and analysed by mass spectroscopy. Small fraction of sample was analysed by Western blotting to analyse the efficiency of the transfections (bottom panel).

Table 4.1 Summary of Mass Spectroscopy studies to detect DISC1-ubiquitin conjugates

Sample (Figure 4.7)	Sample collected at molecular weight	DISC1 peptide detected in the sample	Percentage coverage	PTM detected
Lane 2: Flag DISC1 + HA Ub, DMSO only	100 kDa	^{68}ENTMKY**M**ETL**K**N**K**^{80}	63.8	GlyGLy (K) Oxidation (M)
Lane 2: Flag DISC1 + HA Ub, DMSO only	150 kDa	^{208}LQ**K**LQEDAVENDDYDK^{223}	45	GlyGly (K)
Lane 2: Flag DISC1 + HA Ub, DMSO only	200 kDa	^{9}N**K**LCSC**K**CPLLG**K**VWEADLEACR^{31}	42	GlyGly (K)
Lane 2: Flag DISC1 + HA Ub, DMSO only	250 kDa	^{7}SLNLSL**K**EITT**K**^{18}	40	GlyGly (K)
Lane 4: Flag DISC1 + HA Ub, 2hr MG132	100 kDa	^{68}ENTMKY**M**ETL**K**N**K**^{80}	64	No PTM detected
Lane 4: Flag DISC1 + HA Ub, 2hr MG132	**150 kDa**	**^{360}LQEDAVENDDYDKAETLQQR^{379}**	**42**	**GlyGly (K)** HIGHEST MASCOT SCORE
Lane 4: Flag DISC1 + HA Ub, 2hr MG132	200 kDa	^{7}SLNLSL**K**EITT**K**^{18}	37	GlyGly (K)
Lane 4: Flag DISC1 + HA Ub, 2hr MG132	250 kDa	^{41}LGSV**K**EDYNR^{50}	37	GlyGly (K)

The above Table implies the prescence of DISC1 at various molecular weights which correspond to native DISC1 and post translationally modified (PTM) DISC1 species (Column 1 and 2). Column 3- DISC1 peptides identified in the corresponding samples. Column 4 and 5 indicate the percentage of DISC1 peptide detected in resepective samples and the PTMs identified in corresponding samples. Lysine residues which undergo PTM are represented in bold letters. Column 5- GlyGly indicate Ubiquitination modification on the indicated lysine residue.The sample highlighted in green has been identified to have the highest MASCOT score of 30 of all the samples screened. A higher MASCOT score indicates a more confident match of all the samples analysed. Mass spectroscopy principle, sample preparation protocol was explained in materials and methods section 2.4.8.

The mass spectroscopy studies on DISC1 Ubiquitnation can be summarised as follows:

DISC1- ubiquitin conjugates were detected at various molecular weights above 100kDa (the weight at which native DISC1 migrates). These conjugates were represented as glycine-glycine-lysine linkages (GlyGly(K)) in Table 4.1.

Six lysine residues were identified to be carrying G-G-K modification with variable MASCOT scores. MASCOT scores were generated using a software package from Matrix Science (www.matrixscience.com) that interprets mass spectral data into protein identities. Mascot compares the spectral data to a database of known proteins and scores based on confidence of the match.

Lysine 372 residue was chosen for subsequent work as it was predicted to be the site with higher probability of ubiquitin modification implied by its highest MASCOT score of 30 (Table 4.1).

4.3.4 Analysis of ubiquitination kinetics of the DISC1 K372R mutant (site identified in the Mass spectroscopy studies)

V5 tagged DISC1 lysine to arginine mutant at position 372 (K) was generated and evaluated for ubiquitination at basal levels and in the presence of the proteasome inhibitor MG132. Lysine to arginine substitution mutants are ideal in this situation to retain the net negative charge on the R group (side chain of the amino acid). Immunoprecipitation studies were performed by over expressing V5 tagged DISC1 WT and K372R mutant, to analyse the pattern of ubiquitin chain formation in response to proteasomal inhibition. V5 tag antibody was used as bait to immunoprecipitate the DISC1-ubiquitin protein conjugates.

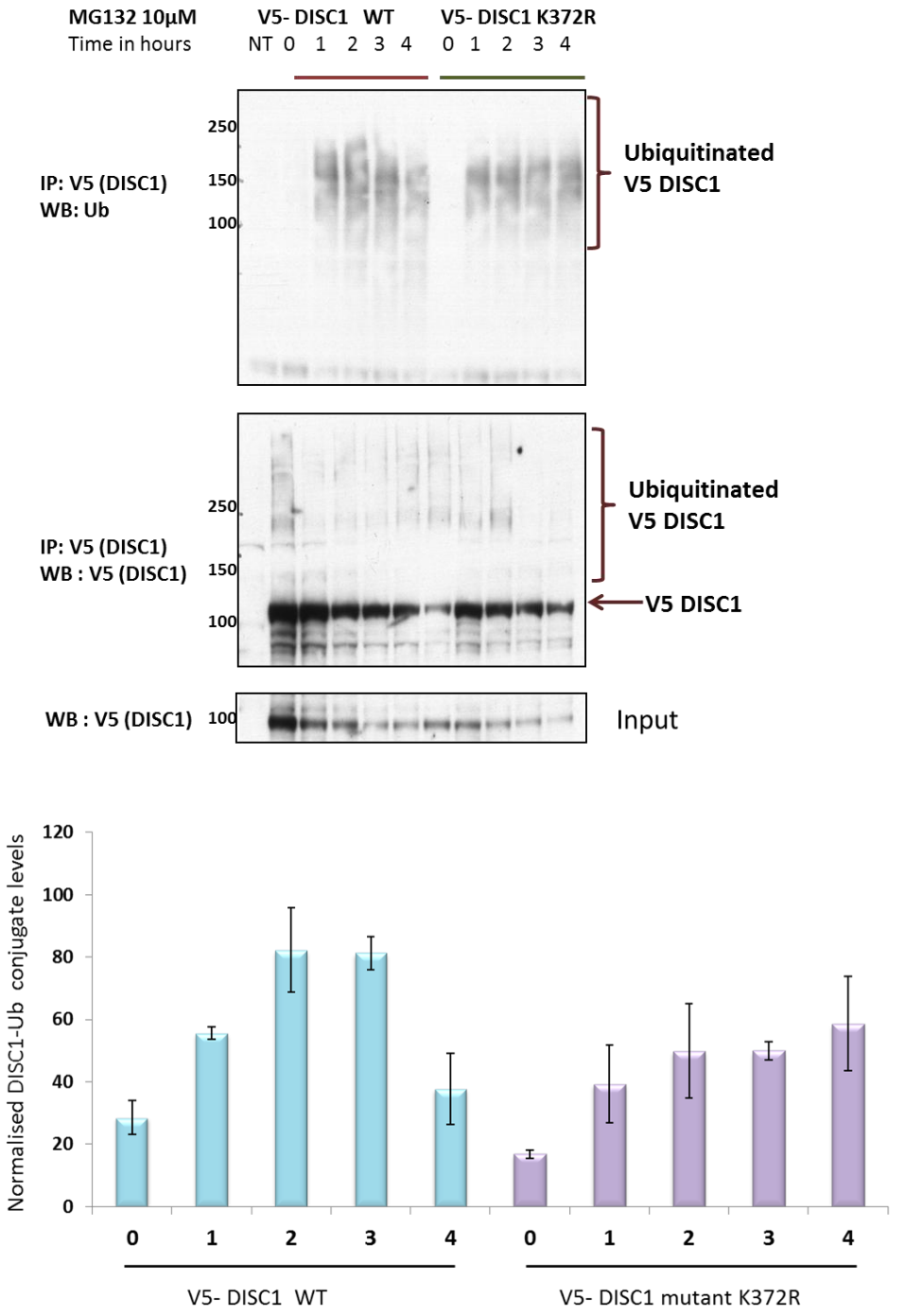


Figure 4.8 Comparative analysis of Ubiquitination of human DISC1 Ubiquitination mutant K372R to that of wild type DISC1.
Wild type V5-DISC1 and K372R V5-DISC1 constructs were overexpressed in HEK293 cells. 48hr post transfection, cultures were treated with MG132 at 10 µM concentration for indicated time points. Immunoprecipitation was performed on the cell lysates using V5 tag antibody as bait. The first lane in all the blots in untransfected HEK293 cell lysate used as negative control. Top panel : Anti ubiquitin antibody. Middle panel: V5 tag antidoy (DISC1). Lower panel: anti V5 tag antibody (DISC1) for input which is the positive control . Graph indicates the statistical representation of densitometric analysis of the amount of DISC1-ubiquitin protein conjugates normalised to unconjugated DISC1 expression levels. Data is represented as ± S.E.M. of three biological repeats.

Interestingly, I discovered that the degradation rate of the V5-DISC1 K372R mutant (represented by the smeary pattern in Figure 4.8, top panel) was reduced compared to that of the WT. This suggests that the K372 site is a *bona fide* ubiquitination site but there are one or more other sites on DISC1 that get modified in this way. This notion is supported by the mass spectroscopy studies which suggest up to five sites. This sort of site specific ubiquitination dependent regulation of a substrate has been reported for other proteins, for example HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase). Though this enzyme has several lysine residues which serve as ubiquitin acceptors, mutational studies show that K^{248} acts as a primary ubiquitin acceptor site (Doolman et al., 2004).

4.3.5 Identification of E3 ligase regulating DISC1 ubiquitination – High Throughput Screen for Fbox proteins

Fbox proteins were initially characterised as components of SCF ubiquitin E3 ligase complexes, which help catalyse the transfer of ubiquitin moiety from the E2 enzyme to the substrate. The Fbox protein is the variable component of the SCF complex where substrate specific Fbox proteins are recruited as a consequence of specific post translational modification such as phosphorylation or SUMOylation (ubiquitination cascade is reviewed in section 1.5). My hypothesis highlights the significance of identifying the putative Fbox protein which regulates DISC1 half-life.

siRNA technology is a widely popular tool which can be used to silence target gene expression. siRNA oligonucleotides can be either introduced to cells by transfection or can be generated within the cell by introducing plasmids which encode shRNAs (short hairpin) which function as precursors of siRNA. siRNA screening has been widely used to investigate the functional role of various proteins and genes. siRNA libraries to screen for specific components such as kinases, E3 ligases and DUBs have now been generated and validated by number of commercial companies and are available in various formats.

One such library has been used in the present study to identify crucial E3 ligases that may catalyse ubiquitination of DISC1. To identify the Fbox protein (substrate recognising component of SCF complex) I used a genome wide screen using siRNA approach. ON-

TARGETplus® siRNA Library (Dharmacon) to all known human Fbox proteins was screened to serve this purpose. This library consists of 96 siRNA pools (four duplexes each) designed to reduce potential off target effects. Owing to the higher transfection efficiency and detectable basal DISC1 protein levels, HEK293 cells were used in the screen. HEK293 cell cultures were transfected with the siRNA following manufacturer's instructions (materials and methods, section 2.2.9). 48hr post transfection, cell lysates were analysed for DISC1 protein levels using the Western blotting technique. Anti GAPDH antibody was used as a loading control. The siRNA knockdown of the putative Fbox for DISC1 should reduce DISC1 ubiquitination, leading to its stabilisation. Increased amounts (vs control) of DISC1 protein can be screened by using Western blots. Putative hits were shortlisted following densitometric quantification of the DISC1 protein bands. These hits were rescreened to eliminate possible technical artifacts. Representative blots of the screen are shown in Figure 4.9 (high throughput screen) and Figure 4.10 (secondary screen). The complete screen (immunoblots) can be found in the appendix of this thesis.

This screen was followed by validation experiments, where the siRNA knockdown was confirmed by the analysis of the protein levels of another known substrate of Fbxw7, c-Myc (Figure 4.11). Gratifyingly, siRNA oligos that down regulated Fbxw7 expression also upregulated the protein levels of DISC1 and c-Myc concomitantly, suggesting that Fbxw7 is indeed involved in DISC1 degradation.

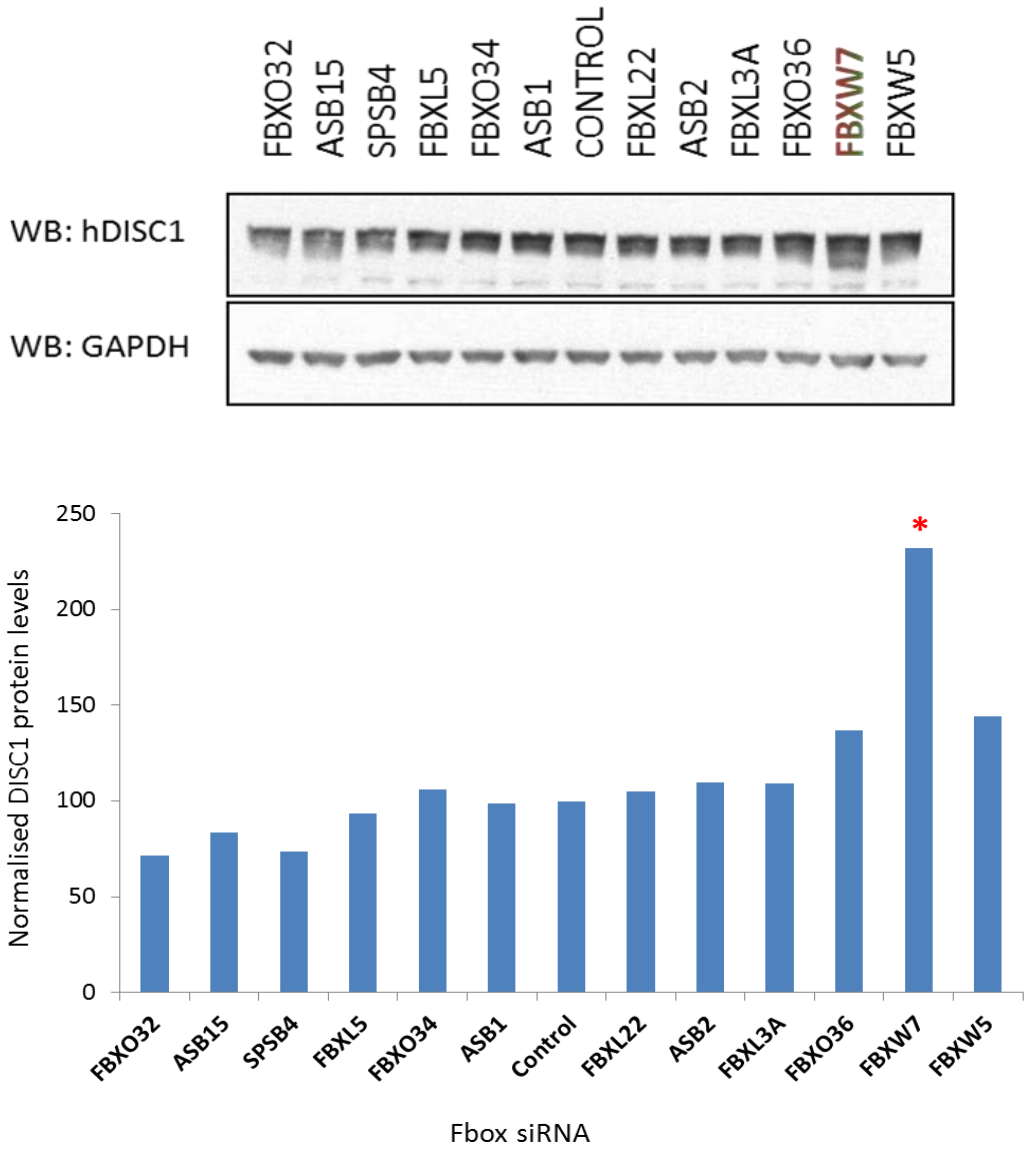


Figure 4.9 Fbox siRNA screen to identify putative Fbox protein that regulates DISC1 ubiquitination – Primary screen
HEK293 cells were transfected with siRNAs targeting the indicated transcripts. Non targetting siRNA was used as a negative control. Upper panel: Anti DISC1 antibody was used. Anti GAPDH antibody was used as a loading control. Lower panel: Densitometric analysis of the amount of DISC1 protein is normalised to GAPDH expression levels.
Putative hit has been identified (Fbxw7) for further analysis, indicated with '*'.

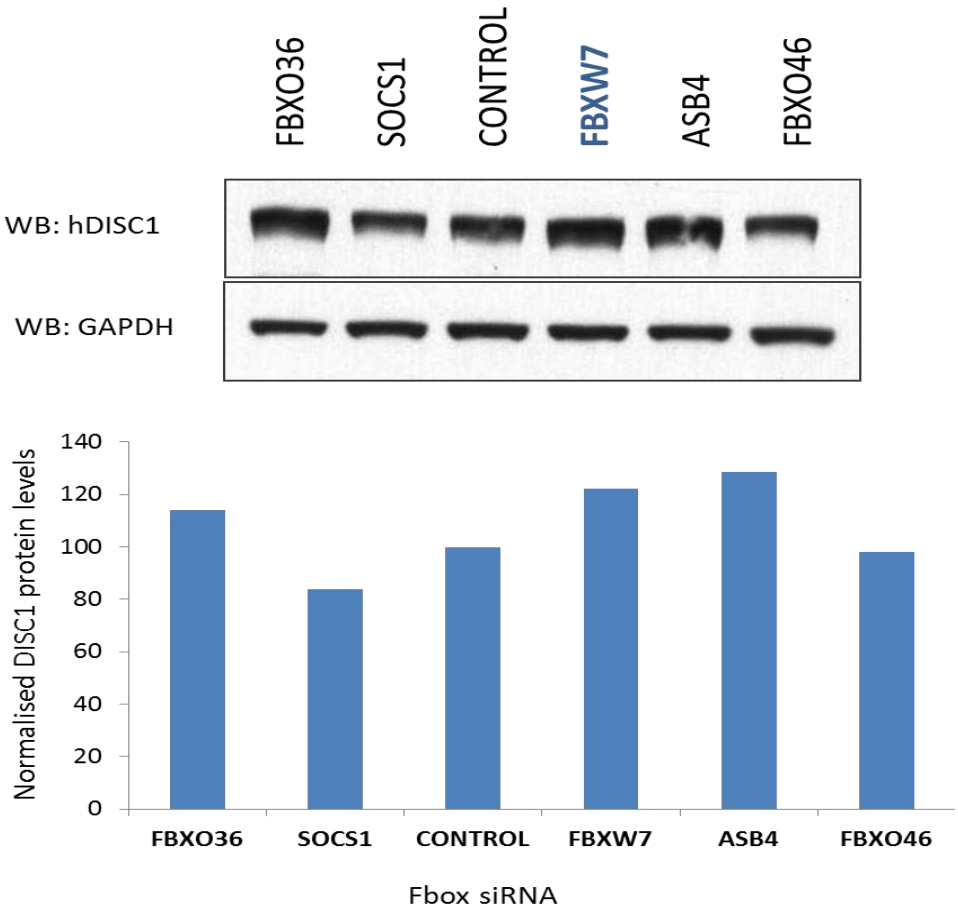


Figure 4.10 Validation of Fbox siRNA screen hit - Secondary Screen
Putative shortlisted Fbox proteins were rescreened. Upper panel: Anti DISC1 antibody was used. Anti GAPDH antibody was used as a loading control. Lower panel: Densitometric analysis of the amount of DISC1 protein is normalised to GAPDH expression levels. Complete information about Fbox siRNA screen with Figures is detailed in appendix. From the above screen, Fbxw7 was identified as a putative hit as its knock down consistently stabilised DISC1 protein levels.

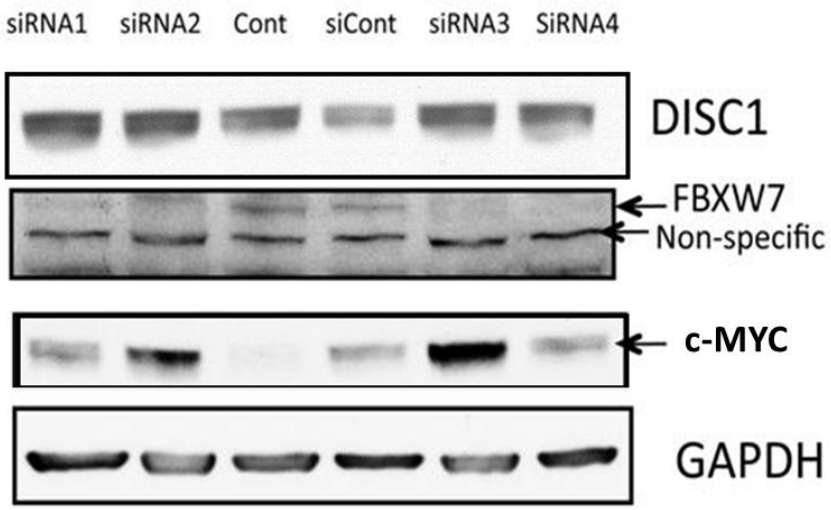


Figure 4.11 siRNA knockdown of Fbxw7 stabilises DISC1 and known substrate, c-Myc – Tertiary screen
HEK293 cell cultures were individually transfected with 4 different siRNA (which constitute the SMART Pool used in the Fbox library screen) targeting different regions on Fbxw7 transcript. DISC1 (protein of interest), Fbxw7 and c-Myc (known Fbxw7 substrate) and GAPDH (loading control) expression levels were evaluated using anti DISC1, anti Fbxw7, anti c-Myc and anti GAPDH antibodies respectively.

4.3.6 Fbxw7/Cdc4 – A Novel interacting partner of DISC1

The siRNA screen detailed earlier provided us with a valuable putative novel interacting partner and E3 ligase for DISC1, Fbxw7. Most of the literature discusses Fbxw7 as a popular cancer drug target and it is interesting to note that many of the characterised Fbxw7 substrates are oncogenes such as c-Myc and c-Jun (Tu et al., 2012, Wang et al., 2012b). Very few studies were conducted to understand the physiological role of Fbxw7 protein in brain. Mice with brain region specific knockdown of Fbxw7 were generated and studies show that Fbxw7 is crucial in regulating neural stem differentiation (Hoeck et al., 2010a). Histological studies on mice with cerebellum specific Fbxw7 deletion revealed reduced Purkinje cell density and a smaller sized cerebellum at P2 (Jandke et al., 2011). Immunohistochemical studies show DISC1 staining of cerebellum, predominantly in Purkinje cells (Schurov et al., 2004a). Fbxw7 was also implicated in the regulation of neural stem cell differentiation via the Notch signalling pathway (Hoeck et al., 2010a).

Hence, we found Fbxw7 to be an interesting putative E3 ligase protein to investigate further.

To validate the results of the Fbxw7 siRNA screen further, biochemical studies were carried out to validate the novel interaction. Co-immunoprecipitation (co-IP) experiments were carried out to isolate a complex of Fbxw7 and DISC1. Due to the unavailability of a suitable Fbxw7 antibody, the putative interacting partners were overexpressed in HEK293 cells. Initially, Flag - Fbxw7 and GFP, V5 - DISC1 were co transfected respectively in 2 different sets of experiments (Figure 4.12 a and b). Using Flag antibody as bait, Flag Fbxw7 immunoprecipitate complexes were collected and analysed for the presence of GFP DISC1 or V5 DISC1. Figure 4.12 a, 4.12b; show that DISC1 and Fbxw7 co-IP. This interaction was also validated by performing reciprocal co-IP, i.e., overexpressing Flag DISC1 and untagged - Fbxw7 followed by immunoprecipitation of Flag DISC1 and probing for the presence of Fbxw7 (Figure 4.12c). Control co-IP experiments were carried out in parallel using IgG antibody which precipitates neither of the interacting partners in all the three different sets of experiments (Figure 4.12).

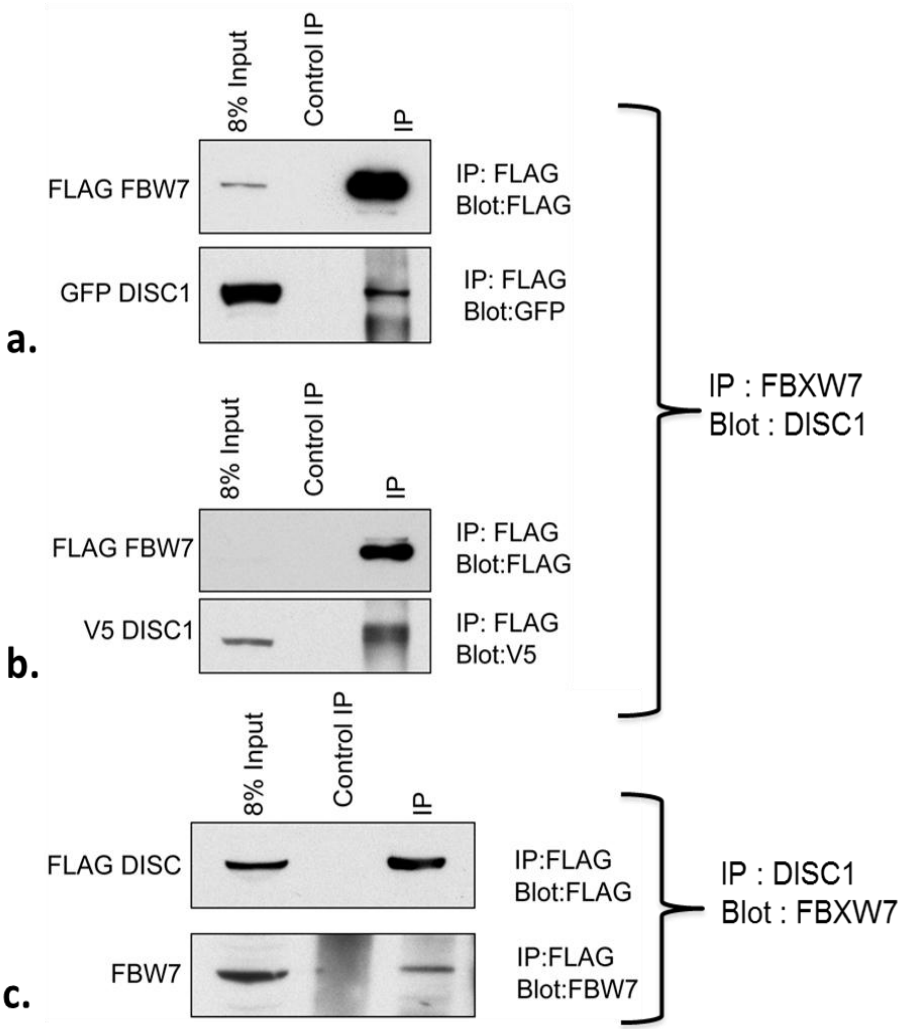


Figure 4.12 Immunoblots showing association of Fbxw7 and DISC1
Representative immunoblot of DISC1-Fbxw7 co-IP. Lysates of HEK293 cells co-expressing - Fbxw7 and DISC1 were immunoprecipitated on to Flag tagged agarose beads. (a) Flag - Fbxw7 and GFP - DISC1 : IP antibody-Flag. (b) Flag - Fbxw7 and V5 - DISC1: IP antibody Flag. (c) Untagged- Fbxw7 and Flag - DISC1 : IP antibody Flag. First lanes in all the blots are 8% input which correspond to the 8% of cell lysate from which the immunoprecipitation was performed (positive control). Second lane represents IP with IgG antibody which serves as a negative control. Third lane represents IP carried out with Flag tagged agarose beads. This work was carried out by Dr. Jon P Day, Gardiner Laboratory, University of Glasgow.

Next, we examined the ability of Fbxw7 to regulate DISC1 protein levels under conditions of translation inhibitor to determine whether Fbxw7 could affect the turnover of DISC1. Cyclohexamide (CHX) is a reversible protein translation inhibitor which exerts its function by blocking the translation elongation step. Flag DISC1 and Fbxw7 were overexpressed in HEK293 cells following by treatment with CHX or DMSO (vehicle) at 50µg/ml concentration for indicated time points and cell lysates were tested using immunoblots.

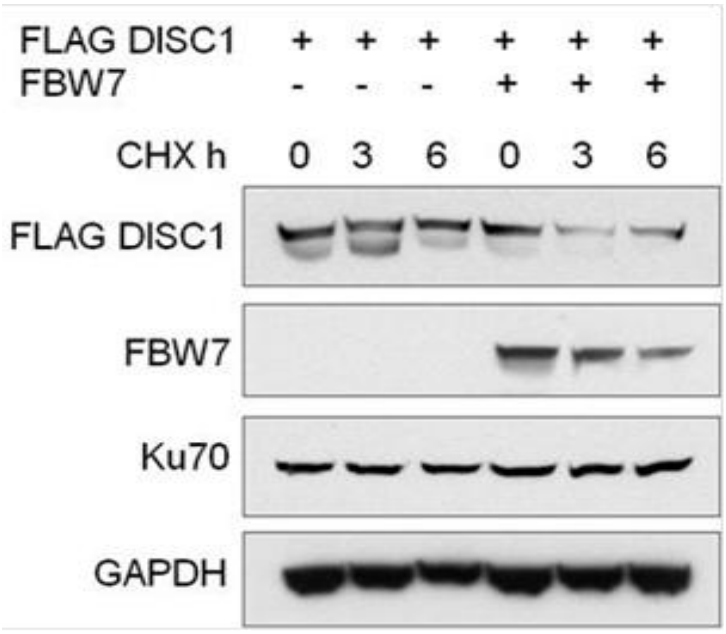


Figure 4.13 Effect of Fbxw7 over-expression on the stability of Flag-DISC1
Flag - DISC1 was co transfected in HEK293 cells with untagged Fbxw7 or an empty vector (negative control). Cells were treated with Cyclohexamide (CHX) at indicated concentration and time and cell lysates are analysed by Western blotting technique using anti DISC1, Fbxw7 antibodies. Ku70 and GAPDH protein expression levels serve as loading controls as they are stable under Cyclohexamide treatment. n=2
This work was carried out by Dr. Jon P Day, Gardiner Laboratory, University of Glasgow.

The decrease in DISC1 levels, induced by CHX, was exacerbated following Fbxw7 expression, as suggested by preliminary studies (Baillie laboratory). This implies that the E3 ligase complex with Fbxw7 promoted the degradation of DISC1. Other proteins which are known for their stability under CHX treatment (Ku70 and GAPDH) were unaffected by either CHX treatment or Fbxw7 expression. These data further validate my hypothesis that Fbxw7 is the E3 ligase for DISC1 (Figure 4.14). These data were supported by the observation that both proteins co-purify in DISC1 immunoprecipitations (Figure 4.12).

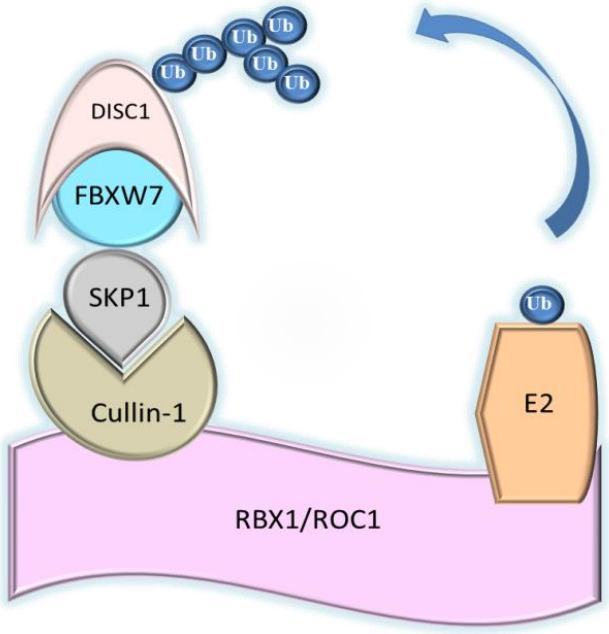


Figure 4.14 Schematic of proposed Fbxw7 (SCF^{Fbxw7}) mediated ubiquitin conjugation of DISC1.
From my study, with the results we have so far I hypothesise that Fbxw7 is the Fbox component of the SCF complex (Skp1, Cullin, RBX1 and Fbox) that orchestrates the ubiquitin transfer from the E2 enzyme to a specific lysine residue on the DISC1 protein.

4.3.7 Fbxw7 - DISC1 interaction is mediated by Cdc4 phosphodegron (CPD)

Substrate degradation by SCF^{Fbxw7} E3 ligase complexes is triggered by a phosphorylation event within a conserved motif known as a CPD (Cdc4 phosphodegron) which physically interacts with Fbxw7 (Orlicky et al., 2003). Substrates such as Cyclin E can have more than one CPD, though they have variable affinities towards SCF binding (Nash et al., 2001). Screening of the DISC1 protein sequence revealed the presence of a putative CPD. Comparison of the DISC1 CPD shows a close resemblance with that of other known characterised Fbxw7 substrates (Figure 4.15). The DISC1 CPD and its interaction with Fbxw7 were further characterised using structure based studies, detailed in chapter 5.

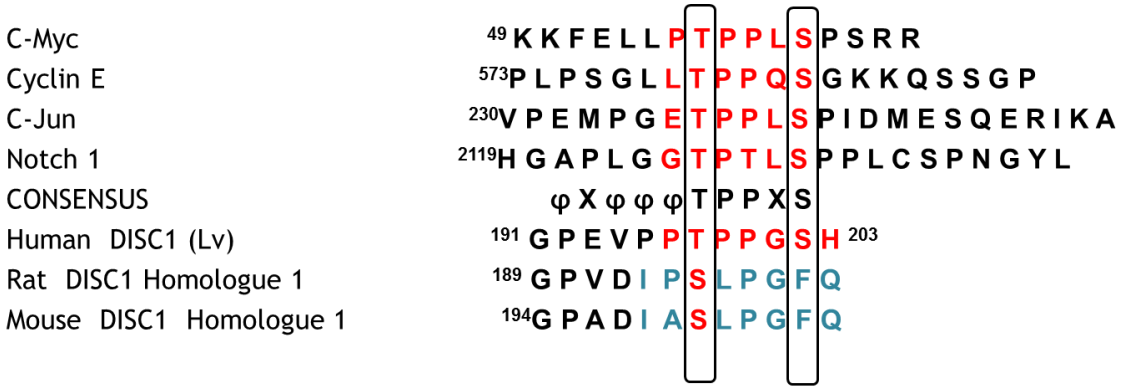


Figure 4.15 SCF^{Fbxw7} substrates with Cdc4 conserved CPD motif, consensus sequence and putative DISC1 CPD.
Boxed sequences represent Threonine and Serine residues which upon phosphorylation recruits the SCF complex for ubiquitination. φ indicates any amino acid.

In order to probe the putative interaction between DISC1 CPD consensus and the SCF Fbxw7 complex, we utilised peptide array technology which is a core technique within the Baillie lab. Peptide libraries consisting of 25-mer peptides containing the DISC1 CPD (with and without phosphorylation on the Thr ^{198} and and Ser ^{202} residues) were synthesised along with a series of amino acid truncations from C terminal and N terminal ends of the peptide. Purified His tagged Skp1-Fbxw7 complex was overlaid onto the peptide arrays and areas of positive interaction were detected with an anti-His antibody (Figure 4.16).

Gratifyingly, robust interaction between the Skp1-Fbxw7 complex and DISC1 CPD consensus was detected. Various characteristics of the interaction site were disclosed through these studies. First, dual phosphorylation within the CPD (Thr^{198} and Ser ^{202}) increases the interaction with the E3 ligase *in vitro*. Secondly, additional binding sites may be provided by the residues at the amino terminal to the CPD such as Valine and Proline. Finally, a strong *in vitro* interaction was achieved by the smallest peptide (showing positive interaction), a 7-mer 'P-pT-P-P-G-pS-H' (spot 31 in Figure 4.16) which is hypothesised to be the minimal interacting surface on DISC1 CPD for successful association with the E3 ligase (Figure 4.16).

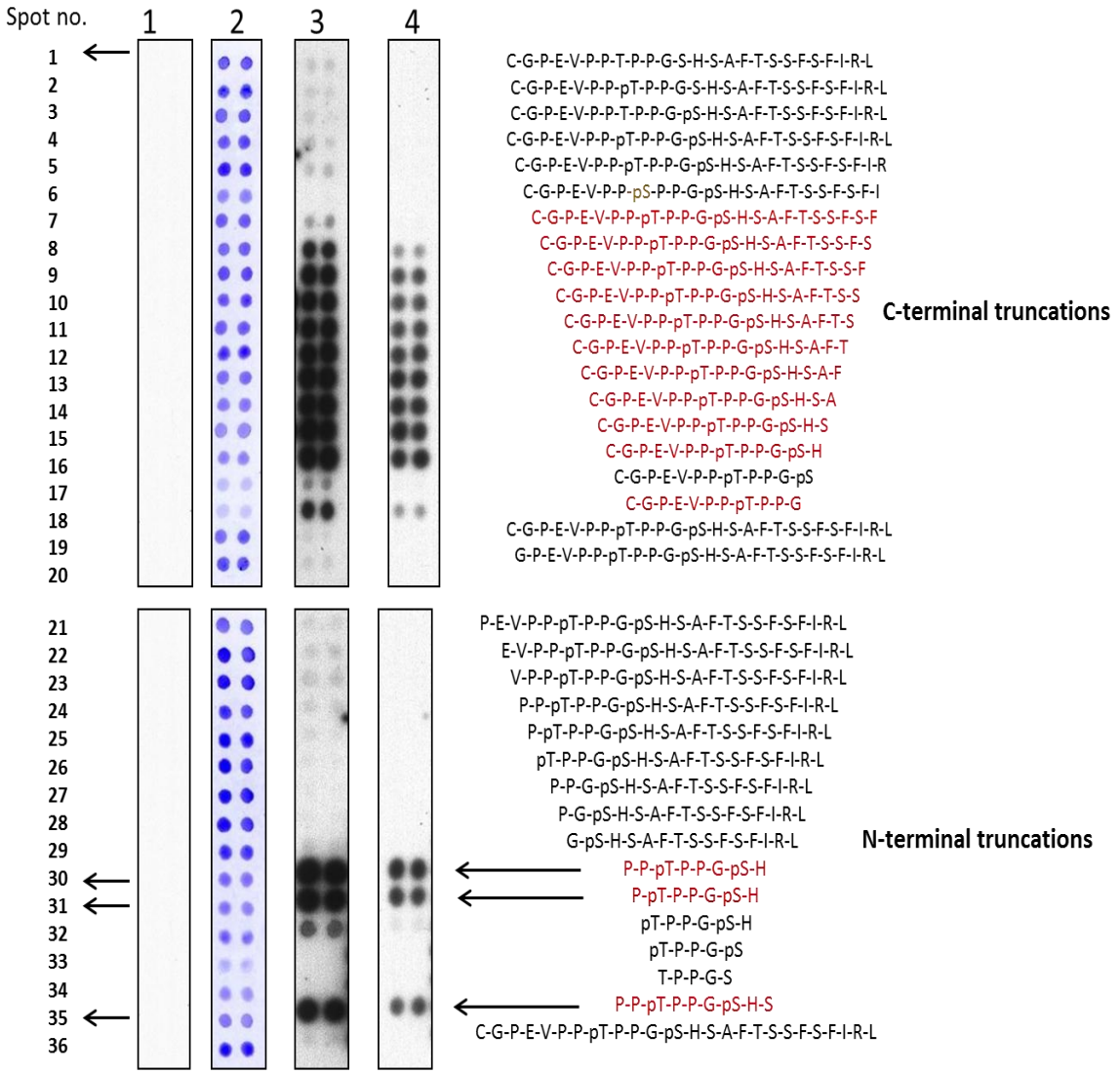


Figure 4.16 Peptide array analysis of DISC1 – SCF^{Fbxw7} interaction through the CPD motif on DISC1.

A DISC1 peptide library consisting of CPD motif and sequentially truncated N terminal and C terminal CPD peptides were spotted on a membrane and analysed for SKp1-Fbxw7 binding using recombinant protein complex. Peptide array analysis is described in materials and methods section 2.5. His tag antibody was used to probe for the interaction. Lanes 1 – Negative Control, probed with antibody only, 2- Coomassie stained array showing the presence of peptides, 3- long exposure of the test array which was overlaid with SCF^{Fbxw7} protein followed probing with His tag antibody. 4- shorter exposure of the above test array. Peptides showing a strong positive interaction were highlighted in red, with arrows marked to the corresponding spots.

4.4 Discussion

4.4.1 Proteasomal regulation of DISC1 protein

The two main proteolytic pathways of mammalian cells, the lysosomal pathway and the UPS, play an important role in maintaining cellular homeostasis of proteins. The role of UPS in neurological disorders has been reviewed in section 1.5.4. Earlier studies have proposed immunocytochemical staining for ubiquitin and its conjugates as a marker for neuro-degeneration (Lowe et al., 1988). These were later identified as ubiquitin rich inclusion bodies (IBs) enriched with deposits of ubiquitin conjugated misfolded proteins. These accumulated proteins may lead to cellular stress and subsequent cell death. IBs are a hallmark of neurodegenerative illnesses such as Alzheimer's disease. Although so far there has been no report of IB formation by DISC1 protein, DISC1 aggregate formation has been widely reported in various cell types (Atkin et al., 2012). The role of these aggregates in DISC1 associated diseases still remains unclear.

My study focused on how DISC1, a putative susceptibility factor of SCZ, is regulated in cells, i.e. what controls the protein levels of DISC1? Initial studies involved characterisation of DISC1 degradation kinetics in the presence of the 26S proteasome inhibitor, MG132. DISC1 was shown to be stabilised and ubiquitin conjugated upon inhibition of its proteasomal degradation. Mass spectroscopy analysis further strengthened my suggested hypothesis that ubiquitin conjugation directs DISC1 proteasomal targeting.

My biochemical studies show the presence of K48 and K63 type ubiquitin chains on DISC1. Antibodies which detect other types of ubiquitin chains (such as K3, K11) were unavailable at the time of this study. Conjugated K48 linked ubiquitin chains are believed to be the signal for proteasome degradation, while conjugation of K63 linked ubiquitin chains is known to serve non proteolytic roles such as such as gene expression regulation and inflammatory responses (Xu et al., 2009). Tetra ubiquitin has been identified as a minimum (shortest) signal required for proteasomal targeting (Ciechanover and Ben-Saadon, 2004). It has been suggested that specific receptors on the 19S complex of the 26S proteasome have the potential to recognise the poly ubiquitin chain assembly formed

by K48 linkage. It has also been proposed that longer ubiquitin chain length may increase the residence time of the substrate. However, the exact mechanism by which the length of ubiquitin chain influences the fate of ubiquitinated substrate has been a long standing debate (Ciechanover and Ben-Saadon, 2004). A Variety of linkages, inter linkages between chains and lengths which in turn assume various confirmations were all known to contribute to the complexity and specificity of the ubiquitination of a substrate and dictate the physiological response (Fushman and Wilkinson, 2011). Though the Mass spectroscopy studies performed in the present study uncovered a putative ubiquitination site on human DISC1 (K372), the type of ubiquitin chains nor the length could not be verified due to the moderate quality of the samples. More investigation to uncover the type of ubiquitin chains would be useful in understanding the physiological consequences of chain formation on DISC1. Technical and biological replicates of Mass spectroscopy studies would be useful, but were not within the scope of this project.

The immunoprecipitation data on ubiquitination of DISC1 K372R mutant suggest reduced ubiquitin conjugation, if not completely abolished. This can be attributed to the fact that substrates might have one or more ubiquitination targeted lysines. Ubiquitin conjugation at different lysines on a substrate may be spatio temporally regulated as well as having different physiological responses (Grabbe et al., 2011). Other experimental studies which may be useful in the further characterising ubiquitination of DISC1 are ones involving expression of an ubiquitin 'lysineless' mutant (Ubiquitin(K0)), in which all the seven lysine residues are mutated to arginine residues. This mutant terminates ubiquitin chain formation, and therefore stabilises the substrate preventing its proteasomal targeting. These mutants should alter the pattern of slow migrating bands on a Western blot as a result of less ubiquitination or shorter chain formation. Lysine at specific sites on ubiquitin can also be mutated to arginines such as K48R or K63R. Overexpressing such mutants would validate the type of chain formed on the substrate protein.

As discussed earlier, the extreme short life of ubiquitin modified substrates due to the reversible nature of the modification (deubiquitination catalysed by DUBs) poses several challenges in biochemical and biophysical characterisation of ubiquitin conjugated

substrates. Although cysteine protease inhibitors (example: Iodoactemide and NEM – N-ethyl maleimide) have been widely used to inhibit DUBs, they were shown to interfere with mass spectroscopy studies (Nielsen et al., 2008). Recent studies have focused on developing strategies to overcome this issue and facilitate a more reliable detection and characterisation of the protein ubiquitination. Tandem-repeated ubiquitin-binding entities (TUBEs) are an example which was developed based on ubiquitin-associated (UBA) domains. TUBEs specifically recognize, interact and thereby protect the tetra ubiquitin by interacting with a higher affinity than individual UBA domains (present on DUBs). TUBEs therefore function as molecular traps and were described as valuable tools for studying PTMs (Hjerpe et al., 2009).

As discussed earlier, many studies have shown that loss of DISC1 expression (RNAi knockdown of DISC1) results in abnormal brain development and function (Niwa et al., 2010, Faulkner et al., 2008, Young-Pearse et al., 2010). Retroviral mediated shRNA knockdown of DISC1 shows that it is a key factor regulating neuronal maturation in adult brain (Faulkner et al., 2008). DISC1 knockdown studies in Zebra fish have revealed abnormal oligodendrocyte and cerebellar neuronal development. RNAi mediated loss of DISC1 expression has also affected neurite outgrowth in PC12 cells (Miyoshi et al., 2003b). All these studies show that the loss of DISC1 function may be critical in the underlying pathophysiology of DISC1 associated psychiatric illnesses. I see this uncovered novel PTM on DISC1 as initial step towards understanding these disease mechanisms, and more investigation and characterisation of this modification would provide more avenues for therapeutic intervention.

4.4.2 Fbxw7 dependent degradation of DISC1

My screening studies uncovered a novel interacting partner for DISC1 i.e., Fbxw7. It is interesting to note that none of the yeast 2 hybrid screens on DISC1 have identified protein as a candidate interacting partner (Camargo et al., 2007). This may be attributed to the transient nature of the DISC1-Fbxw7 interaction - many of the interactions that characterise ubiquitination are extremely short lived. It may also be that a prior post

translational modification such as phosphorylation is required for this interaction, as suggested by my study.

Literature mining revealed that Fbxw7 is mutated in several different types of cancers and its inactivation leads to chromosomal instability (Jandke et al., 2011, Rajagopalan et al., 2004). Recent studies have implicated Fbxw7 role in stem cell differentiation. Fbxw7 sustains hematopoietic stem cell quiescence and regulates their differentiation by regulating transcription factors Notch, c-Jun and mTOR (Reavie et al., 2010, Iriuchishima et al., 2011). Fbxw7 is crucial in neuronal stem cell maintenance and differentiation in brain and is known to exert this function by controlling the abundance of Notch proteins (Hoeck et al., 2010a, Matsumoto et al., 2011). Fbxw7 has been reviewed in section 1.5.3.5.

Several Fbox proteins recognise a short motif within a substrate which upon phosphorylation facilitates recognition and interaction by Fbox protein, known as CPDs (Ye et al., 2004). Many of these proteins have been crystallised with their respective phosphopeptides. While some substrates have only one CPD, many other substrates such as Sic1 and Cln2 have multiple degrons dispersed across the protein sequence. My peptide array studies involving sequential truncation from the C terminal and N terminal ends of the DISC1 CPD also identified the minimal interacting region of Fbxw7 on DISC1 within the CPD (Figure 4.16). As discussed earlier, as in the case of most of the other Fbxw7 substrates, a phosphorylation event followed by an isomerisation might be crucial in triggering this interaction (Zhang and Koepp, 2006, Hao et al., 2007b). Hence, peptide array studies were used to further validate this hypothesis of Fbxw7 mediated DISC1 ubiquitination. As seen in Figure 4.16, SKp1-Fbxw7 complex specifically shows strong interaction with DISC1 CPD peptides carrying dual phosphorylation ($Thr^{198}$and Ser^{202}). This is an important finding which aligns with the observations made by other research groups in other known Fbxw7 substrates. As can be seen in Figure 4.15, DISC1 CPD consensus has a close similarity with Cyclin E's CPD motif. Cyclin E (regulates cell cycle transition from G1 to S phase) is a well characterised Fbxw7 substrate whose degradation is triggered by phosphorylation within multiple degron motifs (Orlicky et al., 2003, Ye et

al., 2004). *In vitro* studies employing Cyclin E CPD peptides and site directed mutagenesis studies have identified doubly phosphorylated degron (Thr^{380}/Ser^{384}) as a high affinity motif and singly phosphorylated degron (Thr^{62}) as a lower affinity motif. More studies are to follow in the next section to further characterise the DISC1 CPD motif. Interestingly the identified CPD is not conserved in mouse and Rat DISC1 homologues (Figure 4.15).

Another noteworthy aspect of Fbxw7 is its isoform specific regulation of its substrates (Grim et al., 2008). As reviewed in section 1.5.3.5, three splice variants of Fbxw7 have been characterised – α, β and γ isoforms, which vary at their amino terminal end. Fbw7-α is predominantly nuclear; Fbw7-β is cytoplasmic; and Fbw7-γ is reported to be localised in the nucleolus (Zhang and Koepp, 2006). Their differential localisation may also dictate their isoform specific physiological role (Welcker et al., 2004). For example, it has been demonstrated that p53 is regulated by the cytoplasmic β isoform (Galli et al., 2010), The nucleolar pool of Fbxw7-γ regulates c-Myc (Welcker et al., 2004) and different degrons with in Cyclin E were shown to differentially interact with three Fbxw7 isoforms (Zhang and Koepp, 2006). The Fbxw7 siRNA used in the Fbox screen was designed to target a common region for all the three isoforms. Fbxw7-γ isoform is absent in brain tissue (embryonic and adult), β isoform is moderately expressed, while the α isoform is the most abundant and ubiquitously expressed isoform of Fbxw7. α isoform is abundant in neuronal stem cells and progenitors ((Hoeck et al., 2010a) (Akhoondi et al., 2010, Grim et al., 2008) and HEK293 cells (cell line used in the present study). Hence I chose to investigate the Fbxw7-α isoform interaction with DISC1 by performing Immunoprecipitation studies. However, the involvement of the other isoforms involvement in DISC1 regulation may not be ruled out and requires further investigation. Positive interaction between FBXW-α isoform and DISC1 may also suggest that DISC1 could be localised in the nucleus, supporting its known centrosomal association (Morris et al., 2003).

The following studies may shed light on the Fbxw7 and DISC1 interaction, and were not within the scope of the present study:

1. Investigating DISC1 – Fbxw7 interaction studies (immunoprecipitation, pull down assays, immunostaining techniques) and Isoform specific knockdown of Fbxw7 and its effect on DISC1 ubiquitination would be useful in understanding if this interaction is isoform specific.

2. The developmental expression profile of DISC1 protein in mice reveals that the 100kDa form peaks at E13.5 and P35, which corresponds to periods of active neurogenesis, supporting its role in active brain development (Lipska et al., 2006a). In humans, the expression is predominant in hippocampus, while the expression is stronger in dentate gyrus in early childhood and gradually rapidly declines with age into adulthood (Lipska et al., 2006b). Fbxw7 has also been implicated in cerebellar development and neuronal stem cell differentiation. However, the expression profile of Fbxw7 in human brain has not been explored yet. It would be intriguing to investigate any expression based inverse correlation between these two proteins in normal subjects and psychiatric patients in the context of my study showing that DISC1 levels are regulated by Fbxw7. This also supports the neurodevelopmental hypothesis of SCZ.

3. GSK3β has been known to be functionally associated with Fbxw7 (source kinase for its substrates). GSK3β is a crucial interacting partner of DISC1 and studies in zebrafish, mouse model systems and mammalian cell lines show that DISC1 variants (A83V, R264Q, and L607F) could not activate Wnt/GSK3β signalling. In this context, it would be interesting to assess the potential of GSK3β to directly phosphorylate DISC1 within the CPD. However, this might prove challenging, as in general, a priming phosphorylation event is required for GSK3β to phosphorylate its substrates.

4. It would be also interesting to investigate how DISC1 common variants and t (1; 11) translocation (truncated DISC1) affect DISC1 stability in cells, which may explain the molecular consequences of the underlying disease mechanism.

4.5 Chapter summary

To summarise this chapter's results, I have provided novel and previously undiscovered information on the regulation of DISC1 turnover in HEK293 cells. The data presented in this chapter indicate that DISC1 protein levels are regulated by ubiquitin mediated proteasomal degradation. Mass spectroscopy studies identified K^{372} as a potential acceptor site while my biochemical data suggest the presence of more acceptor lysine residues at other sites. This is a novel PTM identified on DISC1 and we speculate that this modification has a potential role in the DISC1 pathway. I also propose that DISC1 ubiquitination may play a role in the mechanism of all DISC1 associated neurodegenerative illnesses, as seen in Alzheimer's and Parkinson's diseases where UPS dysfunction has been reported. My studies also uncovered Fbxw7 as a novel interacting partner of DISC1 which regulates DISC1 protein levels by catalysing its ubiquitination as a functional SCF component.

In the following chapter, my studies focused on targeting this newly uncovered PPI (protein- protein interaction) with inhibitors to modulate DISC1 protein levels, exploring a new avenue for therapeutic intervention.

5.

Development of disruptor peptide to modulate DISC1-SCF^{Fbxw7} complex interaction

5.1 Introduction

Association studies, linkage studies and mouse models support DISC1 as one of the most promising candidate susceptibility factors for SCZ and other major mental illnesses (reviewed in detail in (Chubb et al., 2008)). Transgenic mice expressing a truncated dominant-negative form of DISC1 (Tr DISC1), displayed several anatomical and behavioural abnormalities consistent with those observed in patients with SCZ (Hikida et al., 2007, Clapcote et al., 2007, Koike et al., 2006). It has been hypothesised that DISC1 SNPs and mutations such as that of the Scottish C terminal truncation may lead to loss of DISC1 function, which may directly or indirectly contribute to the pathophysiology of psychiatric illnesses. It has been difficult to study human DISC1 protein as the DISC1 protein levels in the lymphoblasts collected from the patients of the Scottish pedigree were shown to be too low to be detected by Western blotting technique. These low protein levels may be attributed to the instability or haploinsufficiency of TrDISC1 (Millar et al., 2005b, Sawa and Snyder, 2005).

To date, there is no evidence that the DISC1 truncated protein (TrDISC1) is translated in lymphocytes in which the translocation has been identified (Scottish translocation) (Millar et al., 2000b). However, there are a few hypotheses for how DISC1 mutation can result in psychiatric disease. Firstly, it has been hypothesized that the translated protein product

might not be stable and is rapidly degraded (Millar et al., 2005b). In a different scenario, the truncated protein may be stable but may interfere with the function of the wild type protein product from the other allele, resulting in loss of function known as a dominant negative effect. Secondly, another hypothesis suggests that the DISC1 truncation could result in haploinsufficiency (definition of haploinsufficiency- both alleles need to be functional for a wild type functional genotype for a particular gene) (Thomson et al., 2005). My proposed project aims to address the DISC1 haploinsufficiency hypothesis and uncover the disease mechanisms involved.

Since DISC1 is ubiquitinated and the E3 ligase component Fbxw7 is crucial in regulating DISC1 protein levels, I hypothesize that a disruptor peptide/small molecule targeting the binding sites of DISC1 and Fbxw7 would abolish their interaction thus interfering with DISC1 ubiquitination and increasing its stability. I propose that the identified and mapped novel protein-protein interaction site could be a future drug target for SCZ and other associated psychiatric illnesses with underlying DISC1 mutations.

5.1.1 Protein – protein interactions (PPI) as drug targets

The present drug market is mostly full of competitive small molecule inhibitors of various enzymes, receptors and ion channels, and over the years the number of targets has dwindled, whilst the developmental costs have soared. Protein-Protein interfaces of large molecular complexes have become attractive drug targets as they have an added advantage of extra specificity and can be easily identified through biochemical co-purification (Arkin and Wells, 2004, Chène, 2006). Until recent years, they have been considered to be 'undruggable' and complex targets. With drugs belonging to this new class already on the market such as Tirofiban and Maraviroc and many others advancing in clinical trials, there has been increasing scope to exploit this new class of drug targets (Vlieghe et al., 2010). In essence, PPI inhibitors represent the next generation of drugs and more of their kind are en route to the market in the near future. PPIs are involved in a wide array of disease pathways that have become attractive drug targets lately, and the ongoing elucidation of the human interactome is opening up avenues in this field of drug discovery. The human interactome has been estimated to have approximately 650,000

interactions, providing an excellent source to develop novel targets for therapeutic drug development (Chène, 2006, White et al., 2008). Having overcome initial technical hurdles and experimental difficulties for characterising PPI drug targets, this class of therapeutic intervention has gathered considerable pace.

5.1.2 DISC1 – Fbxw7 interaction as a drug target

It is estimated that nearly 80% of cellular proteins undergo irreversible ubiquitin conjugated proteolysis by mediation of substrate specific ubiquitin E3 ligases, in response to specific stimuli (Bernassola et al., 2010, Varshavsky, 1997). Most of the characterised E3 ligases are modular protein complexes constituting a core scaffold unit with switchable substrate targeting subunit (Joazeiro and Weissman, 2000, Kipreos and Pagano, 2000). Cullin RING ligase (CRL) ligases, also known as SCF (Skp1-Cullin-Fbox) complexes, are the best characterised complexes which have such architecture (reviewed in 1.5.3.3). The crucial substrate recognition component of the SCF type E3 ligases is the Fbox protein (Skowyra et al., 1997). To date, only 69 human Fbox proteins have their multiple specific target substrates identified and it is hypothesised that there are a number of other Fbox proteins whose substrates are yet to be identified. One Fbox protein which has been implicated in the brain is Fbxw7, which plays a significant role in neural stem cell differentiation (Matsumoto et al., 2011, Wang et al., 2012a). Cultured neurospheres lacking Fbxw7 showed decreased neurogenesis and increased accumulation of cells expressing radial glial markers (Hoeck et al., 2010a). From the above studies, the role of Fbxw7 in neuronal proliferation and maturation is evident, though the precise mechanism is not elucidated yet.

The present study so far uncovered a novel interacting partner of DISC1, Fbxw7, which directly interacts and modulates DISC1 protein levels. This finding is highly significant in the wake of recent reports which identify dysregulation of ubiquitin in neurodegenerative diseases, such as Parkinson's disease, where α-synuclein rich Lewy bodies stain positive for ubiquitin and have been associated with dementia (Al-Mansoori et al., 2013). Since 'haploinsufiency' of DISC1 was one of the proposed disease mechanism of SCZ and other associated chronic mental illnesses (Hikida et al., 2012), the DISC1-Fbxw7 interaction is

considered by our group to be a novel druggable target, and my strategy was to stabilise DISC1 protein levels by disrupting the above complex and hence abolishing its subsequent ubiquitination.

5.2 Experimental aims

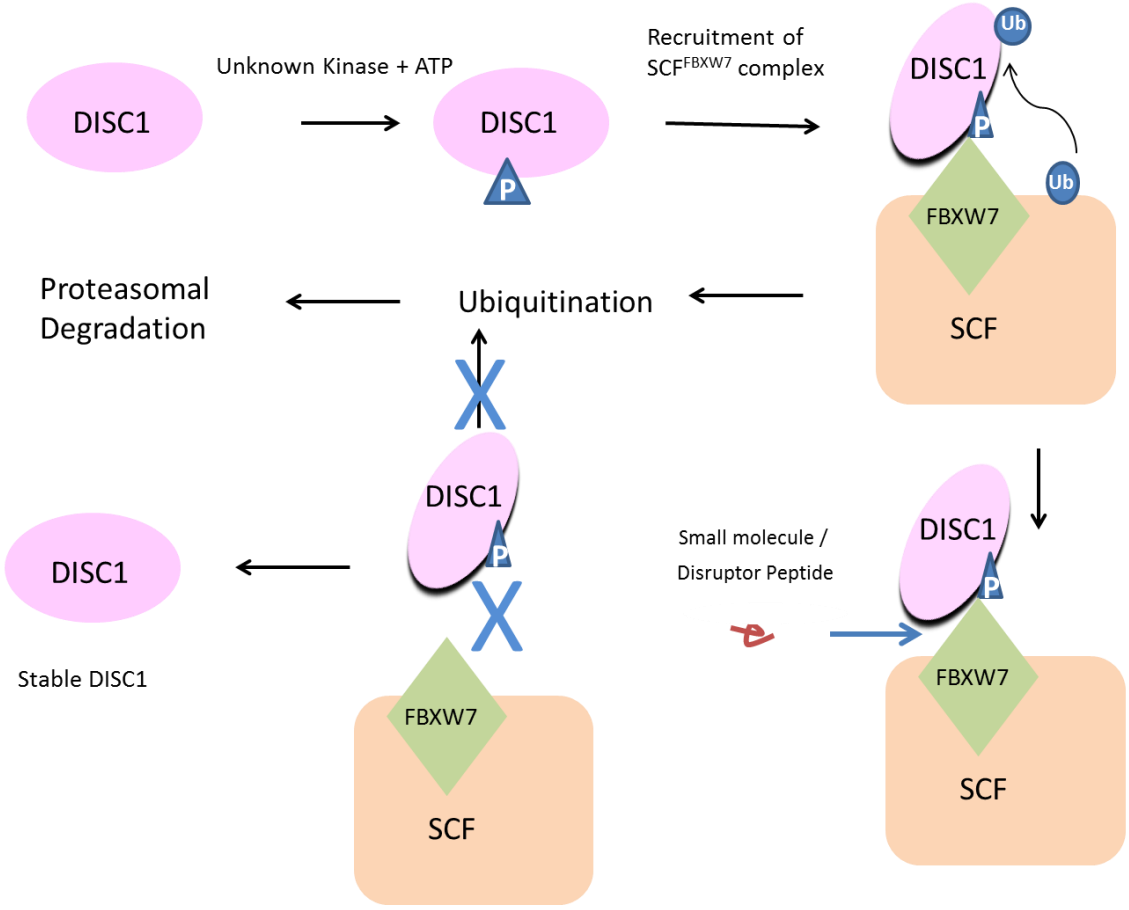


Figure 5.1 Schematic of the proposed hypothesis addressed in the present chapter DISC1 is ubiquitinated and the E3 ligase component Fbxw7 is crucial is regulating DISC1 protein levels. A disruptor peptide targeting the binding sites of DISC1 and Fbxw7 would abolish their interaction interfering with DISC1 ubiquitination and increasing its stability.

I propose that the identified and mapped novel protein-protein interaction site could be a future drug target for DISC1 associated mental illnesses. The developed disruptor peptide has the potential for development into DISC1 - associated neuropsychological disorder

drug treatment and a valuable tool to probe still uncovered functional roles of DISC1 in various signalling pathways and disease mechanisms.

5.3 Results

5.3.1 Peptide array based screening and optimisation of the putative disruptor peptide to increase its activity, specificity and stability

DISC1-Fbxw7 interaction was elucidated by peptide array fine mapping studies in chapter 4, and the precise CPD (Cdc4 Phosphodegron) motif on DISC1 protein was identified. In agreement with other Fbxw7 substrates, Fbxw7 interacts with DISC1 through a preceding phosphorylation event by a yet unknown kinase on a short and well defined motif. The shortest DISC1 CPD motif identified in the peptide array screens was similar to other known Fbxw7 substrates and was selected for further development as a tool to disrupt the protein complex.

Amino acid position	P1	P2	P3	P4	P5	P6	P7
Peptide	**P-pT-P-P-G-pS-H**						

Figure 5.2 Designated amino acid positions on the 7-mer peptide identified and shortlisted for developing a disruptor peptide.
This peptide will be termed as 'peptide T' from here on. pT and pS refer to phosphothreonine and phosphoserine respectively. The remaining amino acids were given single letter amino acid representation.

Peptide arrays have been considered a powerful tool not only to identify and characterise protein interactions but also to develop peptide ligands. In the present study, in-depth structure activity relationship studies (SAR) of peptides were performed in order to facilitate development of a lead peptide disruptor.

Criteria considered while optimising and developing the disruptor peptide were as follows:

1. Most of the naturally occurring amino acids are L isomers and the cellular proteases are specific to peptide bonds linking L amino acids. Therefore, substitution with D isomers of the amino acids increases the stability of the peptide in cells. Peptides flanked by non-natural amino acids also resist proteolysis by exopeptidases.

2. Since side chains of the amino acids play a crucial role in the specificity of enzyme – substrate complex formation, amino acids with capped or modified side chains serve as better substitutes, as they evade proteolysis.

3. When the amino acids are substituted, it is important to maintain the overall charge to mimic the natural peptide, so that the disruptor will act as a peptidomimetic with a higher affinity towards Fbxw7 interaction than that of the natural peptide (DISC1 CPD). Phosphomimetic amino acids such as Glu and Asp retain the charge while replacing the phosphorylated residues pT and pS.

4. These substitution studies also provide information on the role of each amino acid residue and whether the side chains participate in the interaction. Substitution with Ala, which has a methyl group as its side chain, eliminates side chain interactions while the peptide backbone confirmation is retained.

5. Pro with a cyclic side chain containing a nitrogen as a backbone atom, restricts protein confirmation and makes it preferential for inducing β turns in polypeptides sheets (MacArthur and Thornton, 1991). Hence, the amino acid substitutions considered should have a similar backbone while they meet all the above criteria, such as the non-natural amino acids - Pip, Tic, Sar.

6. Shifting the amino group on the peptide back bone, from the α carbon to the β carbon, increases the length of the peptide backbone and increases their stability, since β peptide bonds are non-natural.

7. While considering substitutions for His at P7 in the native peptide, amino acids such as lys and the non-natural amino acid ornithine were considered to retain the negative charge. With an imidazole ring in its side chain and its ability to exist in protonated and de protonated forms depending on the pH, His is known to be crucial in protein

interactions. Replacement with neutral amino acids (no charge on the side chain) such as Trp, Phe, Lys would determine if the side chain of His functions as a charge donor or is involved in pi-stacking (non covalent interactions between aromatic rings in proximity) which is crucial for the interaction under study.

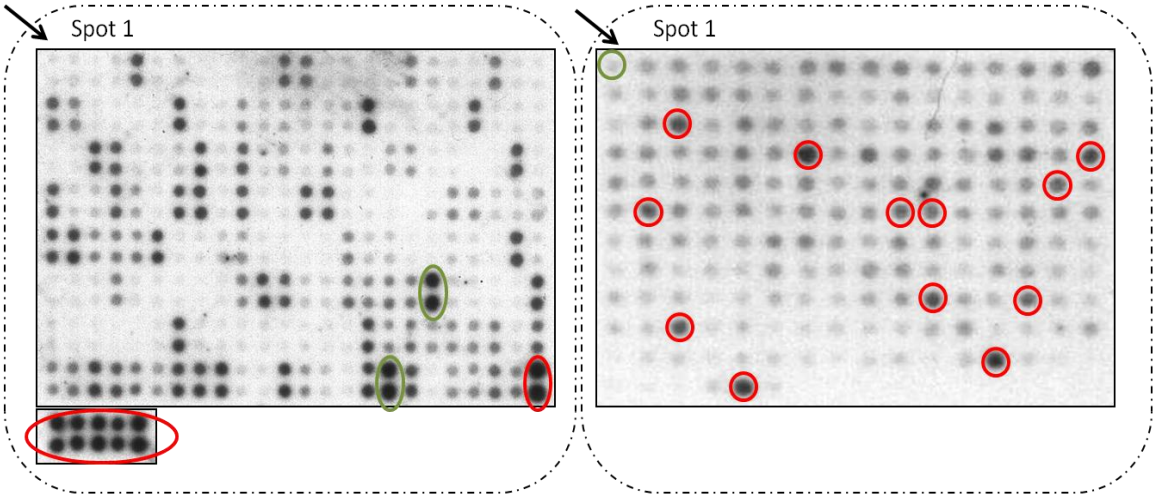
Table 5.1 Recommended amino acid substitutions for disruptor peptide optimisation

Amino acid position in the peptide	Native amino acid	Recommendations for optimisation – Round 1 (single letter representation)	Recommendations for optimisation – Round 2 (single letter representation)
P1	P	**I, L, G, a,** Pip, Tic, A, Sar, β-H-ala, βA	**G**
P2	pT	**A, a, D, E, d, e, mE, mD**	**D, E, d, e, mE, mD,** βE, βD, Q, N
P3	P	**p, G, A**, Sar, β-H-ala, βA, Pip, Tic	-
P4	P	**P, G, A, a,** Sar, β-H-ala, βA, Pip, Tic	-
P5	G	**a, P, Q, q**	-
P6	pS	**A, D, E, d, e**	**mE, mD, e,d, E,D,** βE, βD, **Q, N**
P7	H	**F, f, W, w, h, Y, y, K, k**	-

Non natural amino acids and those with capped/modified side chains are given 3 letter representation. mE – N methyl Glutamic acid, mE – N methyl Aspartic acid, Sar – Sarcosine, L-β-homoalanine, Pip - (S)-N-Fmoc-piperidine-2-carboxylic acid, Tic-N-FMOC-L-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid. Lower case and Upper case single letter represents the D and L enantiomers of the amino acids respectively. Amino acid (used in the optimisation experiments) structures can be found in the appendix. Based on the criteria discussed previously, the above recommendations were considered for developing the disruptor peptide for DISC1-Fbxw7 interaction. Amino acids highlighted in red were only considered as the rest of the amino acids were not available at the time of this study. The above recommendations were suggested by Dr Jeffrey Walton, University of Edinburgh.

To explore the interaction between the DISC1 CPD peptide and Fbxw7, and to develop shorter peptides with improved pharmacological properties, a peptide library was generated containing modified peptides (with substitutions described in Table 5.1). An array produced with this library was screened for interaction with SCF^{Fbxw7} (Figure 5.3 (a)).

Spot intensity was quantified and represented statistically by normalising it to the intensity of the control/native peptide (peptide T) (Figure 5.3 (b), (c)).



Peptide Optimisation part 1

Spot 192 : G-d-P-A-G-mE-K
Spot 193 : G-d-P-a-G-mE-k
Spot 194 : P-d-P-P-G-mE-K
Spot 195 : P-d-P-P-G-mE-k
Spot 196 : G-d-P-P-a-mE-k
Spot 197 : a-d-P-P-a-mE-k

Peptide Optimisation part 2

Spot 35 : P-E-P-P-G-d-H
Spot 55 : P-e-P-P-G-mE-H
Spot 64 : P-E-P-P-G-mD-H
Spot 79 : P-e-P-P-G-mD-H
Spot 82 : P-d-P-P-G-mE-H
Spot 139 : G-mE-P-P-G-mE-H
Spot 142 : G-mD-P-P-G-mE-H
Spot 147 : G-e-P-P-G-mE-H
Spot 173 : G-d-P-P-G-mE-H

Figure 5.3 (a) Peptide array studies to optimise and increase peptide stability and efficacy
A substitution peptide library was generated by substituting amino acids of the core sequence at various positions, as suggested in Table 5.1 above. The peptide library was spotted on a cellulose membrane and incubated with SCF^{Fbxw7} protein complex and probed for His tag with an anti His antibody, which is on the Skp1 component of the complex. Dark spots indicated positive interaction. The left panel represents the first round optimisation while the right indicates the second round. The peptides sequences selected in each round for further optimisations were highlited in red and the sequences were mentioned below the peptide array scans. Control spots were hightlighted in green. Replicate spots were omitted from the list. Control spots (control peptide P-pT-P-P-G-S-H) highlighted in green.

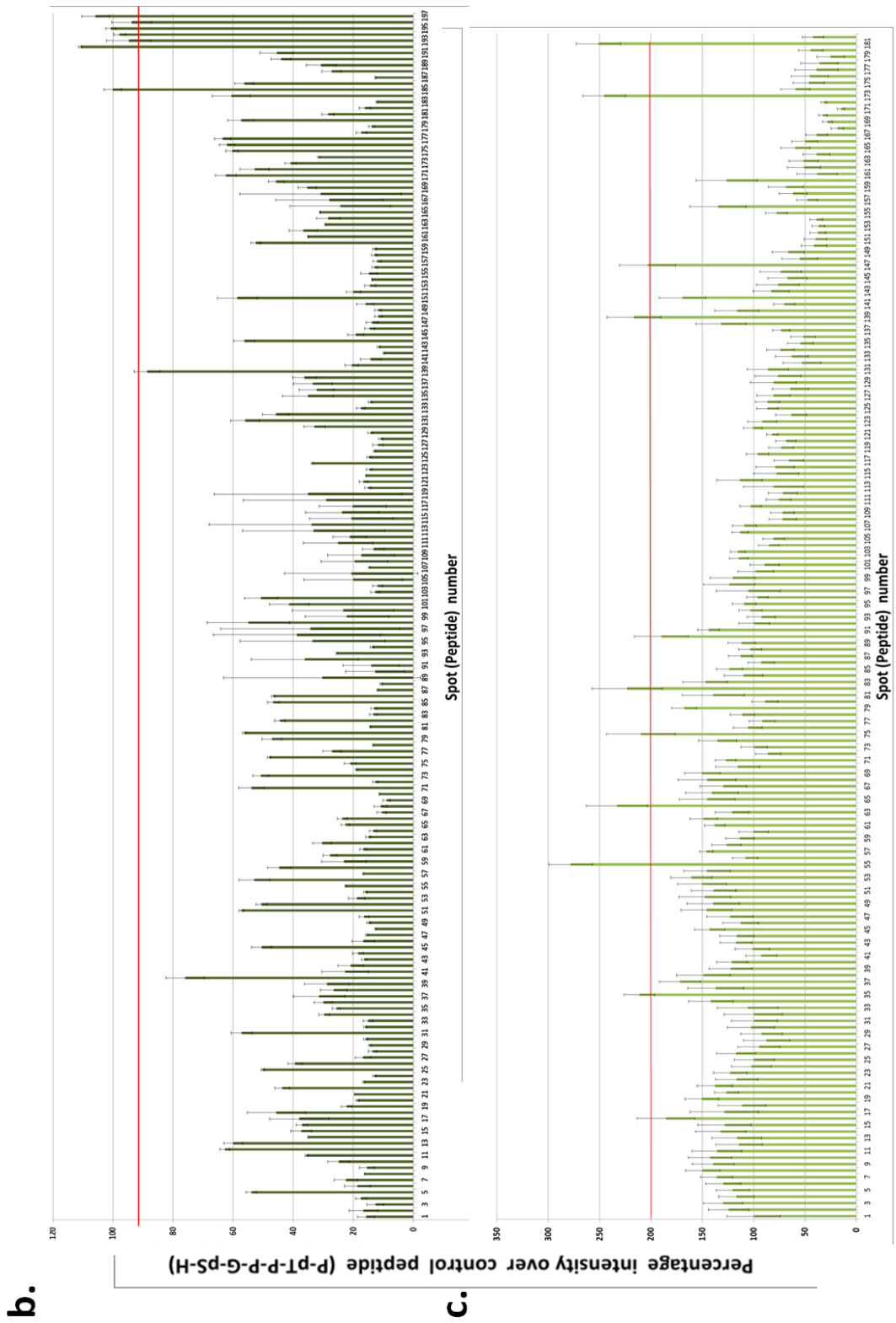


Figure 5.3 (b), (c) Optimisation to increase the efficacy of the disruptor peptide to modulate DISC1-SCF^{Fbxw7} interaction

(b) and (c) - Each spot intensity from the peptide arrays from Figure 5.3a was quantified using Image J and statistically represented as percentage intensity over that of the control spot (native DISC1 CPD peptide, Figure 5.2). Representative data from n=3. Values plotted as mean +/- S.E.M.

After the second optimisation round, peptides showing stronger positive interaction were identified and shortlisted (Figure 5.3a).

5.3.2 Stabilisation of endogenous DISC1 protein by the lead peptides in HEK293 cells

Subsequently, four peptides were selected for further investigation (Figure 5.4) based on the following criteria: (a) Peptides with Gly were selected because Gly at P1 retains the β turn while it replaces Pro in the natural peptide; (b) peptides with non-natural phosphomimetics at P2 and P4 were selected as they maintain the charge while they increase the stability of the peptide. From close observation of the sequence of the peptides, it was evident that the lead peptides selected were phosphomimetic in nature. This also strengthened the proposed hypothesis, which states that the phosphorylation within the DISC1 CPD triggers its association with Fbxw7 (as seen in other known Fbxw7 substrates). Previous work in our lab demonstrated that a stearate group facilitates peptide transport across the cell membrane, owing to its hydrophobic nature (Meng et al., 2009). N terminal stearated peptides (Figure 5.4) were custom made by Think Peptides®, ProImmune Ltd., UK. The peptides were synthesised using FMoc chemistry and purified to > 85% for further cell based testing. Peptides were quality tested using Mass spectroscopy and HPLC to confirm the mass and purity, respectively.

Native peptide: P-pT-P-P-G-pS-H
Peptide Control: St-**G**-**K**-P-P-G-**K**-H
Peptide 139: St-**G**-**mE**-P-P-G-**mE**-H
Peptide 142: St-**G**-**mD**-P-P-G-**mE**-H
Peptide 147: St-**G**-**e**-P-P-G-**mE**-H
Peptide 173: St-**G**-**d**-P-P-G-**mE**-H

Figure 5.4 Optimised putative disruptor peptides selected for testing in cell lines. Peptide sequence is represented from N terminal to the C terminal. St indicates stearated group at the N terminal end of the peptide.

The lead peptides obtained in lyophilised form were carefully resuspended in DMSO, aliquoted and stored at -20°C. The next step in the study was to determine whether the cell permeable stearated 7mer lead peptides stabilise/increase endogenous DISC1 protein levels in HEK293 cells. Initial studies included technical optimisations such as monitoring any possible cell toxicity, concentration of peptide, and the time of peptide treatment. HEK293 cells were treated with the stearated peptides at 100µM concentration for 3hr and cell lysates were analysed for changes in DISC1 protein levels by performing Western blotting.

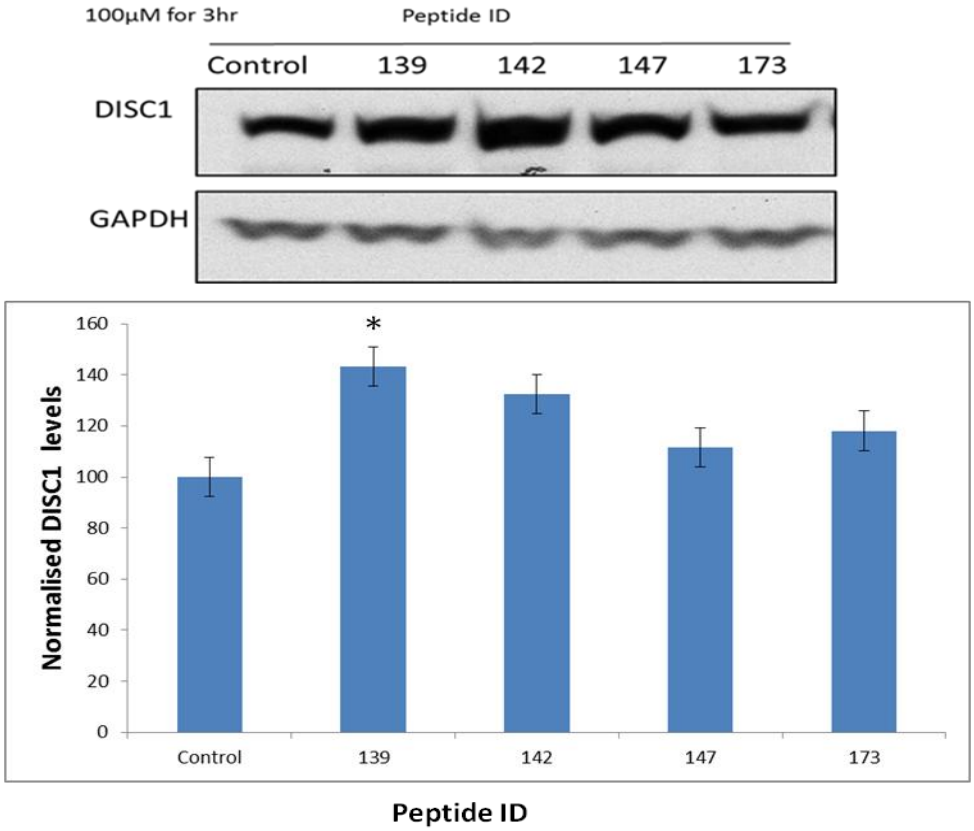


Figure 5.5 Preliminary screen of the lead peptides in HEK293 cells for DISC1 protein stabilisation.
Top panel: Immunoblot analysis of lysates from HEK293 cells treated with 100µM peptide for 3hr. DISC1 expression levels was analysed using anti DISC1 antibody and GAPDH antibody was used to probe for GAPDH expression which served as a loading control. Lower panel: Immune band intensities were quantified by densitometry and are presented as fold change in DISC1 expression normalised to GAPDH. S.E.M. of three biological repeats. Statistical significances between control peptide and each test peptide was calculated using one way analysis of variance (ANOVA). *P < 0.05 values were considered significant.

My preliminary peptide treatments have suggested that only peptide 139 and 142 treatment have stabilised DISC1 protein levels (Increased band intensity over control, determined by densitometric quantification of the blots) in HEK293 cells over control peptide treatments (Figure 5.5).

5.3.3 Application of peptides to cell lines resulted in stabilised endogenous DISC1 protein levels in a dose dependent manner

Peptides 139 and 142, now considered as 'lead peptides', were utilized to perform dose response experiments to determine the dose dependent effect of the peptides on DISC1 stabilisation in HEK293 cells. Cells were treated with the peptides at indicated concentrations for 3hr and cell lysates were subjected to Western blot analysis. To increase the accuracy of the Western blot quantification, the Licor Odyssey infrared imaging system which offers a wider linear dynamic range was used. This imaging system uses secondary antibodies conjugated with infrared fluorophores (Table 2.5), while the primary antibodies remains the same as used in chemiluminescent detection. As evident from Figure 5.6, DISC1 levels increased with increasing concentration of the test peptides.

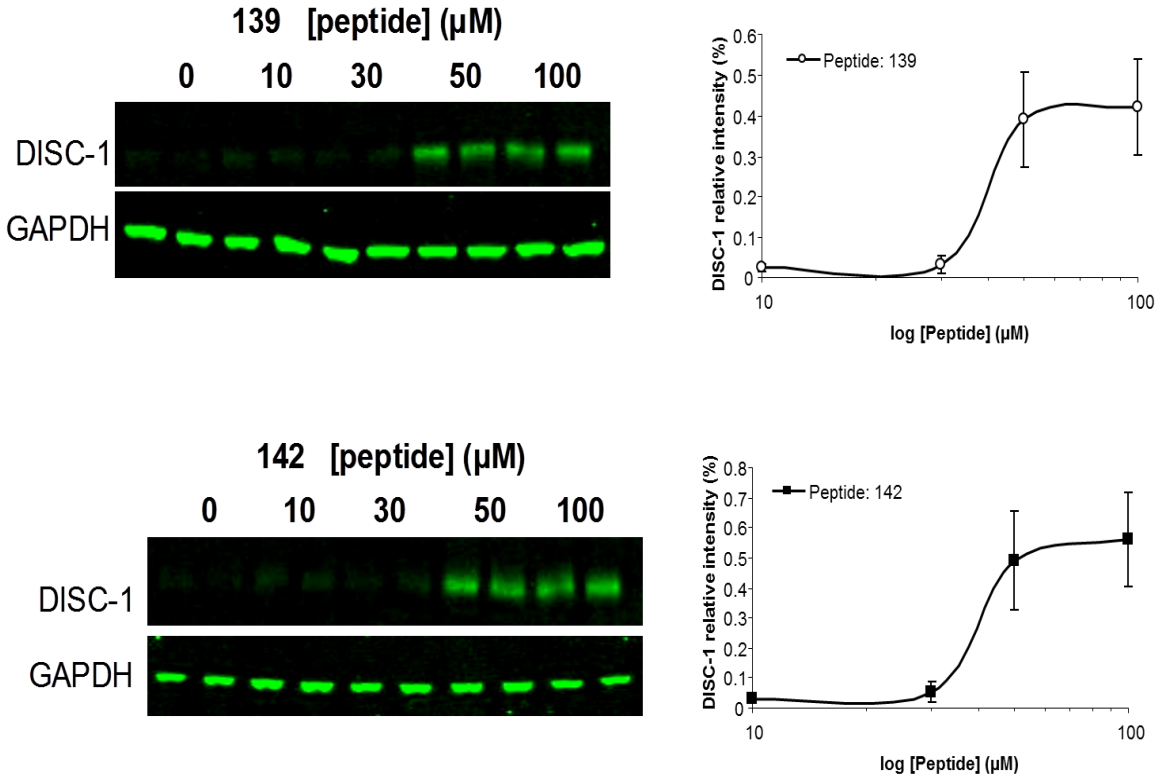


Figure 5.6 Lead peptides stabilised DISC1 protein levels in HEK293 cells in a dose dependent manner.

Peptides 132 and 149 were applied to HEK293 cells with a range of increasing concentrations and the cell lysates were subjected to immuno blot analysis. DISC1 and GAPDH were detected by probing with anti DISC1 and GAPDH antibodies respectively. DISC1 immuno band intensities were quantified by densitometry and presented as fold change in DISC1 expression normalised to GAPDH intensities and plotted on a logarithmic scale with peptide concentration on the X axis and band intensities on the Y axis. Data shown represent the mean ± SEM of 3 separate experiments.
This work was performed by Dr.Christina Elliot, Gardiner Laboratory, University of Glasgow.

5.3.4 The lead peptides specifically stabilised DISC1 while the other known Fbxw7 substrates protein levels remained unaltered

The most important factor to be considered in drug development is specificity. While the DISC1 –Fbxw7 complex is being considered as a drug target in my study, it should be noted that the interaction is via a consensus motif known as CPD or CPD (CDC4/Fbxw7 CPD Domain). This motif and the mechanism of regulation of Fbxw7 interaction has been well characterised with several other substrates implicated in neuronal differentiation, cell proliferation and cell viability such as Cyclin-E, (Hao et al., 2007a), c-Myc (Yada et al., 2004), Notch1 (Grim et al., 2006) and c-Jun (Hoeck et al., 2010a). Fbxw7, as a tumour suppressor gene, strictly regulates the turnover of the above oncogenes and functional disruption may dysregulate crucial physiological pathways and potentially leads to tumerogenesis (Tu et al., 2012, Wang et al., 2012a). Its functional inactivation by altered expression, gene mutations and other unknown mechanisms lead to impaired oncoprotein regulation. As described in Figure 4.15, human DISC1 CPD shares a close similarity with the CPD of other known Fbxw7 substrates. While peptides 139 and 142 stabilised endogenous DISC1 protein levels in HEK293 cells, it was important to determine that the disruptor peptide was specific for DISC1-Fbxw7 interaction and does not interfere with the Fbxw7 mediated regulation of other substrates. Peptide treated HEK293 cell lysates were probed for the expression pattern of Notch1, c-Myc, c-Jun and Cyclin E along with DISC1 using immunoblot analysis.

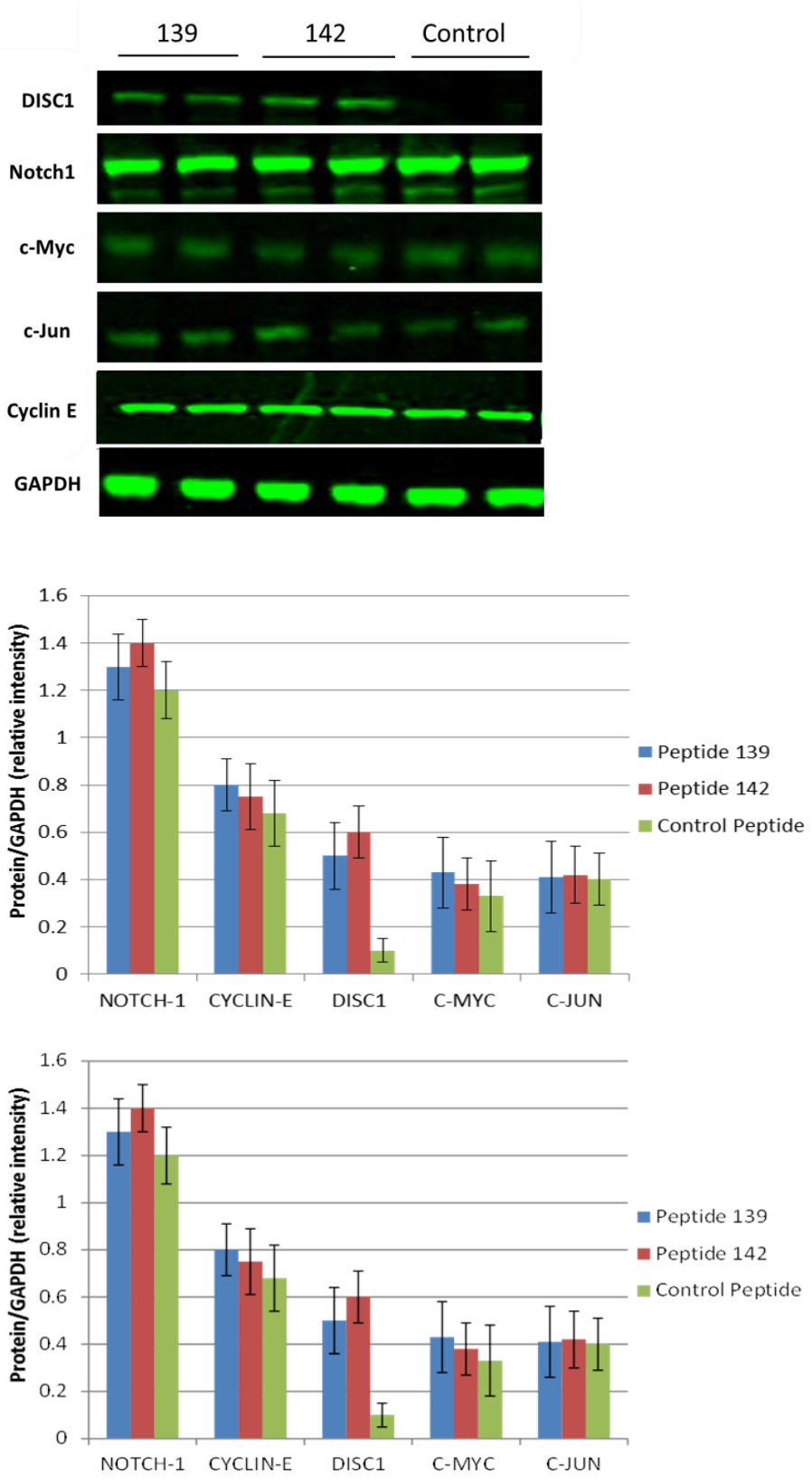


Figure 5.7 Expression of alternative Fbxw7 substrates remained unaltered in response to the lead peptide treatment.

Cell lysates of HEK293 cells treated with the lead peptides 139 and 142 were analysed by Western blotting technique. Protein expression of various Fbxw7 substrates – DISC1, Notch1, c-Myc, c-Jun, and Cyclin E, were detected by probing with anti DISC1, Notch1, c-Myc, c-Jun, and Cyclin E antibodies respectively. GAPDH antibody was used as loading control. Data shown represent the mean ± SEM of 3 separate biological repeats.

Band intensities were quantified by using Odyssey software and statisticaly represented in the graphs. This work was carried out by Dr.Christina Elliot, Gardiner Laboratory, University of Glasgow.

Cell lysates from HEK293 cells treated with the lead peptides (139 and 142) were probed for other known Fbxw7 substrates. Immunoblots (Figure 5.7) show that the peptides specifically stabilised endogenous DISC1 protein levels while the other substrates (Notch1, c-Myc, c-Jun, Cyclin E) largely remained unchanged implying that the lead peptides specifically disrupt Fbxw7-DISC1 interaction and do not interfere with other crucial interactions of Fbxw7.

5.3.5 Induced Pluripotent Stem cells as a cell line model

The exprimental work described in the section has been carried out as part of a 10 week Ph.D. placement at Pfizer Neuroscience Research Unit, Cambridge, Boston, MA in

February – April 2013.

5.3.5.1 DISC1 animal models – Limitations

Identification of DISC1 in the Scottish pedigree has garnered much attention by scientists who have tried to uncover the molecular mechanisms behind the biological phenomenon in order to develop therapeutics from a most promising susceptibility gene for SCZ and other associated mental illnesses. However, research in psychiatric illnesses is severely hampered by the lack of availability of human brain tissue, especially live neurons from patients.

The mouse has long been an attractive genetic model organism to study psychiatric disease, as it has many advantages such as the availability of a range of inbred strains, completed genome sequences and advancements in technology to genetically manipulate mice in numerous ways. DISC1 mouse models were invaluable and provided developmental and mechanistic insights, which could have been otherwise difficult to obtain. A review by Hanna Jaaro-Peled discusses all the seven DISC1 mouse models generated so far (Jaaro-Peled, 2009). These mouse models displayed a wide range of phenotypes which include both abnormal anatomical changes (example: reduced brain volume and neurite outgrowth) and behavioural phenotypes (example: diminished working memory and pre pulse inhibition (PPI)). Q31L and L100P were two ENU (*N*-ethyl-*N*-nitrosourea) induced mutants generated, which exhibited behavioural phenotypes-depression like and SCZ like respectively (Clapcote et al., 2007). MRI studies in these mice have also exhibited enlarged lateral ventricles, a common observation in SCZ patients (Vita et al., 2006). Koike et al. (2006), reported that 129S6/SvEv mice strain harbour a 25bp deletion in the DISC1 gene which results in a termination codon in exon 7, which abolishes the translation of full length DISC1 protein. Working memory was shown to be

impaired when this variant was transferred to the C57BL/6 genetic background (Koike et al., 2006). A DISC1 knockout mouse would the most useful tool; however, generation of a 'true null' has been challenging so far, as all DISC1 isoforms have not been identified and characterised yet (Brandon et al., 2004, Miyoshi et al., 2003a, James et al., 2004a). Since DISC1 was shown to be crucial in neurodevelopment processes, there is also a possible risk of lethality during development of KO mice (Schurov et al., 2004a) (Ishizuka et al., 2006). A conditional knockout mice in Cre/loxP system was another alternative suggested by experts to circumvent the complexity surrounding the KO mice generation (Ishizuka et al., 2006). Kuroda et al, generated transgenic mice lacking exons 2 and 3 (also known as Disc1 (Δ2-3)) which were shown to have higher threshold for the induction of long-term potentiation (LTP) and showed PPI deficits which are a well-known phenomenon observed in schizophrenia patients and other neuropsychiatric disorders (Kuroda et al., 2011).

However, it should be noted that animal models do not completely translate to the human condition and their use has always been limited in psychiatric research. It is impractical to generate schizophrenic mice and extremely difficult to study the psychotic symptoms such as hallucinations and delusion. To add to the complexity, most of these illnesses are polygenic and involve gene - environment interactions. Research over the last decade failed to develop a cell line/animal disease model for SCZ, which has been a major obstacle in drug development, as drug toxicity may vary with the variable ability of different species to metabolise drugs.

Since most of the drugs are targeted to regulate protein expression or enzyme activity, it is important that the protein of interest is well conserved in the species model. In light of DISC1 being implicated in brain dysfunction, it should be noted that DISC1 protein is poorly conserved between various species which may be considered as a major drawback in considering the use of rodent derived cell lines for DISC1 studies. Primate cerebral cortex differs substantially from that of rodents in many features, such as cerebral cortex size, complexity and the nature and range of stem cell population. Drug discovery for

psychiatric illnesses has also been considerably slow owing to the failure of the prognostic validity of animal models.

Since my present study focuses on the idea of developing the DISC1/Fbxw7 interaction as a putative drug target, it is crucial to choose a relevant cell model for *in vitro* studies. The DISC1 and Fbxw7 interaction is characterised in the present study and the amino acids that are involved in the interaction have been mapped using peptide array studies (covered in chapter 4). Mouse models or mouse primary neuronal cultures are popular choices for most *in vitro* studies in the neuroscience field. In the present study, since protein-protein interaction was being characterised, it was important to consider the extent of evolutionary conservation between mouse, rat and human DISC1 protein sequences.

Species	Phosphodegron sequence	Accession number
Homo *sapiens*	CGPEVPP**TPP**G**S**HSAFTSSFSFIRLSL	NP_061132.2
Pan *troglodytes*	CGPEAPP**TPP**D**S**HSAFTSSFSFIRLSL	XP_001151459.1
Macaca *mulatta*	CGLEDPP**TPP**G**S**HSAFASSFSFIRLSL	AAV87214.1
Canis *lupus familiaris*	SVPKAPP**TP**AG**S**QDAFTSSFSFIRLSL	XP_546088.3
Rattus *norvegicus*	DIP---SL**P**-GFQDTFTSNFSFIRLSL	NP_783186.2
Mus *musculus*	DIA---SL**P**-GFQDTFTSSFSFIQLSL	AAN77091.1
Consensus	**TPP**x**S**	

Figure 5.8 Amino acid homology showing DISC1 CPD alignment within various species. Homology analysis was done using the Basic Local Alignment Search Tool (BLAST) at the NCBI website (http://www.ncbi.nlm.nih.gov/blast). Non conserved CPD motif residues in mouse and rat were highlighted in red.

Mouse and rat orthologues of DISC1 are only about 56**%** identical to the human DISC1 at nucleotide and amino acid levels, a fact that suggests the unusually rapid evolution of DISC1 (Jaaro-Peled, 2009, Ma et al., 2002b). From the above Table, it is quite evident that the CPD motif is barely conserved in mouse and rat when compared with similar sequences of higher primates. This poses a major obstacle in considering cell lines from mouse or rat origin to perform *in vitro* studies to characterise DISC1 CPD.

Until recent years, most of the research on psychiatric disorders relied on brain imaging of patients, genetic studies on lymphocytes from patients and biochemical studies on post mortem brain tissue. Though these approaches led to enormous progress in understanding the disease, they could not efficiently address molecular and cellular aspects leading to the development and progression of the disease.

Inaccessibility of human brain tissue has been a major hurdle for decades in psychiatric research. Peripheral leukocytes and autopsy samples were the alternatives available for investigating the molecular basis of mental illnesses (Altar et al., 2005, Hashimoto et al., 2006, Saetre et al., 2008). However, there has been a long standing debate if the findings from the above sources can be successfully extrapolated to brain specific diseases, since factors such as cause of death, medication, use of alcohol and nicotine may influence gene expression and hence fundamental disease related molecular phenomenon may not be addressed (Lipska et al., 2006a). The advent of induced pluripotent stem cell (iPS cell) technology opened up new avenues in the field of neurodevelopmental disease research.

5.3.5.2 Induced Pluripotent Stem cells (iPSCs)

iPS cells are adult cells that have been genetically reprogrammed to behave like embryonic stem cells by inducing them to express genes (by various methods) that are essential for maintaining the defining properties of embryonic stem cells (Nakagawa et al., 2008). The term pluripotency represents cell types with functional capabilities to generate cell types from three embryonic germ layers endoderm, mesoderm and ectoderm. Patient specific culture of immortal cells would yield invaluable information about disease mechanisms and allow a novel route to investigate these mechanisms for the development of novel drugs. However, until recently this was restricted to cancer research. The Nobel Prize in Physiology or Medicine for 2012 was awarded to Prof.Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent. (Reference http://www.nobelprize.org/nobel_prizes/medicine/laureates/2012/*').*

Yamanaka and colleagues generated mouse iPS cells for the first time in 2006, from mouse embryonic and adult fibroblasts by viral transduction of Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions (Takahashi and Yamanaka, 2006). These four factors were later used to generate iPS cells from human skin fibroblasts (Takahashi et al., 2007). However, there were major drawbacks to the protocols used in these studies, with c-Myc being an onco gene and the associated increased risk of tumor development at a later stage. The same group went on to develop a method, whereby, iPS cells were made without having to transduce the c-myc gene and use drugs for selection (Nakagawa et al., 2008). The method described in the above study also increased the quality of iPS cells and reduced the back ground of non iPS cells (Nakagawa et al., 2008). In later years, integration free reprogramming methods were developed which increased the quality and yield of iPS cells (Cahan and Daley, 2013). These pioneering studies were followed by the successful generation of iPS cells from patients with various genetic disorders including neurological disorders like Huntington's and Parkinson's disease (Zhang et al., 2010, Park et al., 2008). Patient specific stem cells offer an extraordinary opportunity to reiterate both normal and pathological human tissue formation *in vitro*, particularly in case of brain diseases, thereby enabling disease investigation and drug target development (Park et al., 2008). It can be understood that after well over a decade of initial constraints these area have now been overcome and research in this area is currently flourishing.

5.3.5.3 iPS cell technology in brain research

iPS cell technology offers an excellent alternative and is invaluable in neuropsychiatric research. Their ability to differentiate in to three germ layers (ectoderm, endoderm and mesoderm) renders iPS cells as ideal patient specific model cell lines for SCZ, where aetiology still remains unclear. Generation of human and patient specific cell models is advantageous in developing drug targets in that they allow a limitless supply of human patient specific cells that can be differentiated into the cell type of choice where the disease specific phenotype can be recapitulated. This novel research approach facilitates the design and verification of disease models which are otherwise not feasible for psychiatric illnesses.

A number of groups reported the successful generation and use of iPS cells in disease modelling to investigate neurological disorders (Pedrosa et al., 2011, Chiang et al., 2011, Zhang et al., 2010, Wernig et al., 2008, Cherry and Daley, 2013). Brennand et al, (2011) employed this principle and reprogrammed fibroblasts from SCZ patients to human iPSC and subsequently differentiated these pluripotent cells into glutamatergic neurons. They performed extensive gene expression profiling and looked for morphological changes in these disorder specific neurons to investigate the molecular defects in SCZ. Compared to the control neurons, they reported reduced PSD95-protein levels, glutamate receptor expression, abnormal Wnt and cAMP signalling along with morphological changes such as reduced neuronal connectivity and neurite number in SCZ neurons (Brennand et al., 2011).

The ability of iPS cells to efficiently differentiate into any desired cell type such as neurons has been successfully demonstrated (Gao et al., 2013), (Pedrosa et al., 2011). With the addition of specific factors to the culture media, iPS cells have been further differentiated into brain region specific identities including hindbrain, forebrain and spinal cord, permitting large scale production of specific neurons carrying the genetic background from the disease carriers (Brennand and Gage, 2012). This also facilitates analysis of the differentiated cells under controlled conditions. iPSC derived neurons are ideal particularly for neurodevelopmental disorders like SCZ, since they facilitate recapitulation of the early stages of neuronal differentiation in genetic backgrounds which may increase the risk of disease. They also allow the study of the cellular and molecular events triggering the onset of these disorders.

For the purpose of screening the lead peptides (developed in the present study) within a short time frame, expandable neuronal progenitors generated from the iPS cells were used.

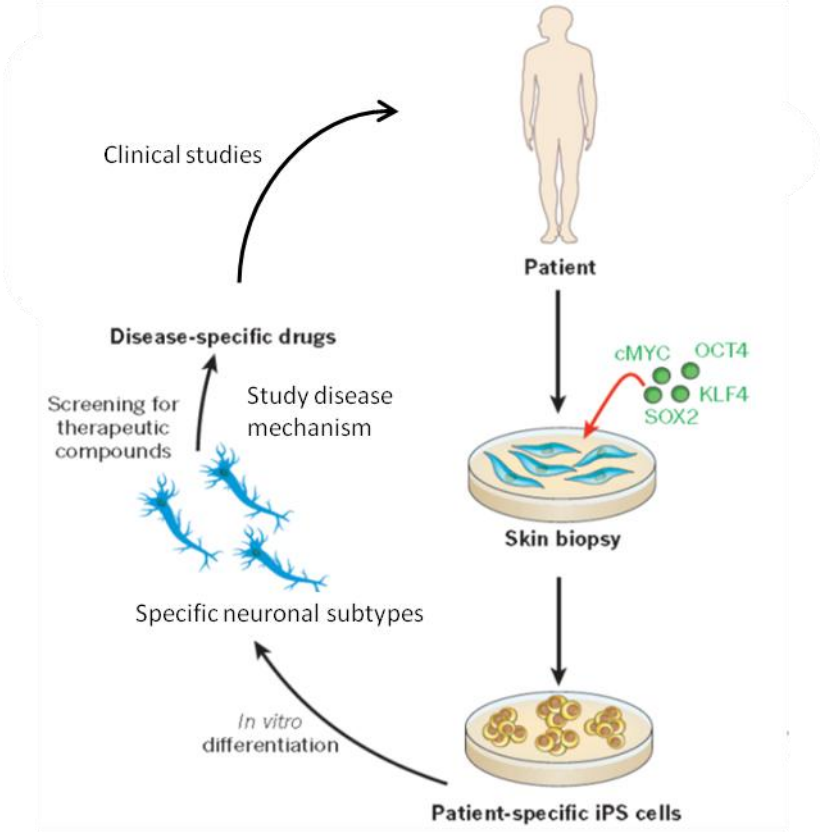


Figure 5.9 Application of iPS cell technology in research and drug development Adapted from (Robinton and Daley, 2012).
This novel technology facilitates reprogramming of easily available patient specific skin fibroblasts into affected neuronal subtypes. These differentiated neurons can be used for patient genotype specific disease modelling *in vitro* and serve as a tool to study drug targets and develop potential drugs.

5.3.5.4 Experimental aims

The aims of this section of the chapter are as follows:

1. Explore iPSc technology for disease modelling by differentiating the accessible iPS cells reprogrammed from SCZ patients and differentiating them to neuronal progenitor cells (NPs).

2. Characterisation of the neuroprogenitor cells (NPs) by screening of pluripotent and neuronal markers.

3. Study DISC1 and Fbxw7 expression profile in NPs.

4. Test the novel hypothesis that DISC1-Fbxw7 interaction controls DISC1 protein levels in NPs.

5.3.5.5 Results

iPS cells generated from skin biopsies of patients diagnosed with SCZ have been used in the present study. The cells were taken from healthy controls (with no known mutations in DISC1 gene) and SCZ patients harbouring a 4bp deletion at the 12th exon-intron boundary in the C-terminus of the DISC1 gene. Neuronal progenitors differentiated from these cells were considered suitable for *in vitro* testing of the putative disruptor peptides for the following reasons:

1. Availability: Recent advancements in the field of iPSc research have enabled the production of these cell lines without specialised equipment.

2. Origin: Since the iPS cells used in the present study were generated from patients diagnosed with SCZ, they allow close recapitulation of the cellular phenomena related to SCZ and are apt for use in investigating the underlying molecular events.

3. Disrupted DISC1 gene: One of the major genetic risk factor of SCZ, DISC1 gene carries a 4bp deletion, resulting in a frame shift mutation which could be translated to a truncated protein. It would be intriguing to investigate if this mutation has a role to play in DISC1 signalling pathway in these cells.

The iPS cells (except Detroit) used in the present study have been described and characterized previously (Chiang et al., 2011, Pedrosa et al., 2011). iPS Detroit cell line were generated by Dr.Sandra Engle, Pfizer Inc. All the cell lines used in this study were karyotyped (for chromosomal aberrations) and screened for the expression markers (stem cells) at Pfizer.

Table 5.2 Description of the iPS cells used in the study

iPS cell line	Assigned name	DISC1 mutation	Origin	Phenotype	Sex	Reference
Control	C1-1	none	Skin biopsy	Normal	M	(Chiang et al., 2011)
Control	Detroit	none	Detroit 551 fibroblasts ATCC (CCL-110)	n/a	n/a	Generated by Dr. Sandra Engle, Pfizer Inc.
Patient	D2-1, D2-2	4bp deletion at 12th intron-exon boundary	Skin biopsy	Chronic paranoid Schizophrenia	F	(Chiang et al., 2011)
Patient	D3-1, D3-2	4bp deletion at 12th intron-exon boundary	Skin biopsy	Chronic undifferentiated Schizophrenia	M	(Chiang et al., 2011)

5.3.5.6 Characterisation of iPS cells and differentiated Neuronal progenitors

Immunocytochemistry based characterisation showed that all of the iPS cell colonies showed compact colony morphology and stained positive for the pluripotent markers Tra-181 and Nanog (Figure 5.12a, 5.12b). With limited availability of reagents, I was able to only screen for Oct4A, a pluripotent marker, using Western blotting analysis (Figure 5.10).

There are four main stages involved in the differentiation process of iPS cell to NPs:
(1) Embryoid body (Ebs) formation, where cell aggregates grow in suspension.
(2) Plating of the embryoid bodies on defined matrices to form neural rosettes and patterning of neural progenitors into specific neuron subtypes.
(3) Expansion and growth of neural progenitor cells, to generate an appropriate precursor population.

(4) Differentiation to specific cell types where neural progenitor cells can develop into neurons of specific subtype (This step was not performed in the present study).

The various stages of neuronal differentiation from iPSc are schematically represented in Figure 5.11. Supplementing minimal growth media with fibroblast growth factor 2 (FGF2), L-glutamine and ß-mercaptoethanol leads to neural stem cell generation, which is characterised by expression of neural markers such as Nestin (Li and Zhang, 2006, Kim et al., 2011). The neuronal progenitors generated, have the potential to be further differentiated in to neurons, astrocytes or oligodendrocytes using specific culture medium supplements (Chojnacki and Weiss, 2008). iPS cells described in Table 5.3, were karyotyped to check for any possible mutations or carcinogenic gene activation which might be possible as a result of episomal gene transfer (Chiang et al., 2011).

The maintenance and differentiation protocol has been optimized and standardized at Pfizer by Dr. Lindsay Wilson, to generate GABAergic neuronal progenitors cells from iPS cells by reprogramming. The detailed protocol has been explained in section 2.3.2. In brief, iPSCs were maintained on Mitomycin C treated (mitotically arrested) MEFs (Mouse Embryonic Fibroblasts) in the presence of 10 ng/mL FGF2. On day 0, iPSc colonies were detached from feeder layers and grown on ultra-low adherence plates in suspension culture and supplemented with ROCK inhibitor to induce embyoid body (EBs) formation in the absence of FGF2. On day 2, EBs were transferred on to matrigel coated plates. The morphology changes with the progression of differentiation were monitored and representative images were detailed in Figure 5.11. The phenotype of the progenitors was also analysed by immunocytochemistry using various neural markers (Figure 5.13 a,b).

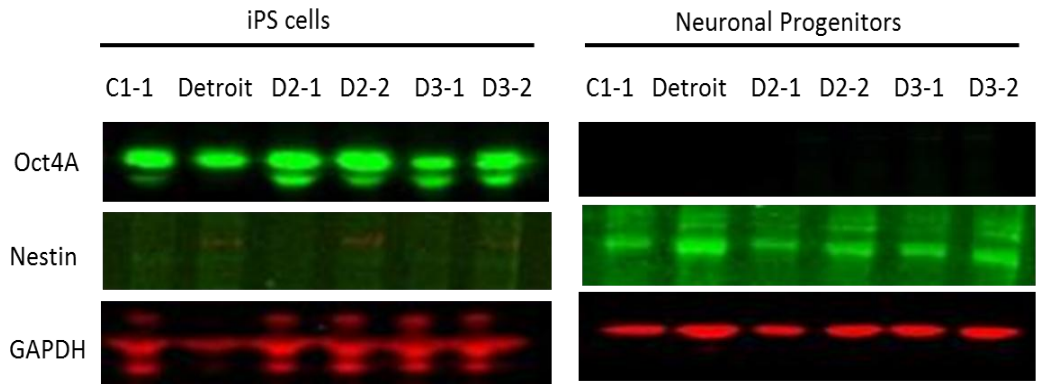


Figure 5.10 Immunoblot analysis of expression of pluripotent and neuronal markers. Cell lysates collected from the iPS cells and neuronal progenitors from all the six cell lines were probed for the presence of pluripotent marker Oct4A and neural progenitor stem cell marker Nestin. GAPDH antibody was used as loading control.

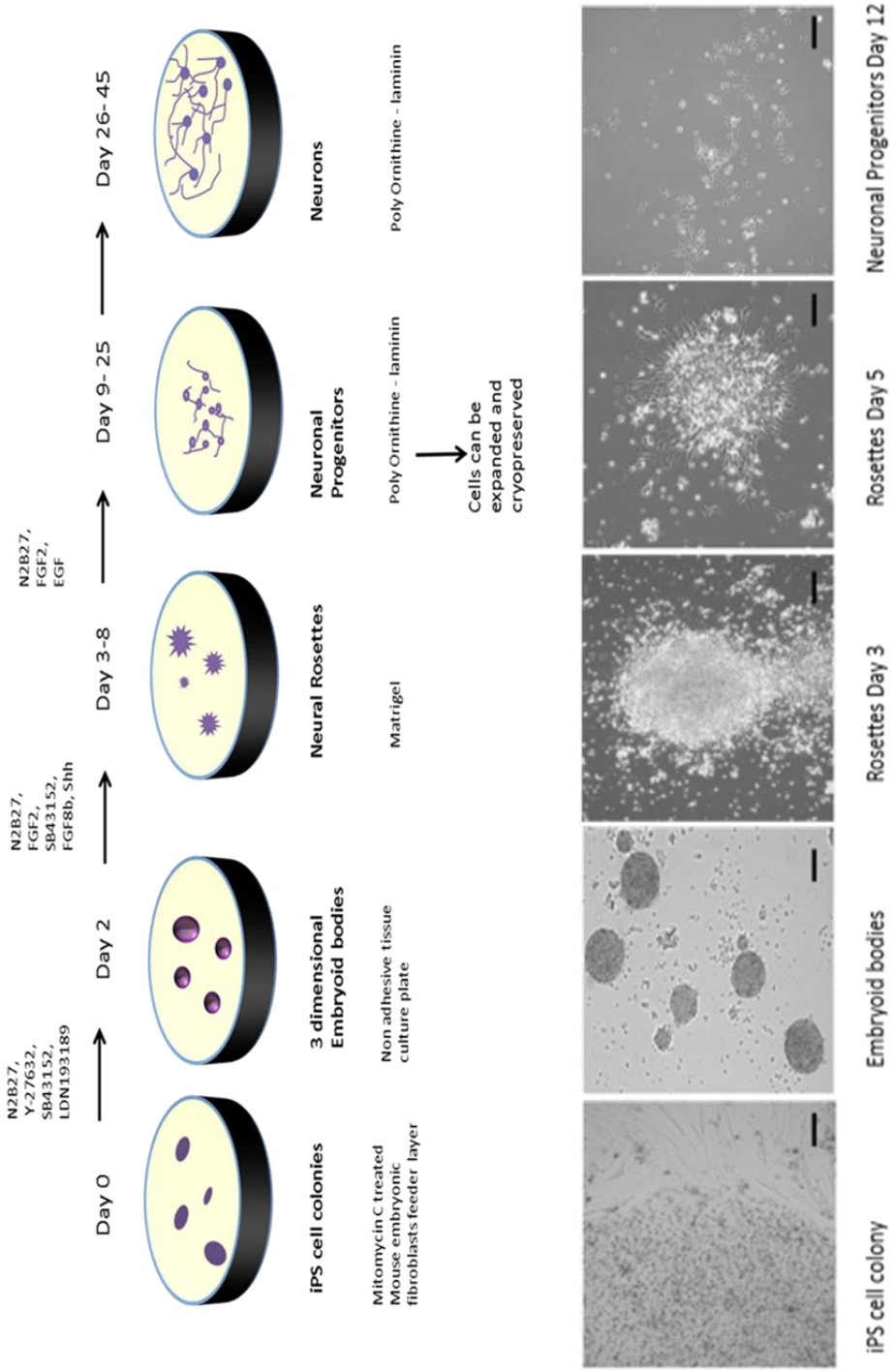


Figure 5.11 Schematic of various stages of iPSc differentiation into NPs
Top panel: Schematic representation of various stages of neuronal differentiation.
Bottom panel: Representative bright field images taken at different stages of differentiation. Scale: 50µM.

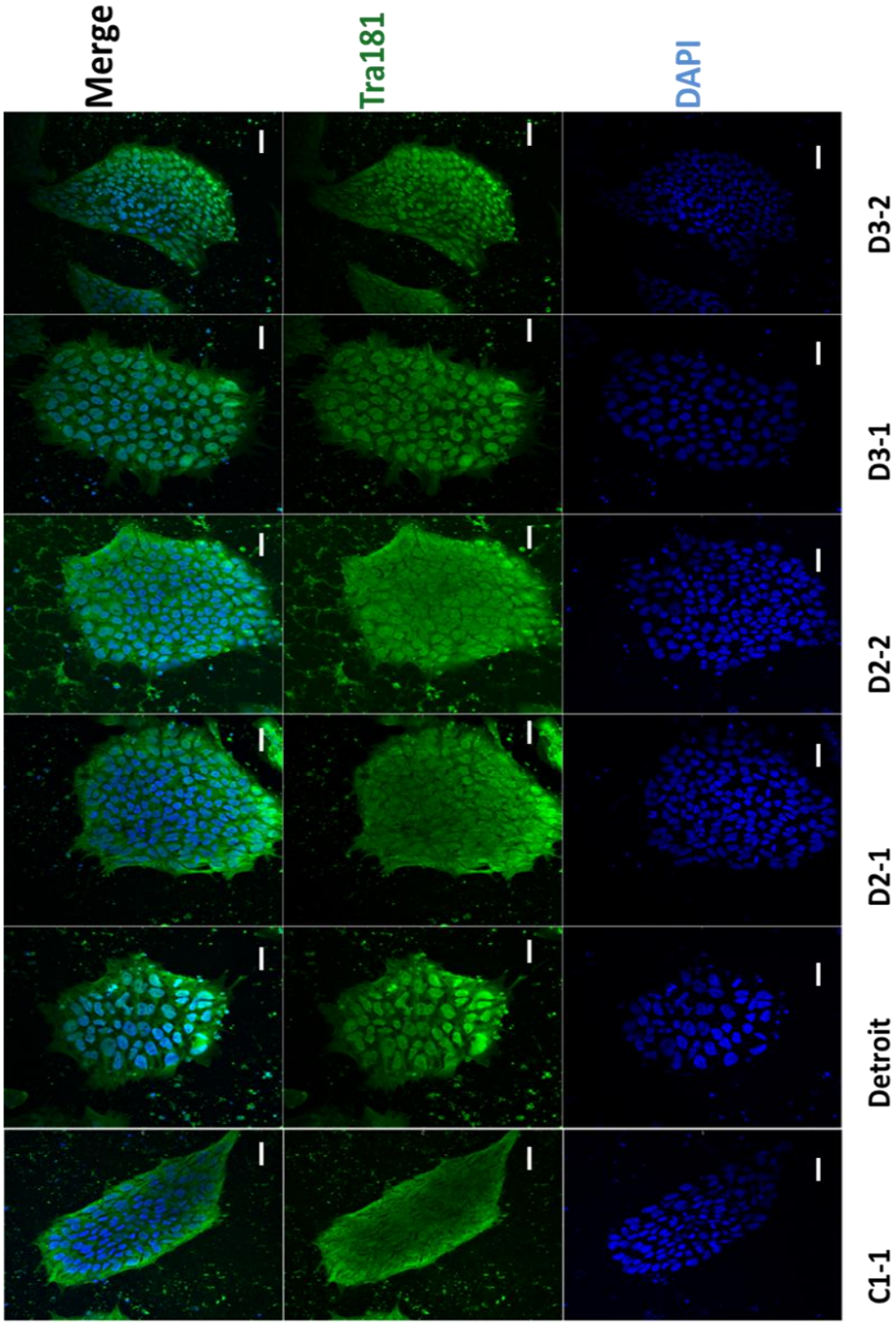


Figure 5.12a Immunocytochemistry on iPS cell colonies showing the expression of pluripotency marker
Top panel: Merge image, Middle panel: Tra181 (pluripotency markers), Bottom panel: DAPI (nuclear stain). Scale bar: 50µM

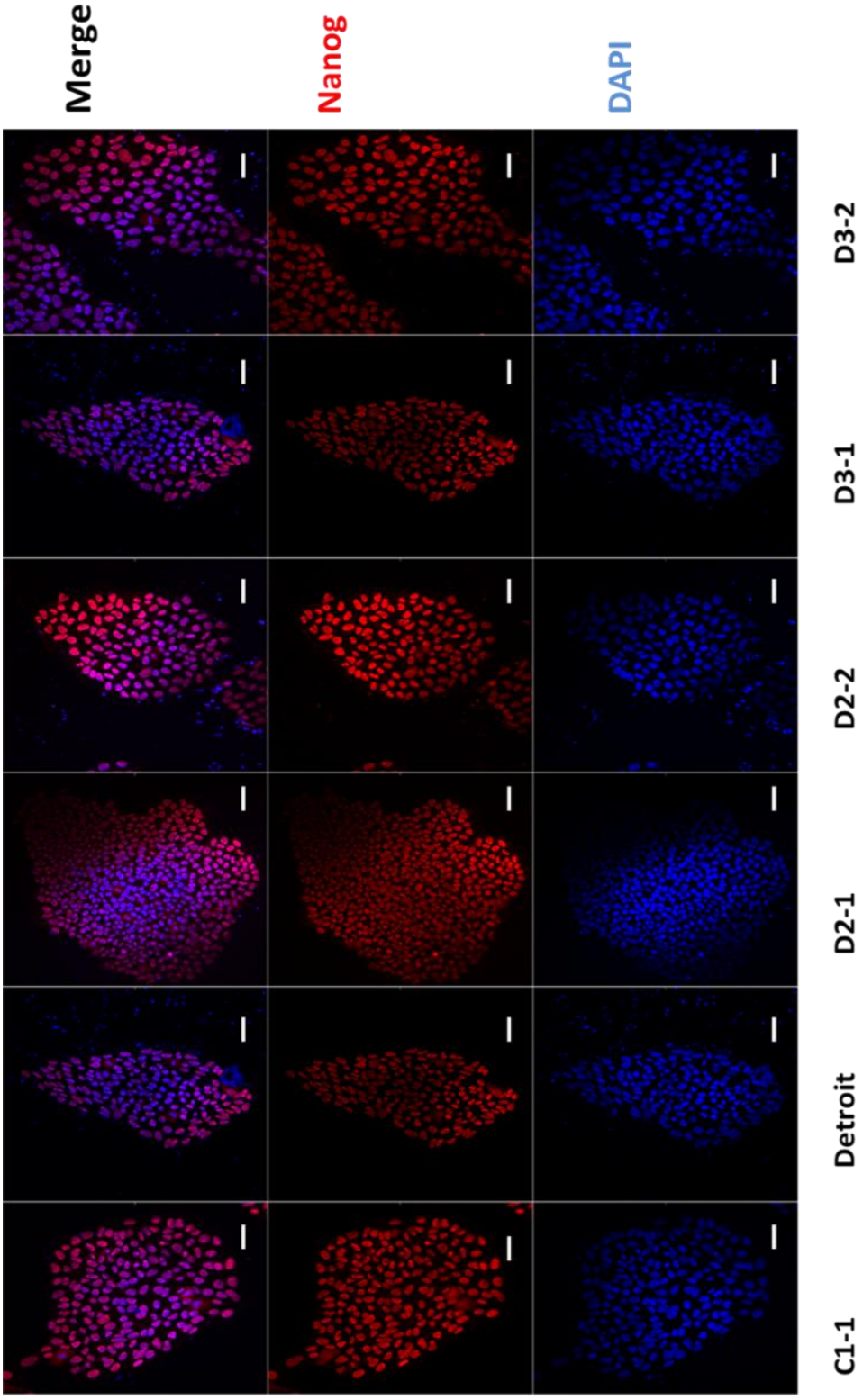


Figure 5.12b Immunocytochemistry on iPS cell colonies showing the expression of pluripotency markers
Top panel: Merge image, Middle panel: Nanog (pluripotency marker), Bottom panel: DAPI (nuclear stain). Scale bar 50µM.

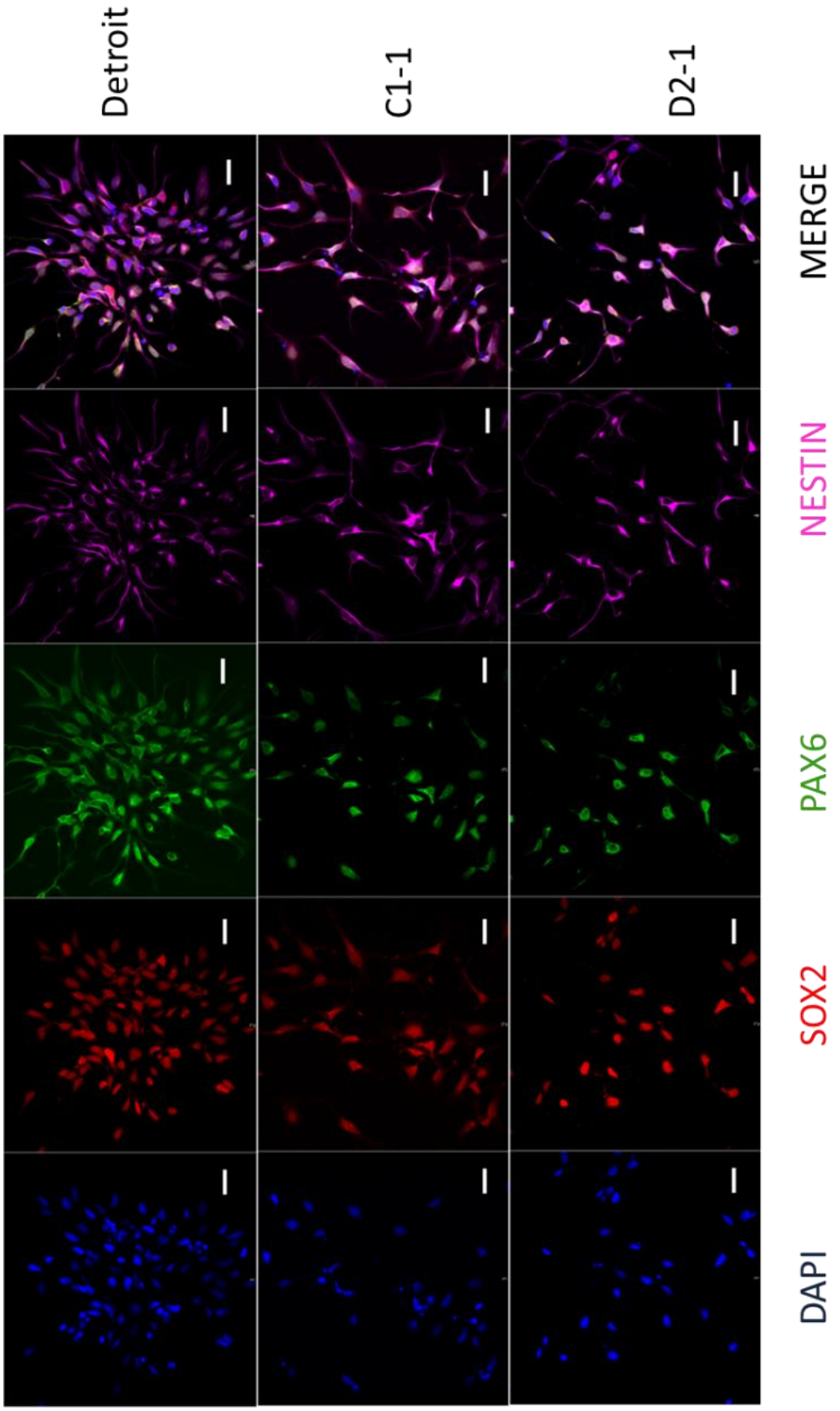


Figure 5.13a Immunocytochemistry on Neuronal progenitors (day 3) showing the expression of neural stem cell markers in cell lines (Detroit, C1-1, D2-1)
NPs stained positive for neural stem cell markers SOX2, PAX6 and NESTIN. Nucleus specific stain DAPI was used. Scale bar: 50µM.

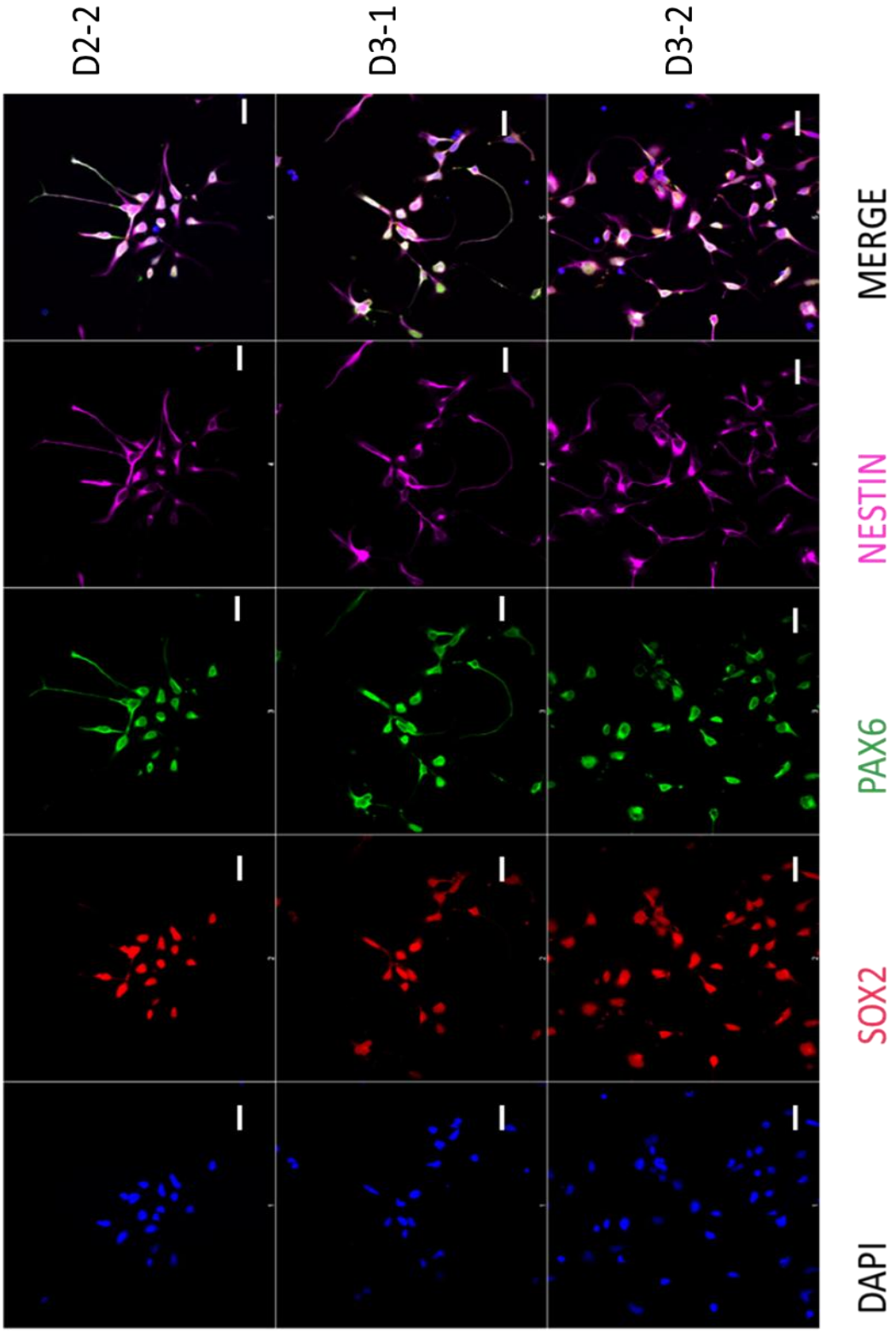


Figure 5.13b Immunocytochemistry on Neuronal progenitors (day 3) showing the expression of neural stem cell markers in cell lines (D2-2, D3-1,D3-2)
NPs stained positive for neural stem cell markers SOX2, PAX6 and NESTIN. Nucleus specific stain DAPI was used. Scale bar: 50µM.

5.3.5.7 DISC1 and Fbxw7 protein and transcript expression profile in the Neuronal progenitors

Before considering the use of these cell lines for testing my hypothesis, it was important to determine whether the cells express DISC1 to levels that can be detected by immuno blotting techniques. DISC1 and Fbxw7 protein expression was quantified in the neuronal progenitors derived from the iPS cells using Western blotting to ensure that they have detecTable levels to aid further biochemical studies. α-DISC1 antibody was used to detect 100kDa isoform of DISC1.

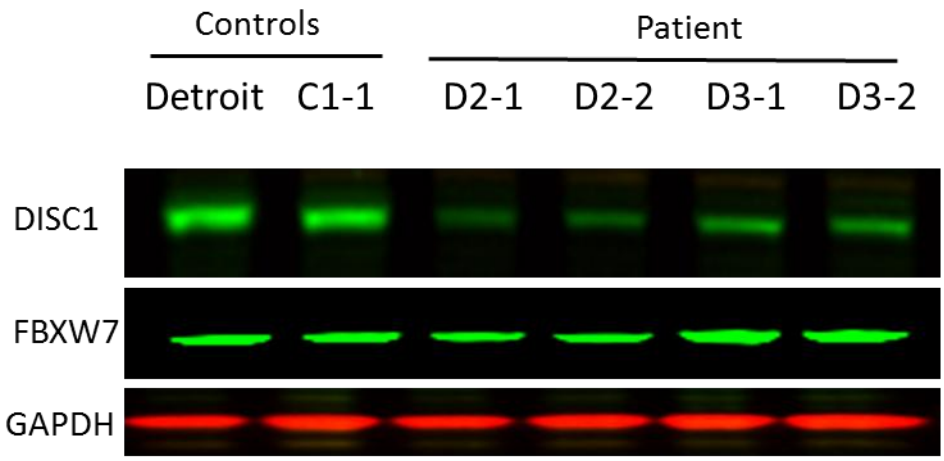


Figure 5.14 Representative immunoblot showing DISC1 and Fbxw7 protein expression profile in the neuronal progenitors differentiated from iPS cells.
Cell lysates from the NPs were analysed using Western blotting by probing with α DISC1 (100kDa) and Fbxw7 (α isoform, 75kDa) antibodies. GAPDH was used as loading control.

From Figure 5.14, it is evident that the NPs from patients carrying a DISC1 deletion (D2-1, D2-2, D3-1, D3-1) have low DISC1 protein levels compared to that of control subjects (Detroit and C1-1). However, no detecTable differences in expression were observed with respect to α-Fbxw7 expression. This may be attributed to the 4bp deletion on one of the alleles of the DISC1 locus which may produce an unstable DISC1 variant.

To assess the difference in mRNA transcript levels between the control and patient NPs, DISC1 and Fbxw7 gene expression was analyzed using real time PCR. The relative expression of DISC1 in the NPs was assessed in two separate cDNA samples. Raw CT values for five reference genes (TBP, PSMB2, PRLI3A, RPN1 and RPLP0) were entered into Normfinder to evaluate the variability of the reference genes (Table 5.3) (Andersen et al., 2004). PRLI3A and RPLP0 genes were omitted from further analysis due to high variability. The gene expression between cell lines was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 5.3 Table representing stability values of five reference genes obtained using Normfinder software

Primer pair	Stability value
TBP	0.007
PSMB2	0.007
PRLI3A	0.011
RPN1	0.008
RPLP0	0.013

The higher the stability value of the reference gene, the higher is the variability of the gene expression. PRLI3A and RPLP0 genes have highest stability of all the reference genes considered in the study and hence omitted from further analysis.

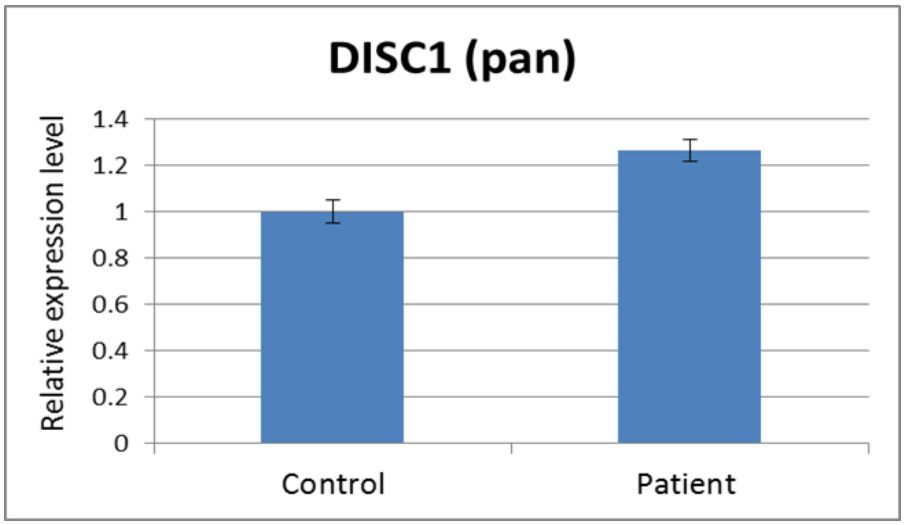


Figure 5.15 Relative expression of DISC1 transcripts in NPs analysed at day 15
Shows relative expression of DISC1 transcripts between NPs from different cell lines (Detroit and C1-1 cell line were grouped in to 'control', D2-1, D2-2, D3-1, D3-2 cell lines

were grouped in to patient) determined by real time PCR using Pan DISC1 primers (Life technologies) which detect and amplify DISC1 transcripts of all isoforms. n=2.

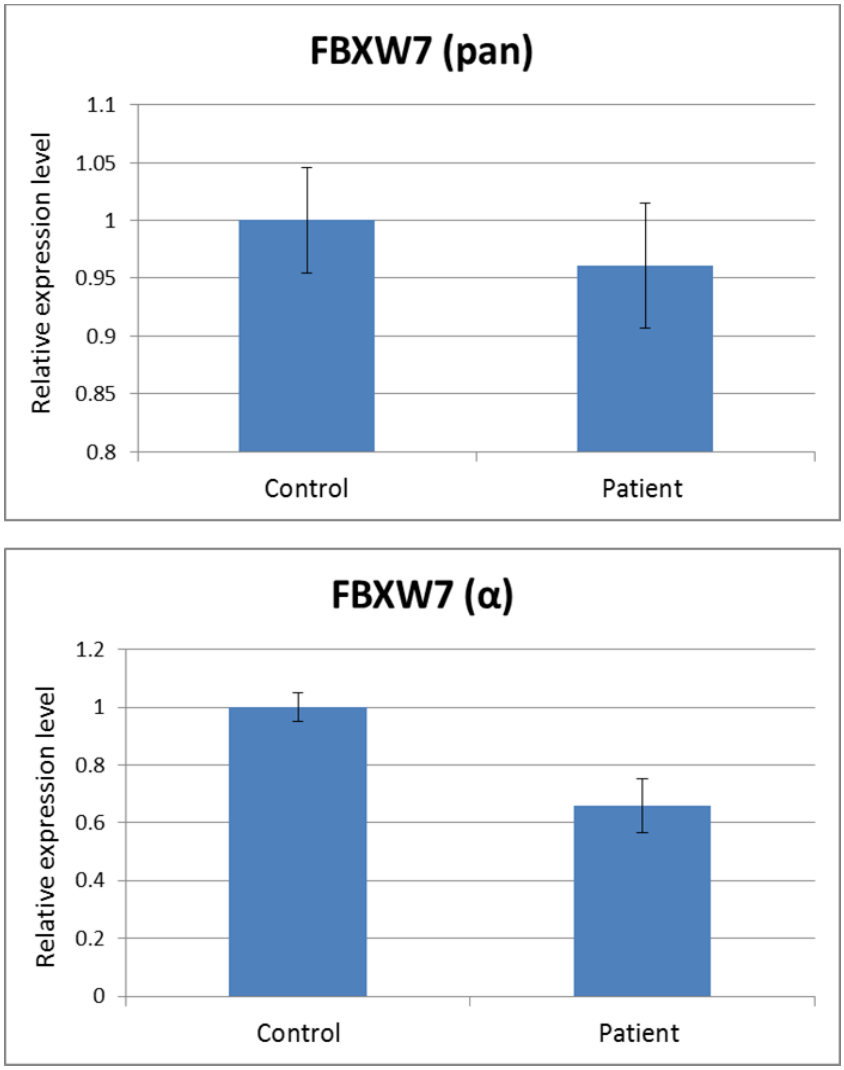


Figure 5.16 Relative expression of Fbxw7 transcripts in NPs analysed at day 15. Top panel: Shows relative expression of Fbxw7 transcripts analyzed using Pan Fbxw7 primers (Life Technologies) which detect and amplify transcripts of all isoforms. Detroit and C1-1 cell line were grouped in to 'control', D2-1, D2-2, D3-1, D3-2 cell lines were grouped in to patient. Bottom Panel: Shows relative expression of Fbxw7 alpha isoform transcripts. n=2.

From the preliminary RT PCR analysis of DISC1 and Fbxw7 transcripts in the NPs generated from the iPS cells, it can be suggested that:

1. DISC1 transcript levels are higher in patient NPs (Detroit and C1-1) over those from control NPs (D2-1, D2-1, D3-1, D3-2) (Figure 5.15). Since all the isoforms of DISC1 are not characterised to date and isoform specific antibodies nor primer probe sets were not

available at the time of this study, the isoform dependent DISC1 expression could not be verified. More biological prepeats should be carried out to gain indepth knowledge on the pattern of DISC1 transcript expression and how it may vary during the neuronal differentiation of iPS cells.

2. Fbxw7 transcript levels were analysed using primer probe sets which were specific for a common region of the 3 isoforms (α, β, γ) and those specific for the α isoform. Though the transcript levels detected by the pan primer-probe sets were not informative with high variation, it was interesting to see that relative mRNA transcript levels of FBXW7 α isoform were lower in patient NPs over those in control NPs.

Although the above data are inconclusive, owing to low biological repeats, it would be intriguing to establish a correlation between the expression of these two proteins to understand their role in NPs differentiation to expand our knowledge on DISC1 role in brain development. Unfortunately, these studies were not within the scope of this project.

5.3.5.8 Proteasomal inhibition causes DISC1 stabilization in NPs

Ubiquitination, a novel post translational modification of DISC1 protein, was reported in the present study (chapter 4). Ubiquitin chain conjugation to DISC1 was characterised and was reported to be readily augmented with proteasome inhibition. Immunoprecipitation studies and subsequent Mass spectroscopy studies confirmed the presence DISC1-ubiquitin conjugates. Since the preliminary studies were performed in HEK293 cells, it was important to examine this hypothesis in a more relevant cell model such as NPs generated from iPS cells, which have an added advantage of being derived from SCZ patients. Due to limited availability of reagents and time constraint, one control line (Detroit) and one patient line (D2-1) were selected for further studies.

NPs derived from Detroit and D2-1 were treated with MG132 at a 10 µM concentration for indicated time points (Figure 5.17), followed by cell lysis and Western blot analysis. DISC1 and GAPDH protein expression was detected using anti DISC1 antibody and anti GAPDH antibodies respectively.

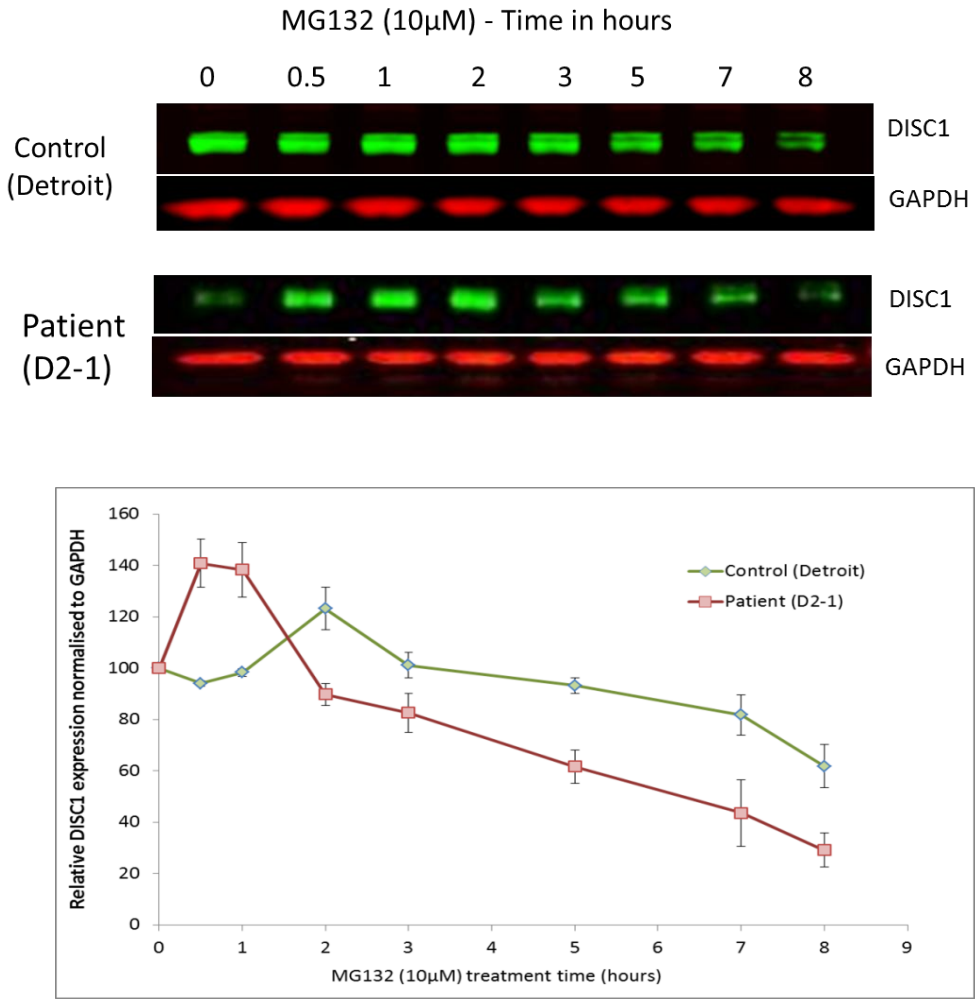


Figure 5.17 Effect of proteasome inhibition (MG132 treatment) on DISC1 protein expression in NPs at day 18.
NP were treated with proteasomal inhibitor MG132 on day 18 at 10 µM for indicated time points followed by Western blot analysis with anti DISC1 (100kDa) and anti GAPDH antibodies (n=2). Quantitative analysis of the immunoblots representing percentage change normalized to GAPDH. Data are mean ± S.E.M. of two experiments on two different sets of NPs collected at two consecutive passages.

Proteasomal inhibition in NPs generated from Detroit (control) and D2-1 (patient) iPS cells resulted in stabilization of DISC1 (100kDa) protein levels within the first 30 min up to 3 hours, consistent with the observation made in HEK293 cells. This indicates that DISC1 turnover is regulated by the UPS system in these cell lines, which therefore can be used as a cell line model to screen the lead peptides.

5.3.5.9 Screening of DISC1-Fbxw7 disruptor peptides on NPs generated from the iPS cells

As a more relevant cell model has been proposed (NPs), we next investigated whether the disruptor peptides developed in the present study (chapter 5) would stabilise DISC1 in these cells. As within the scope of the project, only fundamental experiments were performed by treating the NPs with the peptides that stabilised DISC1 in HEK293 cells (peptide 139 and 142). NPs were treated with peptides at 100µM concentration for 1, 2 and 3hr followed by Western blot analysis of the cell lysates. DISC1 protein expression was determined by probing with α-DISC1 antibody and GAPDH levels served as a loading control.

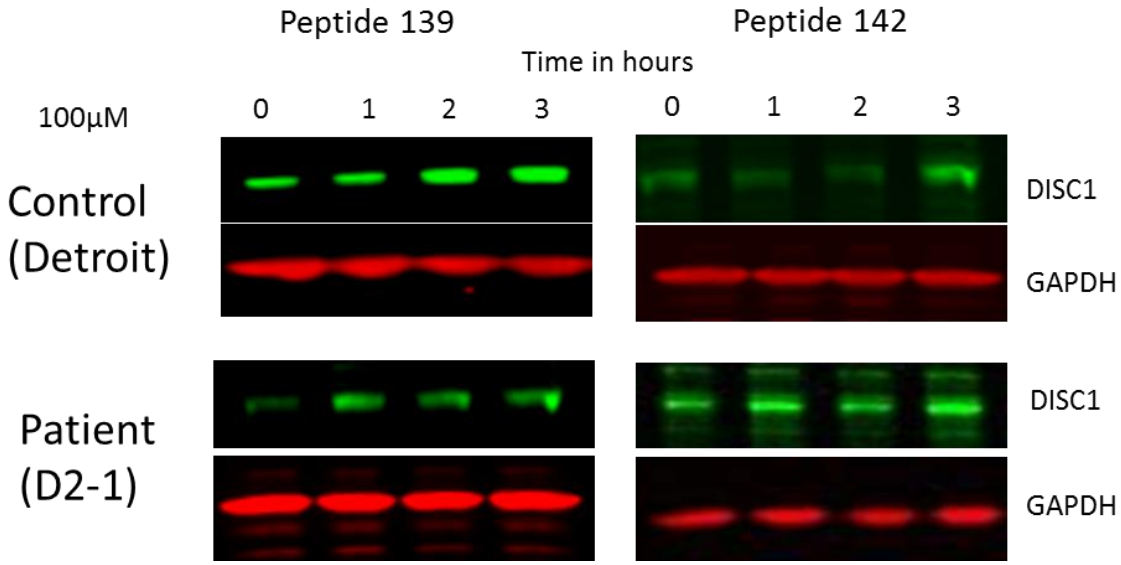


Figure 5.18 Disruptor peptides developed were screened in Neuronal progenitor cells for DISC1 stabilisation using immuno blot analysis.
NPs from Control (Detroit) and Patient (D2-1) cell lines were lysed after treatment with the peptides 139 and 142 at increasing time points from 0 hr (no treatment) up to 3hr at 100µM each. DISC1 protein expression was detected by DISC1 antibody. GAPDH antibody was used as loading control (n=2).

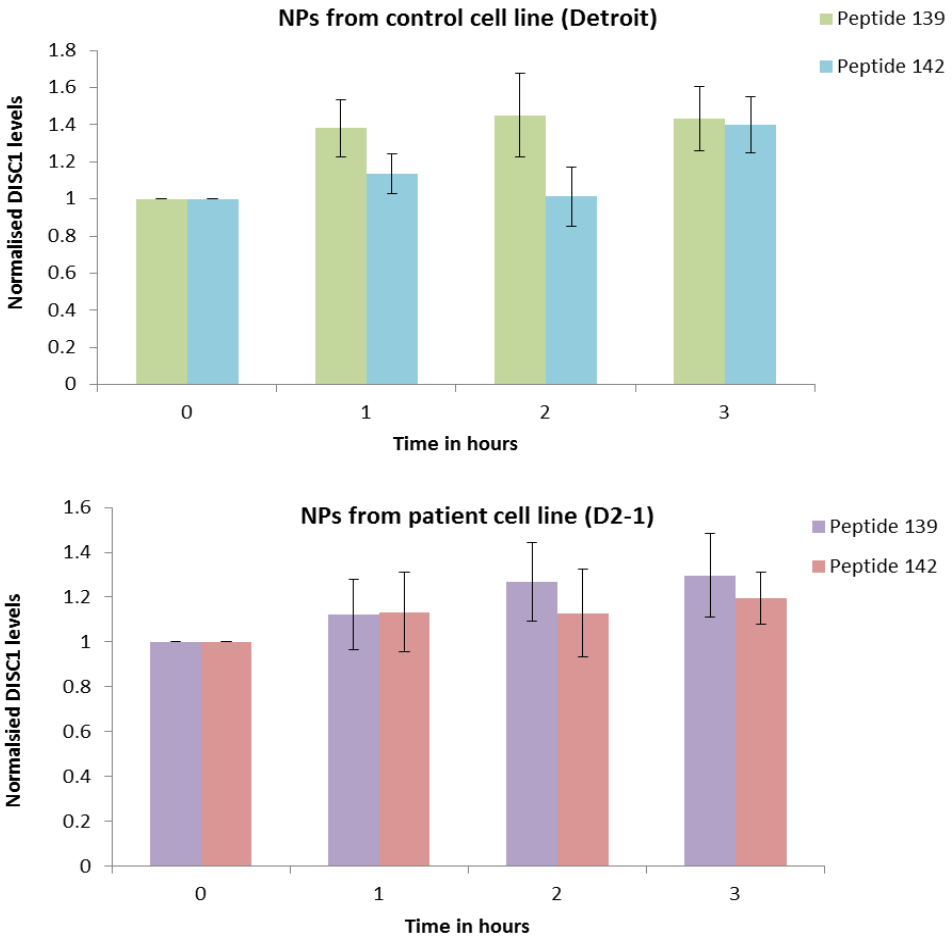


Figure 5.19 Quantitative analysis of immuno blots (from Figure 5.18) showing fold ratio of DISC1 normalized GAPDH.
Data are mean ± S.E.M. of two experiments. Statistical differences could not be determined due to low replicate experiments (n=2).

Lead peptide 139 stabilised DISC1 in both the cells lines as seen in Figure 5.18, left panel within 3hr of peptide treatment at a 100µM concentration. Although peptide 142 stabilised DISC1 protein levels in the Detroit cell line (control) at the 3hr time point, the effect was negligible in D2-1. While the results were promising, more technical and biological repeats could not be performed to ascertain this effect of the lead disruptor peptides due to time and budget restraints.

5.4 Discussion

In summary, the present chapter explores the potential of (1) DISC1 protein complexes for drug discovery and development; (2) peptides that act as substrate-specific Fbox E3 ligase inhibitors; (3) iPS cell derived NPs as a patient specific cell model to investigate disease mechanism and role of DISC1.

A single gene locus may produce multi compartmentalised proteins by mechanisms such as post translational modification, alternative splicing, alternative transcription and translation initiation (Danpure, 1995). Genetic studies on psychiatric patients identified a SCZ proband as carrying a balanced t(1;16)(p31.2;q21) translocation, who had a history of psychotic episodes with hallucinations and delusions (Millar et al., 2005b). Biochemical studies in cells from different individuals with the translocation revealed that DISC1 expression was nearly 50% less both at transcript and protein levels in subjects with the translocation. It has been proposed that the translocation is the likely reason for the observed reduction in the DISC1 expression and for the individuals with the translocation being susceptible to SCZ (Millar et al., 2005b, James et al., 2004a). The haploinsufficiency disease model is one of the widely accepted disease mechanisms in which an individual is heterozygous for a gene mutation or a truncation at a particular locus. RNAi mediated knockdown of DISC1 in mouse embryonic brains delayed neuronal migration and decreased dendritic arborisation (Kamiya et al., 2005b).

Co-Immunoprecipitation and co-crystal structure (data shown in appendix) confirmed complex formation of Fbxw7 and DISC1 (chapter 4). Having identified Fbxw7 as a putative E3 ligase involved in regulating DISC1 turn over, my studies focused on developing the Fbxw7 – DISC1 interaction as a putative drug target. Using advanced peptide array technology, it has been possible to map potential Fbxw7 docking sites on the DISC1 CPD that aligns with a characterised consensus Φ-X-Φ-Φ-T- P-P-X-S (Φ- hydrophobic amino acid, X – any amino acid) (Orlicky et al., 2003). Phosphorylation dependent substrate recognition and SCF E3 ligase recruitment has been a paradigm confirmed by several studies (Orlicky et al., 2003, Hao et al., 2007a, Nash et al., 2001, Skowyra et al., 1997). My

peptide array studies show that *in vitro* DISC1 CPD interaction with Fbxw7 is enhanced following dual phosphorylation (chapter 4), however, the kinase responsible for the phosphorylation is not yet known. As seen in other Fbxw7 substrates it is likely that phosphorylation within the CPD may trigger a conformational change, which makes it accessible to Fbxw7 (Orlicky et al., 2003).

In the present chapter, we explored the potential of peptides as a proof of concept in drug development for psychiatric illness. I proposed that by targeting DISC1-Fbxw7 complex formation and interfering with ubiquitination mediated degradation we could stabilise DISC1 protein. Using structure activity relationship studies, a peptide was developed which had a potential to stabilise DISC1 protein levels in the cell by modulating DISC1-Fbxw7 interaction. Of the 4 lead peptides, two peptides (139 and 142) have shown a significant effect. Intriguingly, these two peptides had mE and mD substitutions at the phopsho sites. It can be hypothesised that the phospho groups are crucial in the interaction and the N methyl capping of the phosphomemitic amino acids (mE and mD) therefore retain the negative charge, increase membrane permeability as well as increase resistance to proteolytic degradation in cells. My studies also show that the peptide is specific to the DISC1-Fbxw7 interaction as the peptide treatments did not alter endogenous levels of other known Fbxw7 substrates such as c-Myc, c-Jun, Notch1 and Cyclin-E. It is crucial to demonstrate this specificity as altering the stability of the other substrates may be tumorigenic which is not desirable. The specificity of the peptide is probably due to the differences in affinity that are seen between Fbxw7 and DISC1 compared with the other substrates. The Baillie lab has calculated the affinity of Fbxw7 for the dually phosphorylated DISC1 as 1µM (using isothermal calorimetry) and this is weak compared with the 65 nM affinity of Fbxw7 for cyclin E (Orlicky et al., 2003). So, it is possible that the weak DISC1-Fbxw7 interaction can be disrupted by the peptide but the others cannot.

It would be interesting to investigate the ability of the peptides to disrupt the DISC1-Fbxw7 interaction in vivo, however, this was not within the scope of this project as peptides are thought not to cross the blood-brain barrier. It should also be noted that all

the recommendations made for amino acid substitutions to increase the peptide efficacy were not possible to screen (in my peptide optimisation screens) as some of the non-natural amino acids (Sar, β-H-ala, βA, Pip, Tic) were not available at the time of this study. Implementation and characterisation of these substitutions may further increase the potency of the peptide.

Synthesis and purification of these synthetic peptides is extremely time consuming and extreme care should be taken while handling and storage as they are susceptible to oxidation and bacterial degradation. Despite the generic complications in developing peptide drugs, they do come with certain advantages such as specificity, potency, smaller size and their cost of production has been improving with advancements in the field of synthetic chemistry making them more accessible. They are also less immunogenic and can have higher tissue penetrance (but probably do not cross blood-brain barrier (Vlieghe and Khrestchatisky, 2010). Since the degradation products of the peptides are amino acids, they pose a minimum risk of systemic toxicity and side effects. The rapid increase in the marketing of synthetic therapeutic peptides is a positive signal, which points to the potential of more research into and preference for this class of drugs by pharmaceutical companies.

The following review provides a list of all the peptide drugs released in to the market to date which include peptides ranging from the size of 2 amino acids (Naaxia for conjunctivitis) to 41 amino acids (Acthrel for Cushing's syndrome) (Vlieghe et al., 2010). However, it is important to consider that a potential CNS drug targeting psychiatric illnesses should ideally cross BBB (blood brain barrier) either by passive diffusion or carrier mediated active transport. Research over the past decade focused on developing a novel drug delivery system to evade BBB, reviewed (Chen and Liu, 2012).

As the present study sheds light on new DISC1 interactions and the significance of DISC1 turnover regulation, it also opens new exciting avenues of research. DISC1 directly interacts and negatively regulates the enzyme activity of GSK3β, preventing it from

phosphorylating β-catenin (De Rienzo et al., 2011). Two different domains (amino acids 1-220 and 356-595) on DISC1 have been mapped to directly interact with purified GSK3β protein using in vitro binding assays (Mao et al., 2009b). This finding is crucial, as the DISC1 CPD (amino acids 196-203) lays well within the proposed GSK3β interacting domain. It is worth noting that GSK3β phosphorylates within the CPD of the other known Fbxw7 substrates triggering subsequent ubiquitin conjugation (Pérez-Benavente et al., 2013, Welcker et al., 2003, Wei et al., 2005, Kitagawa et al., 2009). GSK3β activity has already been implicated in several psychiatric diseases such as SCZ, autism and Alzheimer's disease and has been a common therapeutic target for few decades. 40% reduction in GSK3β protein, transcript levels and activity has been observed in post-mortem frontal cortex of schizophrenic patients (Kozlovsky et al., 2000, Kozlovsky et al., 2001, Kozlovsky et al., 2004). As a kinase with a large number of substrates, it can be hypothesised that GSK3β may also play a crucial role as an upstream regulator of DISC1 ubiquitination; however, much more work is needed to verify this. Further characterisation of the lead peptides developed in the present study is absolutely necessary and may provide insight in to the DISC1 pathway. Exploring how these peptide affect DISC1 interactions with other proteins identified as risk factors for neurological diseases such as DIXDC1, GSK3β, Ndel1 will provide molecular insights which is otherwise impossible to obtain in genetic or clinical studies.

5.4.1 Use of iPS cells and differentiated NPs and neurons as models to facilitate drug discovery

In the last section, my study explored the potential use of iPS cell technology in disease modelling and drug discovery. Until recent years, iPS cell generation and differentiation has been perceived as a strenuous, long and expensive procedure. It should be noted that the first publication on generation of human iPS cells was only 6 years ago and there is still much speculation about their potential in drug development. Considerable advancements have been made lately to improve iPS reprogramming techniques and this new advanced technology can be considered as a boost for neuroscience research. Apart from serving as indefinite supply of human neurons, these cells also serve as a medium that enables the study of human psychiatric illnesses in a neuronal context. Researchers

have successfully employed this approach in modelling other neuronal diseases such as Parkinson's, Alzheimer's, and Huntington diseases (Wernig et al., 2008, Zhang et al., 2010, Yahata et al., 2011). Non clinical drug evaluation is extremely important before advancement into human clinical trials. Employing iPS cells and differentiated cell lines of interest serve this purpose efficiently as they can compare disease and patient specific profiles from a similar genetic makeup.

The present chapter describes the results of a study conducted to utilise iPS cells for studying disease mechanisms involving a DISC1 mutation. I also highlight the possible downstream application of iPSc and their diiferentiated derivatves for drug screening. Interestingly, the proteasome inhibitor MG132 stabilised endogenous DISC1 protein levels in the control and patient neuronal progenitors (NPs). Although, we had seen this in a model line (HEK293), it was reassuring that the phenomenon was recapitulated in the NPs. Immunoprecipitation experiments to show ubiquitin chains in the NPs were not successful and required further technical optimisation to increase the concentration of DISC1-ubiquitin conjugates in the sample to levels that could be detected by biochemical methods.

Gratifyingly, immunoblots and quantitative real time PCR data indicated that Fbxw7 and DISC1 displayed reasonable expression levels, though comparative expression patterns between the control and patient NPs could not be established because of the low number of replicates. Lead peptides which successfully stabilised DISC1 in HEK293 cells (139 and 142), were tested on the NPs generated from control and patient iPS cells. Due to time constraints, only two replicates were possible. These preliminary set of experiments show increased DISC1 levels. However, more biological replicates originating from different sets of neuronal differentiation are crucial in establishing the effect of the peptides.

While the potential use of iPS cell technology is an exciting advance for neuroscience research, crucial challenges need to be addressed. First and foremost, is the possible

genetic variability between iPS cell clones which may be attributed to genetic recombination or integration of episomal vectors or virus in to the chromosomal DNA. Still in its infancy, methods to improve the quality and efficiency of iPS cell generation (reprogramming) are still under way. The protocols employed to differentiate iPS cells into desired cell type (media, serum and growth factors) also plays a crucial role in replicating the cell properties. Future research into development of cell type specific differentiation protocols, phenotype and genotype characterisation is necessary.

5.5 Chapter summary

To summarize, the data presented in this chapter explores the potential of PPIs (substrate-E3 ligase) as therapeutic drug targets. The lead peptides developed, efficiently and specifically disrupted DISC1-Fbxw7 interaction in HEK293 cells. iPSCs differentiated NPs were explored as cell line model as they represent a powerful new approach to capture the genetic background from human individuals with confirmed diagnoses of SCZ. With the provided framework, future experiments should involve complete characterisation of the pateint derived iPS cells and their differentiated derivatives (such as morphology, DISC1 and Fbxw7 relative expression and how their pattern of expression alters in relation to the various stages of differentiaon). I speculate that this may provide crucial information and possibly recapitulate disease onset physiology at the molecular level. Investigating the physiological effects (such as neurite outgrowth, number) of the lead peptides in terminally differentiated neurons derived from the iPS cells may also expand our knowledege on how DISC1 function contributes to neuronal function.

6.

HTS for small molecules that modulate DISC1-Fbxw7 interaction

6.1 Introduction

PPIs (Protein-Protein Interactions) mediate many cellular signal transduction systems, yet notoriously challenging to target due to their large molecular surfaces, lack of profound pockets and paucity of appropriate screening libraries, which can target these interactions. One strategy to overcome these difficulties, is to target so-called hot spots, a recently evolved concept in PPIs (Mullard, 2012). Although the complex nature of PPIs is dictated by physical features and forces that hold binding partners together are challenging to target, certain interactions have a small binding regions, often termed as 'hot spots', on at least one of the partners that can be singled out. The concept of the 'hot spot' was based on an observation that the bulk of free energy is not evenly distributed on the protein surface but concentrated on certain residues (amino acids), which are crucial for the interaction (Lea and Simeonov, 2011). Advancements in protein mapping, crystallography and computational analysis led to identification of PPIs as more approachable and promising drug targets, opposing to the conventional view.

'Small molecules" are organic molecules with a molecular weight less than 900 Daltons , which target certain biological events such as PPI or enzyme activity (Arkin and Wells, 2004). The upper molecular weight limit for the small molecules increases the likelihood of cell permeability and their potential to target intracellular sites. Most of the commonly used drugs are small molecules, as they have an added advantage of metabolic stability and bioavailability over peptide drugs. As an example, the drug RG7388, developed by

Roche Pharmaceuticals to target p53-Mdm2 interaction (RG7388 is a selective, potent Mdm2 antagonist) stabilises and activates p53 and is currently in clinical development for cancer therapy (Ding et al., 2013). Projects such as this, have garnered enthusiasm of pharmaceutical firms for small molecule inhibitors of PPIs.

Once a suiable drug target is identified, different approaches can be employed to identify compound modulators. HTS is one such widely used approach discussed in section 6.2. Functional assays are used in the first round to identify compounds from large chemical libraries which give positive or negative response known as 'hits' that become the starting points of drug discovery. A chemical or compound library consists of stored compounds with associated information about each compound such as structure, molecular weight, purity, and other biophysical properties. Follow up secondary assays are used to test for specific artefacts and false positives identified in the primary screens.

In the previous chapter, using advanced peptide array technology and structure activity relationship studies, we successfully developed and optimised lead peptides, which stabilise DISC1 levels in HEK293 cells. My work so far explored the potential of the novel PPI, DISC1-Fbxw7 as a drug target for psychiatric illnesses. I see this as a promising drug target, as loss of DISC1 function is one of the proposed disease mechanisms (Hikida et al., 2012).

A similar approach has been taken to target Fbxw7 mediated regulation of Cyclin E signalling as a therapeutic intervention for cancer (Hao et al., 2007a). A FP assay was constructed from pure protein and peptides that correspond to the binding interface. This assay was used to perform a HTS on a 50,000 compound library which identified SCF-I2 (assigned name) to cause inhibition of Sic1 (yeast Cdk1 inhibitor) ubiquitination (Orlicky et al., 2010). Crystal structure elucidation revealed that SCFI2 elicits its inhibitory effect by an allosteric mechanism (WD40 domain inhibitor) which was the first of its kind to be identified (Orlicky et al., 2010). Another well-known example where a substrate-E3 ligase interaction was targeted for drug development was that of the p53-Mdm2

complex. Ubiquitination of p53, a tumour suppressor and transcription factor, is tightly regulated by the Mdm2 protein (E3 ligase). Mdm2 gene overexpression leading to inhibition of p53 has been shown in many malignancies. Hence, the disruption of p53-Mdm2 complex represents a suitable drug target for cancer therapy (Vassilev et al., 2004). Close analysis of the crystal structure of Mdm2 revealed the presence of a hydrophobic pocket that was involved in the interaction with the p53 peptide. This area constituted the transactivation domain and was ideal for targeting with small molecules. To this end, a number of HTS studies were undertaken that led to the development of Nutlins (imidazole analogues), which successfully displaced p53 from the Mdm2 leading to subsequent stabilisation of p53 protein (Vassilev et al., 2004).

In the present study, one of the shortest DISC1 peptides identified to be interacting with the SCF^{Fbxw7} complex in the peptide array studies was an 7-residue stretch of DISC1, which is dually phosphorylated at T^{198} and S^{202}. This DISC1 peptide was chosen for *in vitro* assay development in light of the structural model of Skp1-Fbw7-Cyclin E elucidated by Bing Hao and colleagues (Hao et al., 2007a). As a part of this study by Hao et al, purified Skp1-Fbxw7 was co-crystallised with Cyclin E peptides - $CyclinE^{degC}$ peptide ((residues 360–390, phosphorylated at Ser372, Thr380, and Ser384) and $CyclinE^{degN}$ (residues 55–68, phosphorylated at Thr62). Crystallography studies uncovered the residues of these peptides that make contact with the WD40 domain of Fbxw7. Since the DISC1 CPD motif is remarkably similar to that of Cyclin E, the labelled DISC1 peptides to be used as tracer molecules were designed in order to test whether a similar interaction may also be possible with DISC1. I developed an *in vitro* FP assay to validate binding of the identified and optimised DISC1 CPD peptide. The assay was also fit to screen small molecule libraries; a wide spread approach taken in drug discovery and development. Ultimately, identified and validated hits from this approach could then be used to test the hypothesis that by targeting DISC1-Fbxw7, DISC1 protein levels can be regulated.

6.2 High Throughput Screening (HTS)

High Throughput Screening (HTS) is a process of assaying libraries containing large numbers of compounds to identify potential effectors of biological activity against biological events or targets. HTS based studies are widely employed by the pharmaceutical industry in the initial stages of drug discovery and development. They enable testing of large chemical libraries of compounds categorised by their backbone structure, size or specific chemical groups, for their activity in a range of cellular and non-cellular assays. Testing up to 100,000 compounds per day has become feasible by the gradual replacement of cuvettes and dishes with 96 to 3456 well micro well plates and the recent advent of automated robotic liquid handling workstations. This approach has garnered much popularity in recent years due to the increased availability of chemical libraries and the miniaturization of the sensitive assays (Shinohara and Uesugi, 2007) (Duffy et al., 2013). HTS constitutes a primary screen to identify 'hits' (positive response) from the compound library, with suitable positive and negative controls. In general, primary screens are less quantitative, tested only in singlets or doublets, usually within a concentration range of 1-10 µM (which may again vary depending on the type of assay and the biological event being monitored). The short listed compound hits are then re-tested in secondary assays, which are more accurate and used for quantification and to determine the IC_{50} following construction of dose response curves and time response read outs depending on the assays. A confirmed hit exhibits the same activity in the secondary assays, which is then taken forward to tertiary screens or counter screens for optimisation. Compounds that hold biological activity after this process are often termed as 'lead compounds'. Secondary screens play a crucial role in excluding false positive hits. Fluorescence polarization/anisotropy (FP) and Fluorescence/Foerster Resonance Energy Transfer (FRET) are homogenous assays preferred by screening groups, as they are solution-phase assays. Having fewer assay components, simple assay steps and protocols, they are easier to develop than heterogeneous assays such as ELISA (Enzyme Linked Immuno Sorbent Assay) which has multiple components, incubations and wash steps. Following the identification of a suitable drug target, it is important that the assay selected for HTS meets certain criteria, such as being executable in 384 or 1536 well plate format and response readout via a single parameter (Lea and Simeonov, 2011).

6.2.1 Fluorescence Polarisation Assay (FP)

This assay was first described by Jean Perrin in1926 (F, 1926) (Lea and Simeonov, 2011) and gradually expanded by Gregorio Weber and colleagues in the 1950's. This technique has since been in use to characterise antigen-antibody, hormone –receptor and ligand-protein interactions. Since mid-1990 FP assay has been adapted for use in high throughput screening.

Fluorescence based screening methods are rapidly gaining attention in assay development. In recent years, FP assays have become increasingly popular and been used for quantitative analysis of enzyme activity (Zhang et al., 2007) and various molecular interactions (Kimple et al., 2008). The format of this assay, which is mix-read with no wash steps, also makes it a popular choice in the field of drug development. This assay is homogenous, solution based and highly sensitive and sometimes can use just Pico molar concentrations of the fluorescent probes.

6.2.1.1 Principle of FP assay

The principle of a FP assay is the measurement of the molecular size-dependent change in the polarisation of plane polarised light. When plane polarised light is passed through a sample, the fluorescent molecules in the sample are excited and emit light in the same plane if the fluorescent probe is stationary. However, if the fluorescent molecule tumbles and rotates in various planes in its excitation state, it tends to emit light in a different plane than the one in which it was excited. Larger molecules tend to rotate and tumble at a slower rate, hence the emitted light, following their excitation remains highly polarised. If a molecule is smaller, rotation and tumbling is faster and hence the light is depolarised relative to the excitation plane. The extent of polarisation is read out as polarisation units/ milli polarisation units (mP) (Lea and Simeonov, 2011).

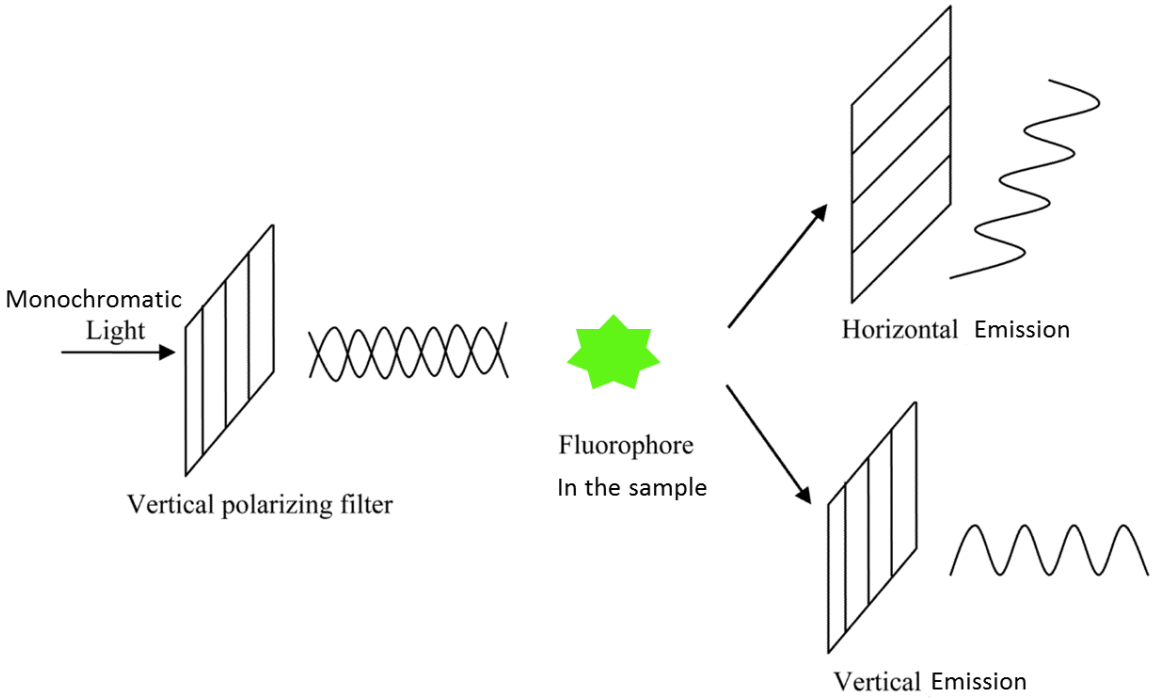


Figure 6.1 Schematic representation of FP detection system.
After monochromatic light passes through a vertical polarizing filter, it excites the fluorescent molecules (also known as tracer) present in the sample. Only molecules oriented vertically to the plane polarised light absorb this light, become excited and subsequently emit this light. The emitted light is measured in horizontal and vertical planes. Adapted from (Lea and Simeonov, 2011).

The molecular weight of the fluorophore tagged molecule (tracer) is directly proportional to the emitted polarisation values. When the fluorescent molecule is unbound, it rotates faster in the excitation phase and hence, the emitted light has low polarisation values. However, when it is bound to the protein complex, it rotates slower and hence the polarisation values are high. The extent of molecular rotation of the tracer during excitation and emission is measured in polarisation units (1P polarisation unit = 1000 mP units) which is calculated as shown in the equation below.

$$\text{Polarisation Value (P)} = \frac{\text{Intensity (vertical)} - \text{Intensity (horizontal)}}{\text{Intensity (vertical)} + \text{Intensity (horizontal)}}$$

.

Equation 6.1

In the above equation, Intensity (vertical and horizontal) represent intensity of light emitted, vertical and horizontal to the excitation plane. Applying the principle of FP, we set out to develop an assay to monitor the displacement of FITC (Fluorescein isothiocyanate) labelled DISC1 CPD peptide from the Skp1-Fbxw7 complex.

6.2.2 Ligand-Receptor kinetics - Basics

Before discussing the results, it is important to understand the ligand-receptor kinetics and the relevant terminology. Ligand-receptor binding is generally explained on the basis of the 'law of mass action', which assumes that ligand (L) binds to free receptor (R) to form a Ligand –Receptor complex [LR]. Assuming this binding is reversible and not taking potential non-specific binding into account, the rate of formation of [LR] is equal to rate of dissociation of [LR] at equilibrium.

$$[L] + [R] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [LR]$$

Equation 6.2

[L] and [R] represent free ligand and receptor respectively. k_{on} and k_{off} represent the kinetic association and dissociation constants. It is important to note that this equation is valid under the assumption that all receptors and ligands are equally accessible, while the binding does not alter either of them.

Equilibrium can thus be represented as following:

$$\frac{[L] + [R]}{[LR]} = \frac{k_{on}}{k_{off}} = K_d$$

Equation 6.3

The concentration of the free ligand and free receptor are related at chemical equilibrium, determined by K_d. K_d is the equilibrium dissociation constant, which can be

defined as the concentration of the ligand at which half of the receptor sites on the protein are occupied. The K_d of a ligand is inversely proportional to its affinity towards the receptor, i.e., the smaller the value of K_d the stronger is the affinity.

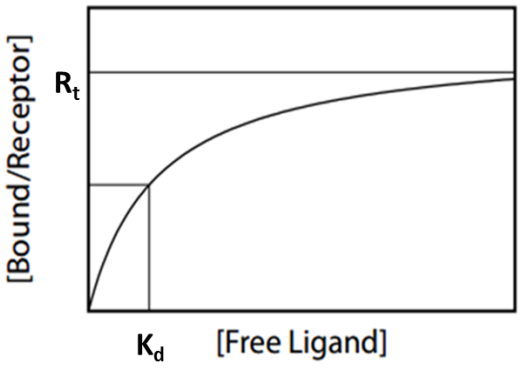


Figure 6.2 Simulated hyperbolic curve representing the binding kinetics of receptor and ligand.
Rt – Total receptor concentration, Kd – Dissociation constant.

Depending on the nature of the inhibitor, the following types of scenarios could be possible:

Competitive inhibition occurs when the inhibitor binds to the receptor's active site reversibly, by competing with the ligand via steric hindrance according to the law of mass action. Competitive inhibitors can be 'isosteric' when they bind at the ligand binding site of the receptor or allosteric if they bind at a proximal ligand binding site, affecting ligand binding at another site by inducing a conformational change in the receptor. The IC_{50} is defined as inhibitor concentration at which 50% of the bound ligand has been displaced form the receptor binding sites. IC_{50} values are variable depending on the concentration of the ligand, inhibitor and that of the receptor. In the presence of a competitive inhibitor, K_d of a ligand effectively increases. Most of the modern drugs are being developed on the basis of competitive inhibition of enzyme activity or ion channels. ATP mimetic protein kinase inhibitors belong to this class. In the present study, a competitive FP assay has been used where an unlabelled ligand (peptide or test compound) and a fluorescently labelled DISC1 CPD peptide compete for binding to a Skp1-Fbxw7 protein complex.

Non-competitive inhibition is also a type of reversible inhibition, which occurs when the inhibitor binds to a different site on the receptor than the natural ligand and inhibits the biological function of the receptor. This type of inhibition results in formation of [LRI] and [RI] complexes which are non-functional; (R Receptor, L ligand, I inhibitor).

The following references would be useful for a review of this concept in depth - (Krohn and Link, 2003, Prystay et al., 2001, Fang, 2012).

6.2 Experimental aims

Studies outlined previously in this thesis have demonstrated that the conserved CPD motif in DISC1 is responsible for DISC1 interaction with the Fbxw7 protein of the Skp1-Fbxw7 protein complex (chapter 5). In addition, peptide array studies also revealed that dual phosphorylation within this motif enhances Fbxw7 interaction *in vitro*. As we targeted this novel PPI with disruptor peptides in the previous chapter, here we investigated if this can be targeted by small molecules.

The aims of the experimental work detailed in this chapter were:

1. To develop and optimise a quantitative, reliable FP assay to quantify and compare the binding affinities of DISC1 CPD peptides (singly and double phosphorylated).

2. Adapt the optimised FP assay to screen large compound libraries in High throughput screens (HTS) to identify inhibitors and activators targeting DISC1-Fbxw7 interaction.

3. Perform secondary screens to confirm the hits identified in the HTS.

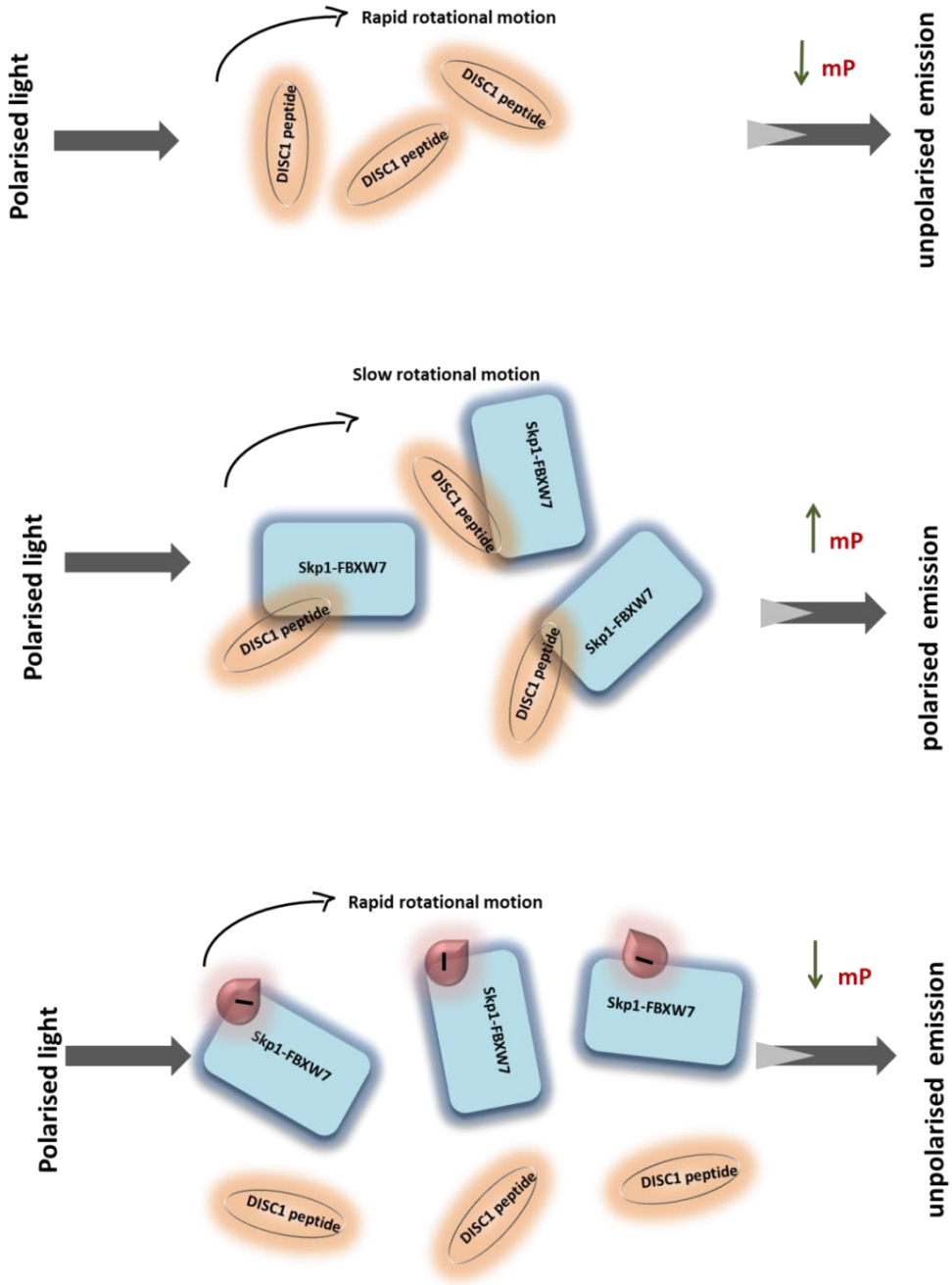


Figure 6.3 Schematic of targeting DISC1-FBXW7 with small molecule inhibitors. Schematic illustration of the FP assay principle in relation to protein-peptide or protein–ligand interactions (Skp1-Fbxw7 complex with fluorescent DISC1 peptide or small molecule inhibitor (I-red sphere)).

In general, a macromolecule is designated as the receptor [R], which is the Skp1-Fbxw7 protein complex in the present scenario and the binding counterpart as ligand [L], labelled DISC1 peptide. The ligand, DISC1 CPD peptide, has been developed and

characterised (chapter 4) after mapping the precise binding sites using peptide array technology. This DISC1 peptide, labelled with the FITC fluorophore, gives low mP values (milli polarisation units) as a read out when free in solution (unbound state) and high mP values when bound to the Skp1-Fbxw7 protein complex. In competitive inhibition, mP values decrease proportionately with the displacement of the fluorescently labelled DISC1 peptide by an unlabelled competing peptide or small molecule inhibitor.

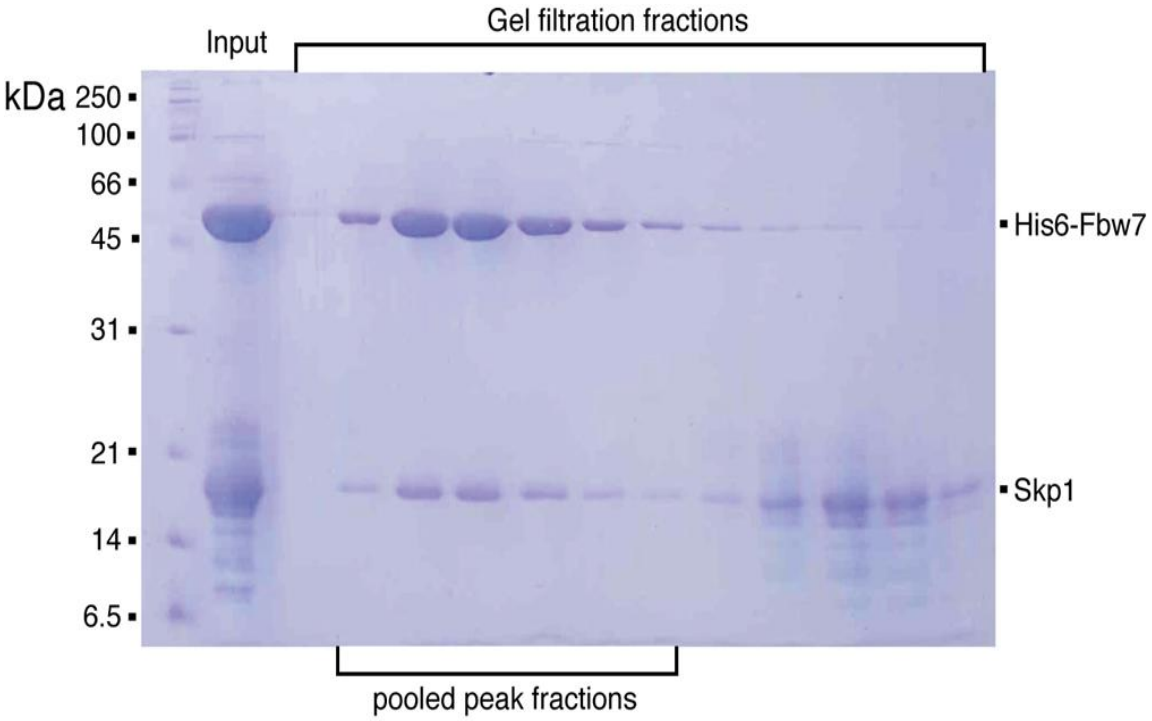


Figure 6.4 Purified recombinant Skp1-Fbxw7
Coomassie stained gel picture showing resolved 6His-tagged Skp1-Fbxw7 recombinant protein complex (in denaturing conditions). Each column represents one fraction collected from column gel filtration. The His6 tag on Fbxw7 was then cleaved by TEV protease (a 27 kDa catalytic domain of the Nuclear Inclusion a (NIa) protein encoded by the Tobacco Etch Virus). This protein complex serves as a receptor to which the DISC1 CPD peptide binds in the FP assay. This protein complex was purified by Yunfeng Li, Prof. Bing Hao's laboratory, University of Connecticut, USA.

6.3 Results

6.3.1 Development and optimisation of a FP assay based on the DISC1 peptide -Skp1-Fbxw7 complex

Initial binding experiments were performed to evaluate the linearity of the assay, which is in turn dictated by the stoichiometric ratio of the ligand and the receptor. High concentrations of the sample would inhibit the passage of light, which causes excitation of the tracer molecules. Low concentrations may cause the molecules closer to the light to absorb too much light so that little is available for the rest of the molecules in the sample. This could affect the linearity of the assay. The following peptides (Table 6.1) were labelled with the FITC fluorophore to determine their binding affinities to the Skp1-Fbxw7 protein complex.

Table 6.1 Labelled peptides used in the present study

Peptide	**Sequence**	**Phosphorylation status**	**Reference**
DISC1 pTpS	FITC-V^{195}PP**pT**PPG**pS**H^{203}	Doubly phosphorylated	
DISC1 pT	FITC-V^{195}PP**pT**PPGSH^{203}	Singly phosphorylated	
Cyclin E	FITC- G^{377}LL**pT**PPQSG^{385}	Singly phosphorylated	(Hao et al., 2007a)

The peptide sequences are represented from N to C terminus in bold letters with FITC tag on the N terminus. The amino acid residues were indicated in high case, lower case indicates phospho group on the following amino acid (p). Cyclin E CPD peptide was used as a positive control to analyse the efficiency of the assay as this peptide has been characterised in a previous study by Bing Hao and colleagues (Hao et al., 2007a).

Saturation binding experiments also determine the measure of specific receptor (protein complex) binding of labelled ligand (peptides in the present study) at equilibrium. By measuring the labelled ligand concentration required to saturate the receptor binding sites, these experiments are used to determine K_d (Equilibrium dissociation constant).

For the binding experiments, a range of concentrations of recombinant Skp1-Fbxw7 protein complex (Figure 6.4) was prepared by serial dilution in the assay buffer (PBS with 0.25% Tween20). 5 µL of the dilutions of Skp1-FXW7 protein complex were pipetted in quadruplicates into a black, low binding, round bottomed 384 well plate. This was followed by the addition of 5 µL of 0.1 µM labelled peptides (Table 6.1) making the total assay volume 10 µL. Plates were then read using a Mithras plate reader using a fluorescence polarisation protocol program which measures the polarisation values at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The concentration range of the protein and that of the tracer peptides were chosen to achieve a reasonable signal to noise ratio in the HTS screens. The concentration of the labelled peptide is one of the influencing factors of the assay efficiency.

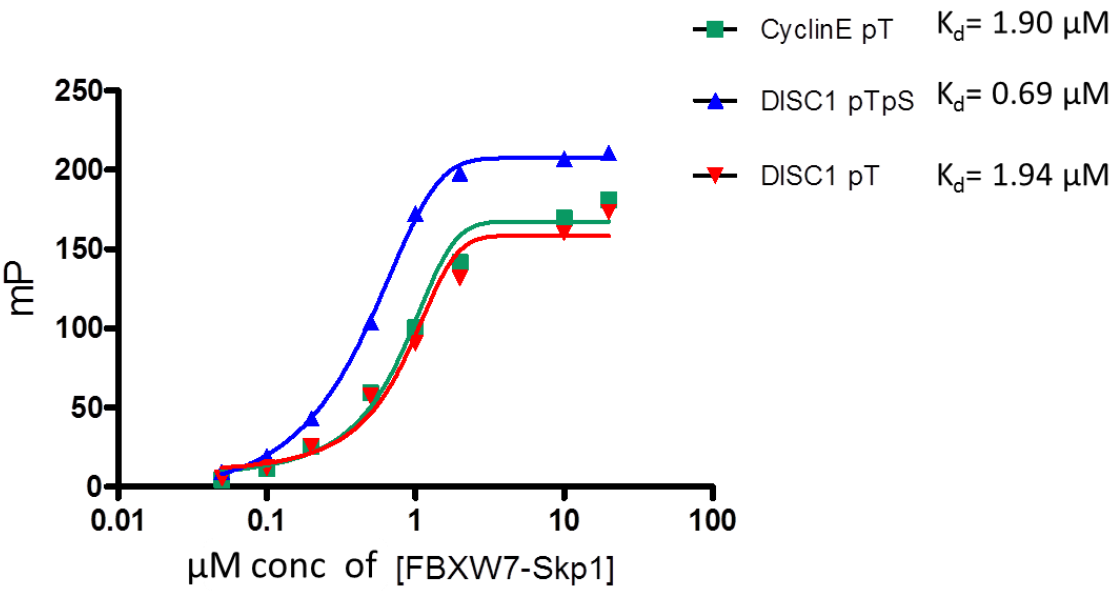


Figure 6.5 Saturation experiments and K_d determination
Saturation experiments were performed by titrating each tracer (FITC labelled peptides from Table 6.1) at 0.1 µM with increasing concentration of Skp1-Fbxw7 protein complex ranging from 0.05µM to 20 µM in the assay buffer (PBS with 0.25% Tween 20) with 0.001% DMSO. mP values are plotted as a function of Skp1-Fbxw7 concentration on a logirthmic scale. K_d values were calculated using non-linear least squares analysis, Prism5 software. n=3.

From the saturation binding experiments, a K_d value was determined for each tracer molecule as represented in Figure 6.5. Previous *in vitro* studies identified the doubly phosphorylated pThr380/pSer384 Cyclin E motif as high-affinity degron and a singly phosphorylated pThr62 motif as a low-affinity degron (Hao et al., 2007). Since Cyclin E CPD has close sequence similarity to that of DISC1, we expected similar binding affinities with DISC1 peptides. As expected, dually phosphorylated DISC1 CPD peptide (pT^{198}/pS202) (K_d 0.69) had 2.8 times more binding affinity over the singly phosphorylated DISC1 CPD peptide (pT^{198}/S^{202}) (K_d 1.94). The binding affinity of Cyclin E (pT) (K_d 1.90) peptide was also close to that of DISC1 pT peptide (K_d 1.94).

6.3.2 Competitive inhibition experiments

Competitive binding studies were performed to determine the concentration of a ligand/inhibitor required to reduce the specific binding of the labelled ligand to the receptor by half (50%) represented by the term Inhibitory Concentration or IC_{50}. IC_{50} is inversely proportional to the receptor binding affinity of the ligand. By determining and comparing the IC_{50} values of various ligands (compounds) determined in a particular experiment, the compounds can be ranked based on their relative inhibitory capacity and this measure can be used to identify the lead compounds for further optimisation and development. My initial experiments involved testing the ability of unlabelled DISC1 peptide T (P^{197}pTPPGpSH^{203} - peptide from which disruptor peptides was developed, chapter 5) to displace the labelled DISC1 peptide (DISC1pTpS) from Skp1-Fbxw7 protein complex. Competitive inhibitory effects of the lead disruptor peptides 139 and 142 (discussed in chapter 5) were also investigated using the principle discussed below. [Peptide 139 – G-mE-P-P-G-mE-H; Peptide 142 - G-mD-P-P-G-mE-H].

A disruptor peptide or small molecule inhibitor (represented as 'I' in the Figure 6.3) displaces the labelled peptide (bound) from the complex which then goes back in to solution (unbound) indicated by low mP values (schematic representation in Figure 6.3). Inhibitors that bind specifically to the protein complex at the labelled peptide binding site, as well as allosteric inhibitors, can be identified when they result in low polarisation

values (high depolarisation). Effective competitive inhibition by such an unlabelled peptide also suggests the suitability of the assay to identify other inhibitors.

The Optimised assay protocol for determining the IC_{50} values for the control (peptide T) and lead peptides (139 and 142) is as follows:

1. A range of concentrations of the competing peptide (test disruptor peptide) was prepared by serial dilutions ranging from 1000 µM to 0.20 µM (final concentration in the assay) in the assay buffer. 5 µL/well was pipetted in quadruplicates in a 384-well black, low-binding, round-bottomed plate. Plates were read for background fluorescence.

2. 5 µL of Skp1-Fbxw7 complex (0.4 µM final concentration in well) and fluorescent DISC1 peptide mix (FITC-VPPpTPPGpSH, 0.1 µM final concentration in well) in the assay buffer was added.

3. Plates were read in a Mithras plate-reader using a fluorescence polarisation protocol program (excitation and emission wavelengths of 485 nm and 535 nm respectively).

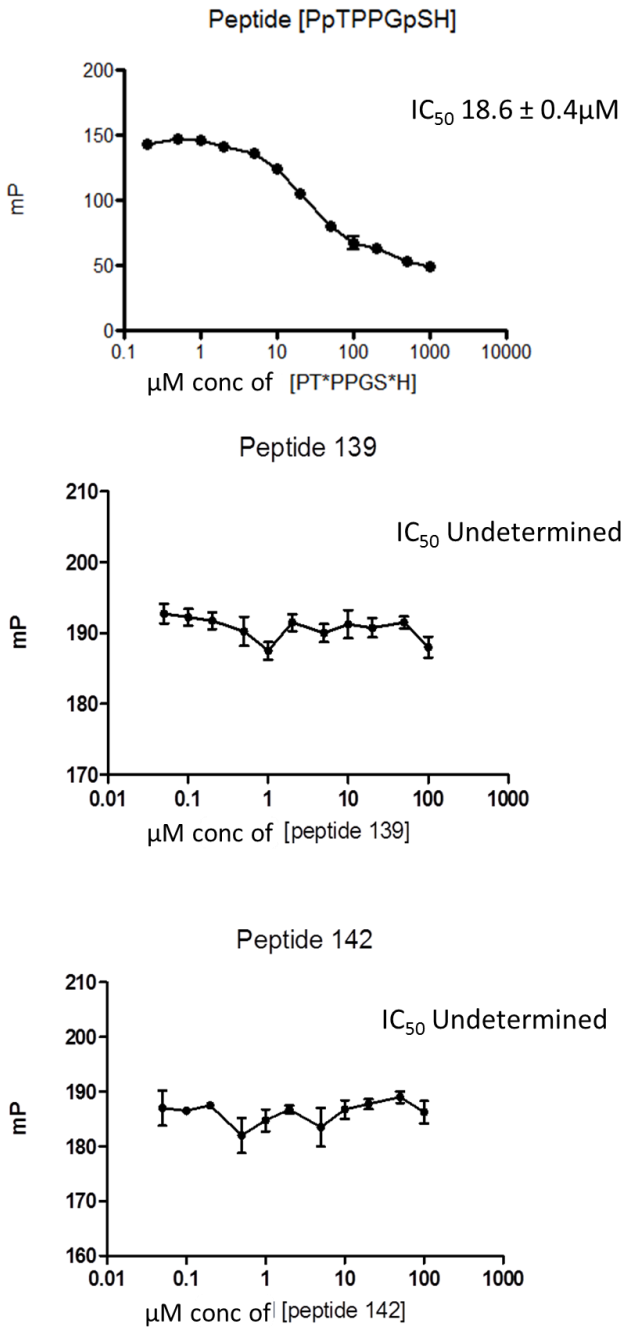


Figure 6.6 Competitive binding experiments
Competitive binding FP assay experiments were performed with unlabelled test peptide T, 139 and 142. A range of concentrations chosen for titration of competitive disruptor peptides were represented on X axis on logarithmic scale. IC_{50} values were determined by non-linear least squares analysis, Prism5 Software.

From Figure 6.6, it is evident that unlabelled DISC1 peptide T competitively inhibited the binding of labelled DISC1 pTpS peptide in a dose dependent manner as expected, with an IC_{50} of 18.6±0.4 µM (top graph). However, the lead peptides 139 and 142 (middle and bottom graphs) (discussed in chapter 5) did not disrupt the DISC1 peptide tracer from the Skp1-Fbxw7 complex. Though its unclear at this point why these peptides couldn't

displace the tracer molecule, we speculate that low affinites of the lead peptides could be one possible explanation.

6.3.3 Determination of Z’ factor

Before we proceed to employ this developed assay in HTS, it is mportant to determine the Z’ factor. A screening window coefficient denoted as Z’ factor determines the suitability of a given FP assay for HTS. Taking replicate error and signal to noise ratio in to account, the Z’ factor is determined by the equation below (Zhang et al., 1999).

$$Z' = 1 - \frac{3(SD_p + SD_n)}{|\mu_p - \mu_n|}$$

Equation 6.4

SD_p and SD_n are the standard deviation of the signals for positive and negative controls and μ_p and μ_n are the mean values for the positive and negative controls respectively.

This factor is defined by the means of positive and negative control and the data variation represented by the standard deviation associated with the control (positive and negative) measurements. The Z’ factor therefore indicates the capability of hit identification for an assay under defined conditions of screening and is a characteristic parameter of the assay quality independent of the test compounds. The Z’ factor value within a range of $0 < Z' \leq 1$ is meaningful and the higher the value, higher is the data quality of the assay, dictating the suitability of the assay (Z’> 0.5 is a good assay while a value of 1 indicates a perfect assay). Factors such as assay protocol and the efficiency of the instrument influence Z' factor and hence, before progressing to perform HTS is it important to optimise the assay for conditions, reagents, instrument, protocol and the binding kinetics which can be judged by Z' factor (Zhang et al., 1999).

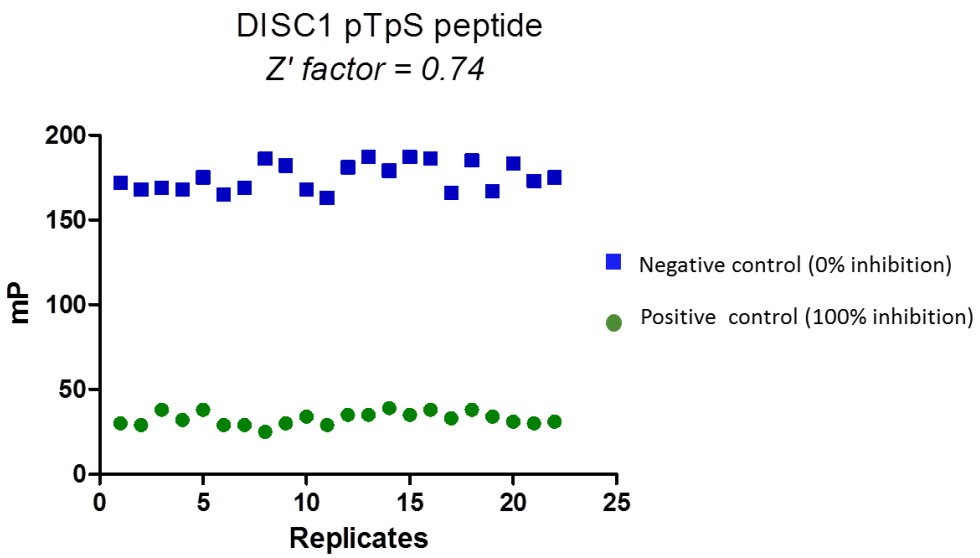


Figure 6.7 Calculated Z'-factor for competitive FP-based assays
Controls and Z'-factor for original competitive FP assay using labelled DISC1pTpS peptide (0.1µM) and Skp1-Fbxw7 protein complex (0.4µM). Positive controls representing 100% inhibition contain unbound labelled DISC1 peptide, while the Skp1-Fbxw7 protein bound labelled DISC1 peptide representing 0% inhibition was used as negative control. Each point represents a replicate assay to determine Z' factor.

Solving the equation 6.4 with the data from Figure 6.7, a Z' factor value of 0.74 was obtained which indicates that the designed assay and the conditions optimised are suiTable for the use of it for HTS (Zhang et al., 1999).

6.3.4 Adapted FP assay protocol for HTS

For the above described optimisation experiments, the following protocol was designed to perform HTS, also taking into consideration, reagent availability (purified recombinant protein and peptide) and the size of the compound library.

1) 5µL of each compound (10 µM final concentration in 1% DMSO) was added to each well (with a liquid handler availabe at respective screening facilities) in a 384-well black, low-binding, round-bottomed plate using a liquid handler. Plates were read for background Fluorescence.

2) 5 µL of Skp1-Fbxw7 complex (0.4 µM final concentration in well) and labelled DISC1 peptide mix (FITC-VPPpTPPGpSH, 0.1 µM final concentration in well) in the assay buffer was added.

3) Plates were read in a Mithras plate-reader using Fluorescence polarisation protocol program (excitation and emission wavelengths of with 485 nm and 535 nm respectively).

6.3.5 High Throughput Assay 1

Using my initial FP-based competitive binding assay, the assay conditions were optimised making this assay protocol amenable for identification of non-peptide, small-molecule inhibitors through HTS.

The Prestwick Chemical Library of 1200 small molecules which are approved drugs (FDA), were screened to uncover yet unrecognised targets. Prestwick Phytochemical library of 320 natural products mostly derived from plants were also screened in our first HTS. This library was screened at Biomolecular Screening & Protein Technologies CRG - Centre for Genomic Regulation, Barcelona, Spain.

Reactions were prepared in assay buffer (PBS with 0.25% Tween 20) and contained 5 µL of 0.4 µM Skp1-Fbxw7 recombinant protein, 0.1 µM of labelled DISC1 pTpS peptide mix and 5 µL of compound (in 1% DMSO) to make a final volume of 10µL. The order of addition of components and the steps involved in the assay is as follows: compound was added to the plate (5 µL each in singlets) and the plate was read for any background fluorescence of the compounds. This was followed by adding protein-tracer mix (total 5 µL) and the plates were immediately read to give the final polarization readout. Each plate had negative controls (equivalent to 0% inhibition) which constitute protein complex-tracer mix (5 µL) and 5 µL of assay buffer while the positive controls had the protein complex-tracer mix (5 µL) and 500 µM peptide T (5 µL) which gave 100% inhibition.

The screen was performed twice on the library on two different days using the same protocol to give two technical replicates. mP values of all the compounds were plotted on an XY scatter plot against the respective assigned compound ID (well number).

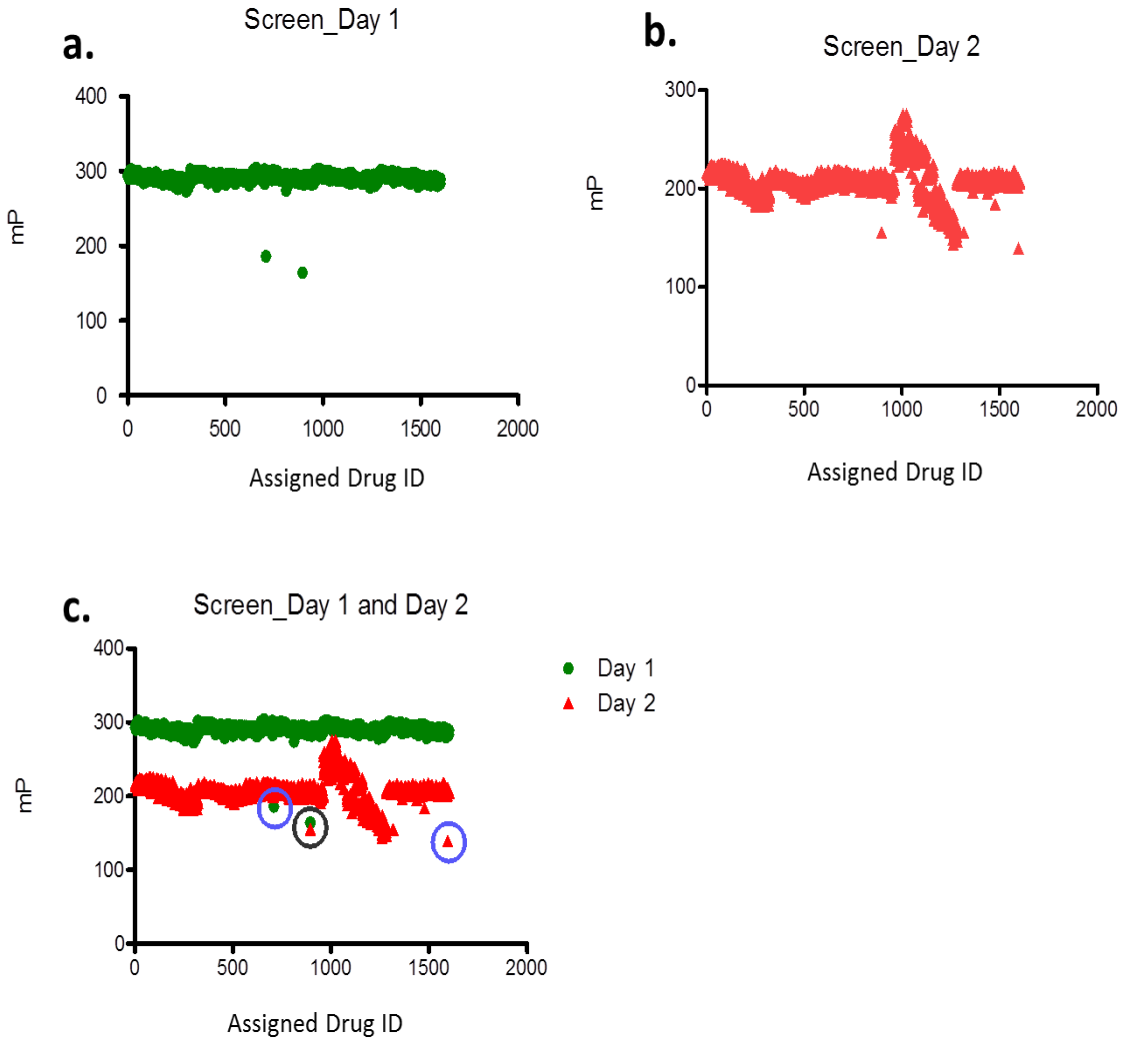


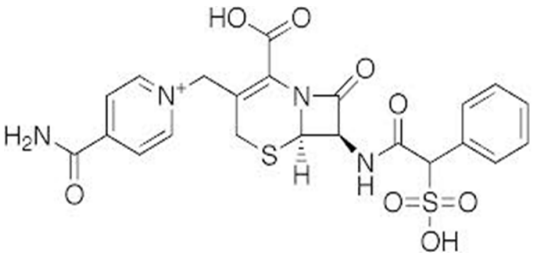
Figure 6.8 Summary of the HTS screen 1 (Prestwick chemical library and Prestwick phytochemical library).
Plot (a) represents data for day 1; plot (b) represents data on day 2. Plot (c) summary of the screen from both the days. mP values were plotted against the corresponding Drug IDs/well numbers on a XY scatter plot. The compound highlighted with green circle was shortlisted as a hit in the replicate screens was identified as Merbromin (CAS number - 129-16-8). The compounds highlighted in blue circle were the hits obtained only on day 2 screen identified as Cefsulodin (CAS number - 52152-93-9) and Fusaric acid (CAS number - 536-69-6).
This screen was performed by Dr.Tamara Martin and Jane Evelyn Findlay, University of Glasgow at Biomolecular Screening & Protein Technologies CRG - Centre for Genomic Regulation, Barcelona, Spain.

CAS number (Chemical Abstracts Service) is a unique numerical code assigned to every known chemical, independent of its structure, chemical nature nor properties. It is more useful in identifying a compound or chemical than its chemical name. Stereoisomers of a compound have different assigned CAS number (http://www.cas.org/content/chemical-substances). After the screen, the mP values were plotted against each well number (also the assigned compound ID) starting from 1. In Figure 6.8, the replicate screens performed were also summarised in a single graph to identify the hits. Compounds that have displaced labelled DISC1 peptide from the Skp1-Fbxw7 complex represented by a drop in mP value by at least 20 units (compared to the positive control) were considered for further analysis. Unfortunately, some distortions in the readouts of 5 plates were observed on the day 2 screen. This can be seen in the plots presented in Figure 6.8. The several other parameters that dictate the efficiency of FP assay have been discussed in detail in section 6.4.

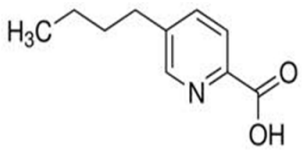
6.3.5.1 Secondary screens

Secondary screens are necessary to identify false positive hits or compounds with low affinities. Dose dependent experiments were performed to generate dose response curves which illustrate the relation between the inhibitor dose and the magnitude of the inhibition.

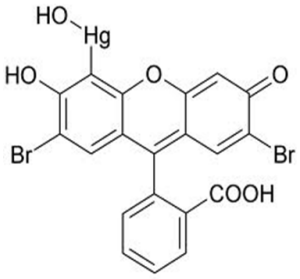
The identified compounds were shortlisted as putative hits (Figure 6.8) from the screen and were purchased from Sigma. Secondary screens which include experiments to identify any quenchers (substances which decrease fluorescence intensity) or false positives (example: auto fluorescent compounds) were undertaken. Compounds showing dose dependent inhibition needed to be identified for further characterisation.



Cefsulodin
IUPAC name: 4-(aminocarbonyl)-1-[((6*R*,7*R*)-2-carboxy-8-oxo-7-{[phenyl(sulfo)acetyl]amino}-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methyl]pyridinium



Fusaric acid
IUPAC name: 5-butylpyridine-2-carboxylic acid



Merbromin
IUPAC name: dibromohydroxymercurifluorescein

Figure 6.9 Chemical structures of the hits identified in HTS screen 1 with their generic and systemic names
(IUPAC -International Union of Pure and Applied Chemistry*).*

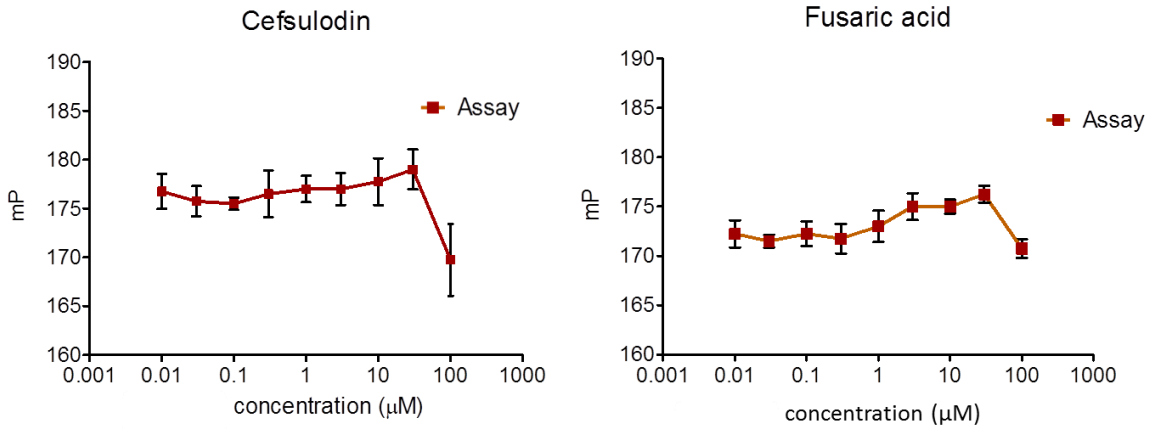


Figure 6.10 Dose response FP assay curves for Cefsulodin and Fusaric acid
mP values of the assay were plotted on Y axis against concentration of the compound on log scale on X axis.

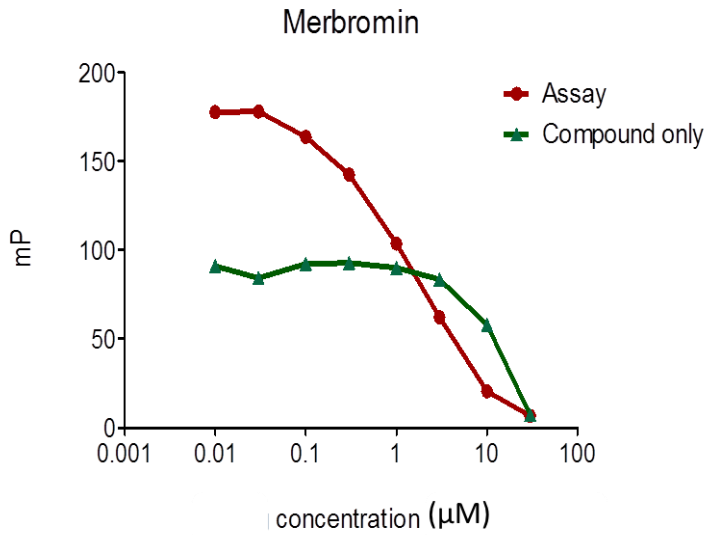


Figure 6.11 Dose response curve for Merbromin
mP values of compound only and assay were plotted on Y axis against concentration of the drug on log scale on X axis.

FP assays were performed according to the protocol described in section 6.3.4 The protein-tracer mix was titrated with various concentrations (0.01 µM – 100 µM) of the compounds and the resulting polarization values (mP, Y axis) were plotted against the

concentration values of the drugs on a log scale. The protein-tracer mix concentration and the volume was maintained the same in all experiments. Negative and positive control assays were also included.

As can be seen in Figure 6.10, neither Cefsulodin nor Fusaric acid produced any inhibition (low polarisation-mP values) of the protein-tracer binding. These drugs were eliminated as false positives, having failed to disrupt the protein-tracer complex concentration-dependently. Merbromin which was identified as a hit in both the replicate screens was investigated for its inhibitory effect. The polarisation values of the compound on its own (no protein or peptide added) decreased with its increasing concentration, which indicates that it is auto fluorescence (Figure 6.11, green curve). However, when the protein-peptide was added to increasing concentration of the compound, polarisation values start at a high value and gradually decreases ((Figure 6.11, red curve). This may indicate the binding of the compound to the protein complex at low concentrations, however, it is difficult to hypothesise on the increased polarization values at higher concentrations of the compound (in the assay) as the auto fluorescent nature of the compound could have a prominent effect on the sensitivity of the assay. A non-fluorescent assay is necessary to clarify the ability of Merbromin to modulate DISC1-Fbxw7 complex. Merbromin was disregarded as a possible lead.

6.3.6 High Throughput Screen 2

A second HTS was performed at Scottish Bioscreening Facility, University of Glasgow. 7000 compounds from Pharmacological Diversity Set (PDS) from Enamine, Kiev have been screened in this HTS. Compounds in the library were pre-plated and maintained as described previously (Duffy et al., 2013). The vendor for all the compounds in the library assured 90% purity (assessed by liquid chromatography–mass spectrometry and proton nuclear magnetic resonance spectrometry). The library was designed based on the predicted pharmacological properties of the compounds and each compound has been predicted to have nearly 3000 different biological activities (Duffy et al., 2013). Compounds were clustered based on their activity and irrespective of their chemical structures and all toxic compounds were excluded (Duffy et al., 2013).

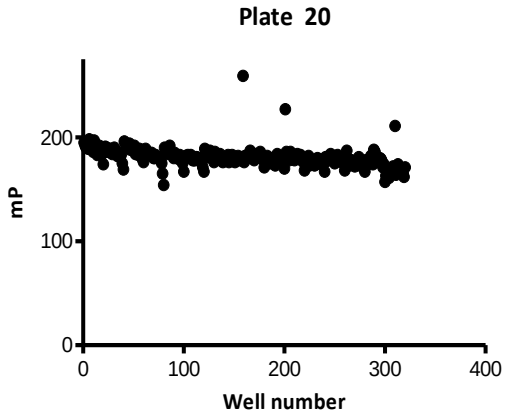


Figure 6.12 Representative graph of the HTS data from one 384 well plate (plate 20). Each well was assigned with a number. mP values (Y axis) were plotted against the corresponding well number (compound) on X axis.

Table 6.2 Putative activators identified in the HTS screen 2 based on fluorescence polarisation.

CAS number	Plate ID	Well ID	MW	mP
T0519-4556	90923-B-27	K11	497.574	220
T0519-5749	90923-B-27	C15	408.048	218
T5657824	90923-B-22	L03	359.475	200
T5682623	90923-B-20	H21	291.353	259
T0508-8329	90923-B-20	K03	277.326	227
T0503-3491	90923-B-20	P12	315.422	211

CAS (Chemical Abstract Service) number, microplate ID, well ID in the corresponding microplate, molecular weight (MW).

The above-mentioned library screen was performed as described in section 6.3.4. The assay conditions were maintained across both the HTS. However, due to limited availability of reagents such as purified protein complex and the peptides at the time the study, the complete library (total library size is 23,000 compounds) could not be screened. Refer to the appendix for the complete screening data.

The lower limit for the mP value read out for the compounds to identify potential hits (inhibitors) was set at 150 (based on the read out of the positive control wells in each plate). Unfortunately, none of the compounds inhibited the interaction. However, certain

compounds caused positive binding of DISC1 and Skp1-Fbxw7 complex, which implies that this PPI is stronger in the presence of these compounds (also termed as activator). However, secondary assays are required to confirm these observations. Table 6.1 depicts the activator hits identified in the screen. As can be seen in the Table, all the hits identified had their polarisation value above the set upper limit set as 200mP based on the positive control.

6.4 Discussion

The novel PPI (protein-protein interaction) of DISC1-Fbxw7, identified in the present study has been validated as a potential therapeutic target. In this chapter, a FP assay was developed, optimised for HTS and employed to identify small molecule inhibitors that disrupt binding of the DISC1 CPD peptide and Skp1-Fbxw7 complex. I propose that blocking the interaction between the two proteins by small molecules would inhibit DISC1 ubiquitination and subsequent proteasomal degradation of DISC1 protein.

My results, outlined in the present chapter support the feasibility of using fluorescence based FP assay to characterise molecular interactions and provides an in vitro assay for the DISC1-Fbxw7 interaction. This can be considered as useful technique to quantify the interaction while also being suitable for HTS. This represents an advance in the field, as one of the interacting partners, DISC1, is hard to purify as it has a high tendency to aggregate (Bader et al., 2012). Since the assay can be performed on a miniature scale, it is cost effective, while also being highly reproducible. Previous work using peptide array technology shows that both singly and dually phosphorylated DISC1 CPD (DISC1 pT198 and DISC1 pT198/pS202 peptides) were bound by the Fbxw7 protein. However, from my FP assays it was evident that the K_d of the DISC1pTpS was lower (0.69 µM) than that of DISC1 pT (1.2µM) suggesting the dual phosphorylation is required for high affinity binding of Fbxw7 enhancing DISC1 degradation. Developing mutants of these phosphorylation sites followed by ubiquitination experiments would be useful in understanding the relevance of this phosphorylation events to DISC1 stability in cells. This data is comparable with the previous findings where Fbxw7 was shown to recognise doubly phosphorylated CPD in CyclinE (Hao et al., 2007a) and β-TrCP1 (Wu et al., 2003). This

data also supports the proposed hypothesis of multi phosphorylated CPD mediated DISC1 interaction with Fbxw7and its subsequent degradation. Fbxw7 has a binding pocket within the WD40 domain and additional pockets on the surface exposed to the solvent. These pockets were found to make crucial intermolecular contacts with the phospho residues and the moieties flanking them (Wu et al., 2003, Hao et al., 2007a). Since the DISC1 CPD has a close resemblance to that of Cyclin E, we propose a similar binding model for DISC1 involving the phospho-residues.

Orlicky et al employed a FP assay to identify small molecule inhibitors of substrate recognition component of CDC4 (yeast homologue of Fbxw7) (Orlicky et al., 2010). A similar approach was taken in the present study to identify small molecule inhibitors of DISC1-Fbxw7 complex. During my optimisation experiments, control peptide (Peptide T) and the lead peptides (139 and 142) were tested for their ability to displace the labelled peptide from the receptor (Skp1-Fbxw7). Although, the control peptide nicely displaced the labelled DISC1 peptide in a dose dependent manner with an IC_{50} of 18.6±0.4 µM, lead peptides 139 and 142, which stabilised DISC1 levels in HEK293 cells (chapter 5), failed to disrupt the peptide-protein interaction. While this does not necessarily mean that the peptides do not target the PPI under study, we propose that more intense characterisation is needed and it is necessary to explore the factors that may interfere with the assay. In this context, it is important to note that FP may not be ideal for identifying weak interactions/inhibitors and is dependent on the peptide-protein K_d (Huang, 2003). This may be difficult to overcome by using high inhibitor or disruptor peptide concentrations, as it may lead to abnormal polarisation values due to aggregation or nonspecific binding (Lea and Simeonov, 2011). Hydrophobicity of the peptides may also have an influence on their activity and it has been shown that at high concentrations they tend to form micelle like particles (Lea and Simeonov, 2011). While peptide purification is a laborious task, it is important to note that their purity is crucial in dictating the sensitivity of an in vitro assay. The labelled peptides were obtained at the purity of about 85% and hence experiments using higher purity grade should be performed to evaluate the efficiency of the HTS (determined by Z' factor). Practical limitations such as limited purified protein availability restricted further *in vitro* characterisation of the lead peptides 139 and 142 in the present study.

In our first HTS (HTS1 – section-6.3.5) three compounds (Cefsulodin, Fusaric acid, Merbromin) were identified as hits and taken forward for secondary screens. As a complementary approach, a literature search was carried out which revealed that Cefsulodin is a narrow spectrum third generation analogue of cephalosporin, which is active only against Pseudomonas aeruginosa (Tsuda et al., 1988). It has been proposed that penicillin binding protein 3 (PBP3) is the principle target for this drug though it has not been characterised properly. Earlier studies implicated its use in combination with other antibiotics in treating haematological malignancies; other potential therapeutic uses could not be established (Tsuda et al., 1988). Fusaric acid is a mycotoxin produced by several species in genus Fusarium (belong to the family of filamentous fungi). It has been known to decrease plant viability and toxic to certain gram positive bacteria and mycobacterium (Fischer et al., 1980). However, its clinical use as antimicrobial is restricted. Its mode of action has not been established yet. A review by Hexiang Wang and T.B.Ng detailed the pharmacological effects of Fusaric acid on various physiological systems. One such effect is its potential to elevate dopamine levels while decreasing norepinephrine levels supporting its role as a dopamine β-hydroxylase (DBH) inhibitor (Wang and Ng, 1999, Fischer et al., 1980). DBH is a key enzyme involved in catalysing the conversion of dopamine to noradrenalin. Clinical studies have established an association between DBH gene polymorphism and smoking status within SCZ population (Zhang et al., 2012). I considered this as attractive hit as dopamine signalling dysfunction has been reported to play a key role in the aetiology of SCZ associated disorders as proposed by Stein and Wise, also known as the Dopamine hypothesis (Stein and Wise, 1971, Davis et al., 1991, Cubells et al., 2011, Ella et al., 2012, Zhang et al., 2012). The compound shortlisted as a hit in the two replicates of HTS1 is Merbromin (trade name mercurochrome). It is used as a topical antiseptic. It has been identified as a pan active inhibitor of various Protein methyltransferases (PMT) in an independent HTS screen (Ibanez et al., 2012).

In the secondary screens, Cefsulodin and Fusaric acid failed to displace the labelled DISC1 peptide from the protein complex and were considered as false positives. However, Merbromin was identified as an autofluorescent compound, evident from low mP values

with increasing concentration of compound on its own (with no protein-peptide mix added). As suggested earlier, a non-fluorescent assay should be performed before omitting this compound as a potential hit.

In the second HTS (HTS2, section 6.3.6) 7000 compounds were screened from Pharmacological Diversity Set (PDS) from Enamine (Kiev). Unfortunately, none of the compounds inhibited Skp1-Fbxw7 binding of DISC1. However, certain compounds (listed in Table 6.1) have shown increased polarisation (compared to the positive control), which may infer that in the presence of these compounds, the binding was stronger (or more receptor sites were occupied by the ligand). Assuming that these compounds may increase the DISC1-Fbxw7 interaction, they were shortlisted. It can be hypothesised that the hits may have a potential to directly or allosterically enhance DISC1-Fbxw7 interaction, which can only be confirmed after further secondary screens and characterisation. Due to financial restrictions and time constraint further secondary screens for the putative hits could not be performed. A gradual fall in the polarisation values was observed with increasing well number in all the plates in HTS screen 2 (data presented in the appendix of this thesis). I speculate that as it took nearly 20 minutes to read each plate, time drift may have caused a temperature increase leading to assay reagent evaporation which may have inturn resulted in a fall in the mP values.

From the two HTS campaigns carried out for this study, no dose-dependently active and chemically meaningful hits were obtained. Possible reasons for this are: Quality of the chemical library has a crucial role to play in the number of the hits of the screen. Only 8500 compounds were screened (total in screen 1 and 2) in this study, which is relatively small compared to the libraries screened by pharmaceutical companies constituting millions of compounds. Screening compound libraries posed major challenges such as auto fluorescence, quenchers, temperature control and evaporation due to low assay volumes.

A possible alternative to resource-demanding HTS of small molecule libraries is 'drug design'. Increasing availability of crystallographic protein structures and advancements in computers technology allows for virtual screening and in silico modelling. There has been much focus on developing algorithms which allow one to identify possible ligands. These can then be chemically synthesised or purchased if commercially available, and their binding properties analysed using conventional assays. Hits may then be optimized using further rounds of in silico screening/modelling and conventional assays. In simple terms, computer aided drug design has successfully led to the development of a number of FDA approved drugs (Durrant et al., 2009). This approach could be an alternative way to develop DISC1-Fbxw7 inhibitors, given that Skp1-Fbxw7 crystal structure has been solved (Hao et al., 2007a, Wang et al., 2004, Orlicky et al., 2010) and a suitable assay to test possible hits has been developed in this work. DISC1 expression levels were shown to be high during active brain developmental and to regulate neurogenesis and this supports the role of abnormal levels of DISC1 in the pathophysiology of associated neurodevelopmental disorders. Hence, I propose that modulation of DISC1 protein levels is an attractive potential mechanism.

6.5 Chapter summary

To summarize, in this chapter, we developed a reliable, quantitative FP assay using which we characterised and quantified DISC1- [Skp1-Fbxw7] interaction. Using this assay, we have shown that doubly phosphorylated DISC1 CPD has a higher affinity over the singly phosphorylated CPD peptide providing in depth information on DISC1-Fbxw7 binding affinity. Furthermore, the FP assay was used in two HTS campaigns to screen a total of 8520 small molecules to identify potential modulators of the interaction. Although no confident inhibitors of the interaction were found, contemplated potential activators were shortlisted for future analysis. I propose that more intense optimisation of the assay and screening of more libraries would be fruitful.

7.

Final Discussion

7.1 Background

Since the discovery of DISC1 in a Scottish pedigree, carrying a highly penetrant balanced translocation (1q42.1; 11q14.3), there has been accumulating evidence to support its multiple roles in neurogenesis, migration and synapse signalling. These aspects make it a promising candidate gene for SCZ (Bradshaw et al., 2008, Bradshaw and Porteous, 2010, Maher and LoTurco, 2012). DISC1's multi compartmental localisation and a broad interactome support its involvement in multiple signalling pathways. Recent studies have associated DISC1 with other major chronic mental disorders such as autism, depression and bipolar disorder and it has been proposed that all DISC1 related illnesses should be categorised as 'DISCopathies' (Korth, 2009, Korth, 2012).

Although several DISC1 SNPs and haplotypes were identified to be associated with these neurological disorders, there has been no established consensus as to the disease causing mutation (Lipska et al., 2006b, Saetre et al., 2008). Moreover, the role of DISC1 in subjects with no known mutations has garnered much attention. While, DISC1 haploinsufficiency was one of the initially proposed mechanisms in the Scottish translocation, there have been no reports of low levels of mRNA transcripts or protein levels in SCZ patients (Leliveld et al., 2008, Lipska et al., 2006b). However, short splice variants were shown to be specifically upregulated in SCZ patients (Nakata et al., 2009). This is intriguing as these reported short isoforms resemble the Scottish truncation gene product, which may suggest that regulation of specific alternative splicing of these splice variants could be one of underlying disease mechanisms.

A classical hallmark of neurological disorders is abnormal processing or misfolding of proteins, which in some cases, leads to toxic aggregate formation (Taylor et al., 2002). DISC1 protein aggregates have been reported by several groups and high expression of heat shock proteins (molecular chaperones) have been found to be up regulated in SCZ brains. Hence disruption in DISC1 processing was one of the other disease mechanisms suggested (Hikida et al., 2012, Leliveld et al., 2008).

Post translational modifications are crucial in maintaining protein integrity, folding and cellular turnover, and UPS dysfunction has been previously reported in psychiatric illnesses (Lehman, 2009, Layfield et al., 2005). A more recent study also reported UPS dysfunction and brain specific abnormalities in UPS related mRNA transcripts in patients suffering from SCZ (Rubio et al., 2013, Bousman et al., 2010, Altar et al., 2005). Owing to the overlapping nature of UPS related abnormalities and phenotypes observed with loss of DISC1 function, we proposed that exploring and uncovering the yet unknown PTMs on DISC1 would provide answers, as well as open new avenues to understand the underlying pathophysiology on DISC1 associated disorders.

In the following sections, I briefly review my results and discuss the wider implications of my intriguing findings.

7.2 Consequences of DISC1 SUMOylation on its neurological functions

My initial work (results chapter 3) focussed on investigating DISC1 as a putative SUMO substrate. SUMOylation is a Ubl (ubiquitin Like). Posttranslational modification, which plays a vital role in neuronal signalling pathways contributing to synapse formation, ion channel activity, spine morphogenesis and synaptic transmission; reviewed in (Martin et al., 2007, Wilkinson and Henley, 2010). For the first time I provide the experimental

evidence that DISC1 can be SUMOylated. I identified potential SUMO acceptor lysine (K643), although the presence of other sites may not be ruled out. From previous reports, mitochondrial and neuronal fractions had enriched SUMOylation of substrates and since DISC1 localisation and regulation of mitochondrial dynamics has been studied and its dysfunction has been observed in SCZ and other neuropsychiatric disorders (James et al., 2004b, Millar et al., 2005a), I propose that in-depth characterisation of this PTM may provide answers to questions concerning the mechanistic behind DISC1's involvement in mitochondrial dysfunction.

Transgenic mice expressing mutant DISC1 (C terminal truncated DISC1), under control of CaMKII promoter, displayed abnormal phenotypes consistent with those observed in SCZ patients, such as enlarged ventricles, altered spine density and fewer parvalbumin-positive neurons (Hikida et al., 2007, Ayhan et al., 2011). This established a strong association between DISC1 with post synaptic physiology. A direct correlation has been established between the presynaptic neuronal expression of DISC1 and presynaptic glutamate release (Maher and LoTurco, 2012). SUMOylation of synapse associated substrates regulates presynaptic glutamate release depending on the stimulus. Different stimuli may promote SUMOylation or deSUMOylation of different subsets of proteins, which in turn affects the release the neurotransmitter (Feligioni et al., 2009). It would be interesting to investigate the SUMOylation status of DISC1 at the synapse (one approach could be the use of SUMO "dead" mutant of DISC1) to understand the precise role of DISC1 in synapse signalling.

I also provide preliminary evidence of one of many possible consequences of this modification on DISC1, such as altered protein interactions, in this case with DIXDC1. Although inconclusive, if this indeed is true, this could have major physiological consequences as this protein complex regulates neurogenesis and cortical development (Singh et al., 2010). This PTM event on DISC1 may act to regulate neuronal progenitor proliferation via its interaction with DIXDC1. As a multifunctional scaffold protein, DISC1 has the ability to orchestrate multiple signalling pathways via its broad "interactome" and we speculate that SUMO conjugation would have a major impact by either creating new

binding surfaces or inhibiting interactions possibly mediated by conformational changes on DISC1.

One of the other consequences, is altered sub cellular localisation regulated by reversible dynamic PTMs such as SUMOylation and Ubiquitination. Another well studied protein SUMO substrate is PML (Promyelocytic leukemia protein), which is SUMO conjugated and localised in a sub nuclear structures known as PML nuclear bodies. Many other SUMO-modified proteins have been shown to be co-localised in these transcription PML nuclear bodies (Zhong et al., 2000, Gill, 2004). Since DISC1 also partially co-localises with PML nuclear bodies (Sawamura et al., 2008), I speculate that SUMO modified DISC1 may be targeted to these PML bodies which are associated with cell proliferation and differentiation (Zhong et al., 2000). DISC1 interacts with ATF4 (Activating Transcription Factor) and modulates cAMP-dependent cAMP-response element (CRE)-mediated transcription. DISC1 variants 37W and 607F disrupted nuclear targeting of WT DISC1 in a dominant negative manner (Malavasi et al., 2012). It can be speculated that these variants could have an altered propensity to undergo PTM (either SUMOylation or ubiquitination) which may alter their sub cellular targeting. The above mentioned are just a few of examples of the possible consequences of DISC1 SUMOylation and I propose that it may have a more complex role in neuronal signalling.

The above points may be addressed by further experimental work which could not be done during my studies due to time constraints:

1. Detection of SUMOylated DISC1 using the UFDS method in a more relevant cell line, such as primary neuronal cultures or iPSc originated neurons. Immunostaining could be performed to identify the localisation and distribution pattern of SUMO conjugated DISC1 and its tendency to form aggregates.

2. Mass spectroscopy studies with high quality samples followed by site directed mutagenesis would provide firm evidence of all possible SUMO acceptor sites on DISC1.

3. Although DISC1 is less conserved in human and rodents, the SUMO site (K643) site is conserved in both. Hence, investigating the SUMOylation status of DISC1 in DISC1 mouse models would be useful. It is intriguing that this site is missing in the C terminal truncation (Scottish translocation) and hence the lack of this PTM may contribute to its dominant negative character.

Other PTMs such as phosphorylation may be required as a triggering event to initiate DISC1 SUMOylation. PKA mediated phosphorylation at S710 (S713 in human) has been shown to regulate neuronal migration during cortical development (Ishizuka et al., 2011b). SUMOylation on phosphor dead (S710A) and phosphor mimicking (S710D) DISC1 mutants, using the UFDS method, may identify a possible physiological link between these two PTM events.

7.3 DISC1 turn over regulation via ubiquitin mediated proteasomal degradation

As discussed in chapter 5, I demonstrate for the first time that the turnover of DISC1 is mediated by the UPS pathway. My data suggests that DISC1 is ubiquitinated and its degradation is inhibited in the presence of proteasome inhibitor, MG132 in a time and dose dependent manner. Previous studies have reported that DISC1 antibodies consistently detect high molecular weight bands (above 100kDa), especially in developing cerebral cortex (Morris et al., 2003, James et al., 2004a, Sawamura et al., 2005a). Although, these DISC1 associated high molecular weight bands were proposed to be DISC1 dimers, from my findings, these bands may also relate to the SUMOylated or ubiquitinated DISC1 species. Spatial and temporal regulation of DISC1 protein expression during brain development also suggest that complex regulatory mechanisms may be involved in regulating DISC1 turnover (Brandon et al., 2004, Miyoshi et al., 2003a, Ozeki et al., 2003a). Since, many of the proteins functioning at the synapse are either activated, inactivated or degraded upon suitable stimuli; an upstream deficiency in the UPS would

disrupt the synapse signalling reported in SCZ (Vawter et al., 2001, Altar et al., 2005). I speculate DISC1 could be one such crucial substrate involved.

Although, my work primarily focused on the proteolytic role of the UPS, it is important to note that ubiquitin conjugation to DISC1 may have other potential functional consequences. Until recently, a common notion existed that substrates with K48 chains are normally targeted for proteasomal degradation while those with K63 chains to lysosomes and DNA repair mechanisms (Nagao et al., 2006) (Nathan et al., 2013). However, there has been emerging evidence supporting the role of these chain formations in other cellular events. For example, Met4 (transcriptional activator) is conjugated by single K48 type Ubiquitin chain catalysed by E3 ligase, Cdc34–SCF^{Met30} (Flick et al., 2004). This conjugation inactivates Met4 activity instead of targeting it for degradation. As such, this study provided the first line of evidence for the non proteolytic role of K48 type ubiquitin chain conjugation (Flick et al., 2004). Several mechanisms have been proposed to explain the differential fate of the ub conjugated substrates dictated by the type of chains (Nathan et al., 2013). One mechanism that was widely accepted was the presence of various classes of DUB's which specifically recognise the substrates and promote their deubiquitination (Finley, 2009).

Although through my findings, I address some important aspects of DISC1 protein regulation, my work also raises some interesting questions such as:

1. What is the stimulus that triggers DISC1 ubiquitination? How does the developmental profile of ubiquitinated DISC1 vary? What is the role of DISC1 ubiquitination in neurogenesis mediated by its protein interactions?

2. Is there cross-talk between the UPS and SUMOylation pathways with respect to DISC1? Do they compete for same acceptor lysine residues or promote or inhibit other PTMs? These are questions that should be addressed if further time and funding can be sought.

7.3.1. Significance of Fbxw7 dependent DISC1 protein turn over

As discussed in chapter 4, siRNA library screen identified Fbxw7 as a potential Fbox protein, which regulated DISC1 ubiquitination as a component of an SCF E3 ligase complex. Also known as hCdc4 and hSel-10, scientific literature emphasizes Fbxw7 as a therapeutic target for cancer therapy (Kanei-Ishii et al., 2008, Wang et al., 2012a, Iriuchishima et al., 2011). With the increasing identification of neuronal proteins as Fbxw7 substrates, its role brain specific physiological pathways have garnered much attention lately.

High levels of Fbxw7 mRNA transcripts were detected from E13.5 with predominant expression in granule cell (GC) layers (Jandke et al., 2011). Fbxw7 expression was also identified to be significantly high in adult brain regions such as hippocampus and cerebellum. This is interesting in the context of strong expression and association of DISC1 with hippocampus structure and function (Lipska et al., 2006a, Faulkner et al., 2008, Meyer and Morris, 2008, Austin et al., 2004). Regional specific shRNA knockdown of DISC1 hampers the migration of early born GC in the dentate gyrus (Meyer and Morris, 2009), while its knockdown was shown to enhance migration of adult born granule cells in the dentate gyrus (Kvajo et al., 2008). However, literature searches did not reveal any inverse correlation of DISC1 and Fbxw7 expression during development as we expected. While the complexity surrounding the isoform specific regulation of these proteins is one possible reason, it can also be speculated that Fbxw7 may also target yet unknown upstream substrates of the DISC1 pathway. Hence, the role played by DISC1 may be spatio-temporally distinct, varying with the stimuli which dictates its function.

Two important substrates of Fbxw7 are c-Jun and Notch, which regulate neuronal apoptosis and neurogenesis respectively (Lütolf et al., 2002, Hoeck et al., 2010a). Cerebellum specific deletion of Fbxw7 reduced the size of the cerebellar vermis, with increased Notch 1 and phosphorylated c-Jun levels, implying a crucial role of Fbxw7 in cerebellar morphogenesis (Jandke et al., 2011). Studies also reported that Fbxw7$^{-/-}$ embryos die *in utero* at E10.5-11.5 with reported abnormalities in brain (Zhou et al., 2013b). I speculate that the abnormal anatomical changes observed in Fbxw7 conditional

knockout mice could also be associated with DISC1 turnover, whose protein levels are tightly regulated during early development. My findings support the critical role of Fbxw7 in brain development by identifying DISC1 as its substrate and I suggest a complex interaction between these proteins in regulating various cellular pathways. However, further work is required to elucidate how FBXW-DISC1 interaction contributes to the development of the brain.

7.4 Targeting novel PPI, DISC1-Fbxw7 interaction: scope for therapeutic intervention

Upon identification of a conserved phosphodegron (CPD) on DISC1, I performed peptide array studies and identified the shortest stretch of the CPD domain that might be involved in the interaction with Fbxw7. This sequence within the DISC1 CPD, was optimised using peptide array studies by various substitutions with non-natural amino acids while the net charge of the peptide remained unaltered, to increase the peptides physiological stability and affinity and towards the Fbxw7 substrate binding domain (WD40). Following a series of optimisation rounds, lead peptides 139 and 142 were selected for testing on cells, to investigate their ability to stabilise DISC1 as per my hypothesis. These peptides stabilised DISC1 in a dose and time dependent manner in the HEK293 cell line, while the other substrates of Fbxw7 (c-Myc, Cyclin E and Notch) were unaltered. I propose that the differences in affinity between Fbxw7 and DISC1 (low) compared to other substrates including Cyclin E (high) allows the peptides to be substrate specific.

I suggest that the peptides can be a starting point for the development of novel therapeutics. The lead cell permeable peptides which specifically stabilise DISC1 in cells offer an attractive alternative to proteasome inhibitors which affect a larger pool of substrates leading to side effects. Performing physiological assays of DISC1 function, such as assessing neurite outgrowth and neurite number, following peptide treatment in a relevant neuronal cell line (such as iPS derived neurons) may explain the mechanism of reported disease phenotypes.

Furthermore, the Skp1-Fbxw7 protein complex (Schulman et al., 2000), was crystallized bound to a 15 residue, doubly phosphorylated DISC1 peptide P^{193}EVPPpTPPGpSHSAFT^{207} (X ray crystallography work was performed by Yunfeng Li, Prof. Bing Hao's laboratory, University of Connecticut, USA; Figures in appendix section 7). This crystal structure (Figure in appendix) shows a striking resemblance to that of Cyclin $E^{degronC}$ - residues 360–390, phosphorylated at Ser^{372}, Thr^{380}, and Ser^{384}) (Hao et al., 2007a). My findings therefore suggest that the doubly phosphorylated DISC1 is recognised by Fbxw7 following Cyclin E paradigm.

I also explored the use of patient specific iPS differentiated neurons for potential as disease models. As an emerging revolutionary tool, iPS technology holds a therapeutic application for drug screening. For neurodevelopmental disorders such as SCZ, which involve complex gene environment interactions, the use of the human neurons developed by this method offer a considerable advantage over animal models, as human DISC1 is significantly different from those in rodents. They also offer a unique opportunity to recapitulate the disease phenotype and elucidate the disease mechanism. Patient specific iPS cells have been generated by various groups (Brennand et al., 2011, Brennand and Gage, 2012, Chiang et al., 2011). In the present study, I used neuronal progenitors but not fully differentiated neurons due to the limitations of cell number and time constraints. DISC1 protein levels in the patient derived neuronal progenitors were low compared to those in controls subjects, and were ideal to test out hypothesis of targeting DISC1-Fbxw7 interaction to rescue DISC1 protein levels. My preliminary results were promising as the lead peptides stabilised DISC1 levels, however, more experimental repeats are required to validate this effect. Assessing the pattern of expression of DISC1 and Fbxw7 at various stages of differentiation from iPS cells to neurons and comparative analysis of how this profile varies between control subjects and patients would be intriguing. Although similar studies were performed in post mortem brain samples (Lipska et al., 2006b), they may not resemble the physiological levels in live neurons.

I suggest that the following list of experiments would further enhance our understanding of DISC1 function:

1. Site specific mutational studies within DISC1 CPD, followed by assessment of DISC1 ubiquitination and turnover.

2. Phosphorylation has been shown to be a prerequisite for β-TrCP1 and Fbxw7 to recognise their cognate substrates-β catenin and Cyclin E respectively (Wu et al., 2003, Hao et al., 2007a). My findings of phosphorylated CPD mediated DISC1 ubiquitination is comparable to the models proposed in the above studies. However, I speculate that GSK3β could be the source kinase as for the other Fbxw7 substrates. This may be challenging to explore as a priming phosphorylation of the substrate by a different kinase is often required for GSK3β phosphorylation.

3. It may be useful to investigate the effect of GSK3β activators and inhibitors on DISC1-Fbxw7 interaction since Wnt/GSK3β signalling has been identified as a critical signalling pathway in psychiatric disorders.

4. It would be interesting to identify any DISC1 common or rare mutants falling within DISC1 CPD region. Certain DISC1 variants (A83V, R264Q and L607F) failed to activate Wnt pathway and diminished neuronal progenitor proliferation (Singh et al., 2011). Similarly, a recent study reported that patients carrying R264Q variant were resistant to anti-psychotic medication (Mouaffak et al., 2011). Although the precise mechanism is not known, I speculate that these variants affect DISC1 protein stability and my findings provide a frame work and open new avenues to explore the underlying disease mechanism.

7.4.1. Small molecule modulators of DISC1-Fbxw7 interaction

Experimental work discussed in chapter 6, concerned the process of screening for small molecule modulators that affect via DISC1-Fbxw7 protein interaction. A reliable and quantitative FP assays was developed based on DISC1 CPD mediated interaction with Fbxw7, which was adapted to perform HTS on two different compound libraries to identify modulators of this interaction. The hit identified in the first HTS, a fluorescent

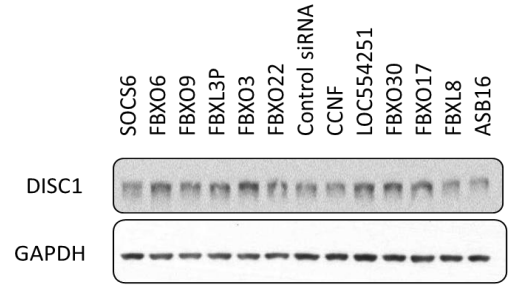
compound, Merbromin requires intense characterisation in secondary screens using non fluorescence based techniques to corroborate its potential to be developed as a drug. Certain compounds in the second screen were identified to increase the DISC1-Fbxw7 interaction (activators). Assessment of their potential to modulate this PPI may identify compounds which may be developed further to act as DISC1 down regulators. These would be useful for the investigation of DISC1 function in animal studies.

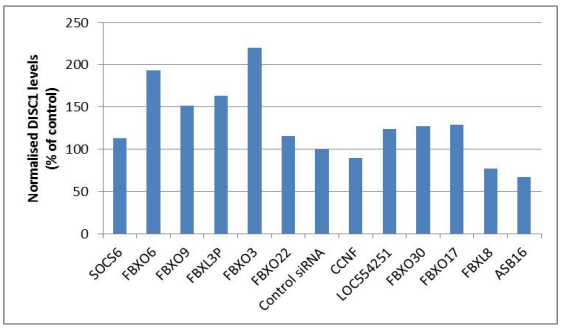
7.5 Final Conclusion

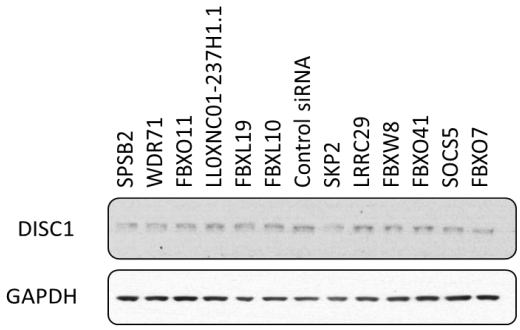
My study identified novel post translational modifications (SUMOylation and Ubiquitination) on DISC1 which would advance the understanding of how DISC1 protein function in various cellular pathways is regulated. With the identification of SCF^{Fbxw7} as the E3 ligase that regulates DISC1 ubiquitination and subsequent elucidation of their binding sites, I uncovered the DISC1-Fbxw7 PPI as a promising drug target. I optimised two lead peptides (139 and 142) to inhibit this interaction and in so doing arrived at a new class of therapeutic approaches directed at the up regulation of DISC1 via inhibition of its destruction by the proteasome. I also developed an *in vitro* assay to identify small molecule disruptors which could provide a novel, targeted therapeutic strategy for the treatment of SCZ and associated disorders which involve loss or gain of DISC1 function. I propose that the modulation of this interaction may in turn modulate its function, targeting the neurodevelopmental processes, underlying the pathophysiology of DISC1 associated illnesses that delay or prevent the onset of the psychiatric illness.

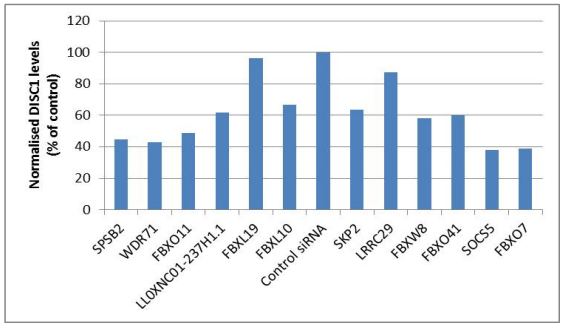
Appendices

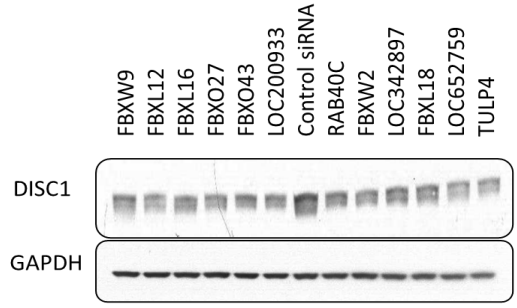
8.1 Fbox siRNA screen data

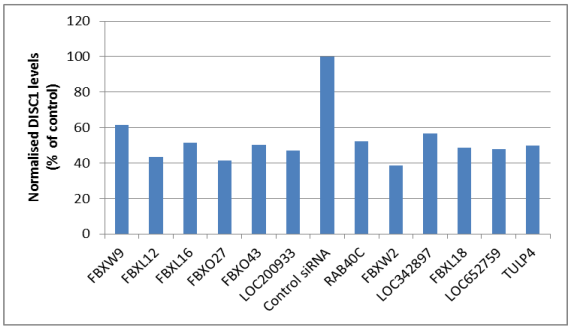


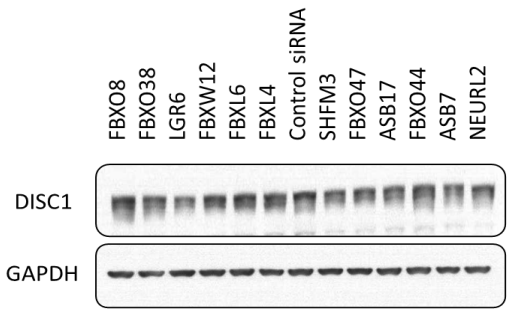


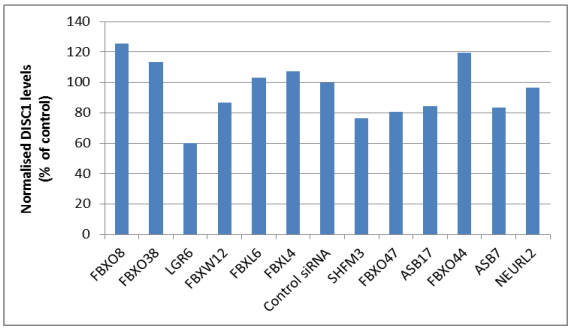












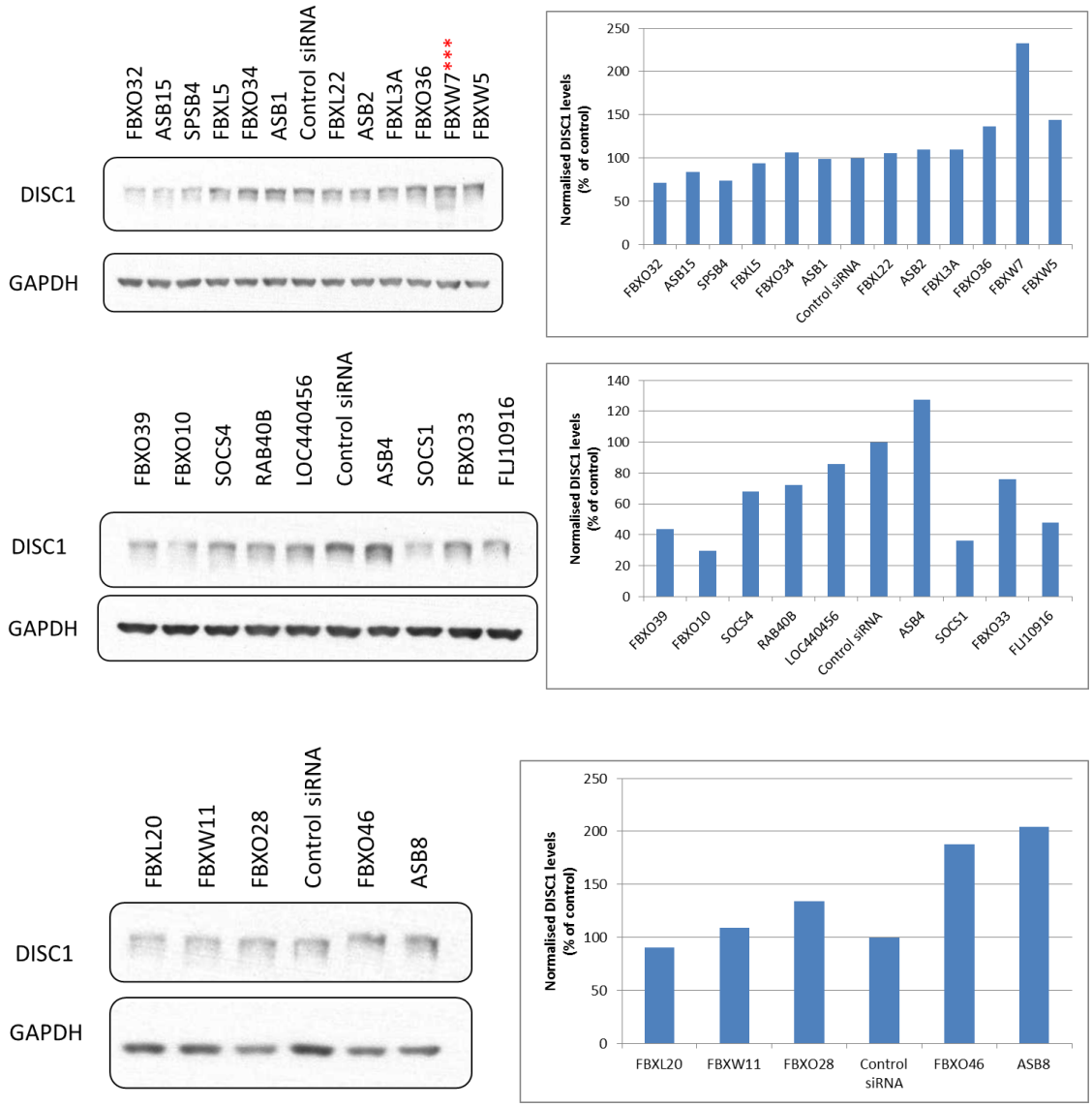


Figure 8.1 Fbox siRNA knock screen in HEK293 cells – Effect on DISC1 protein levels.

8.2 X-ray crystallography studies : Co-crystallisation of DISC1 phosphodegron peptide and Skp1-Fbxw7 complex

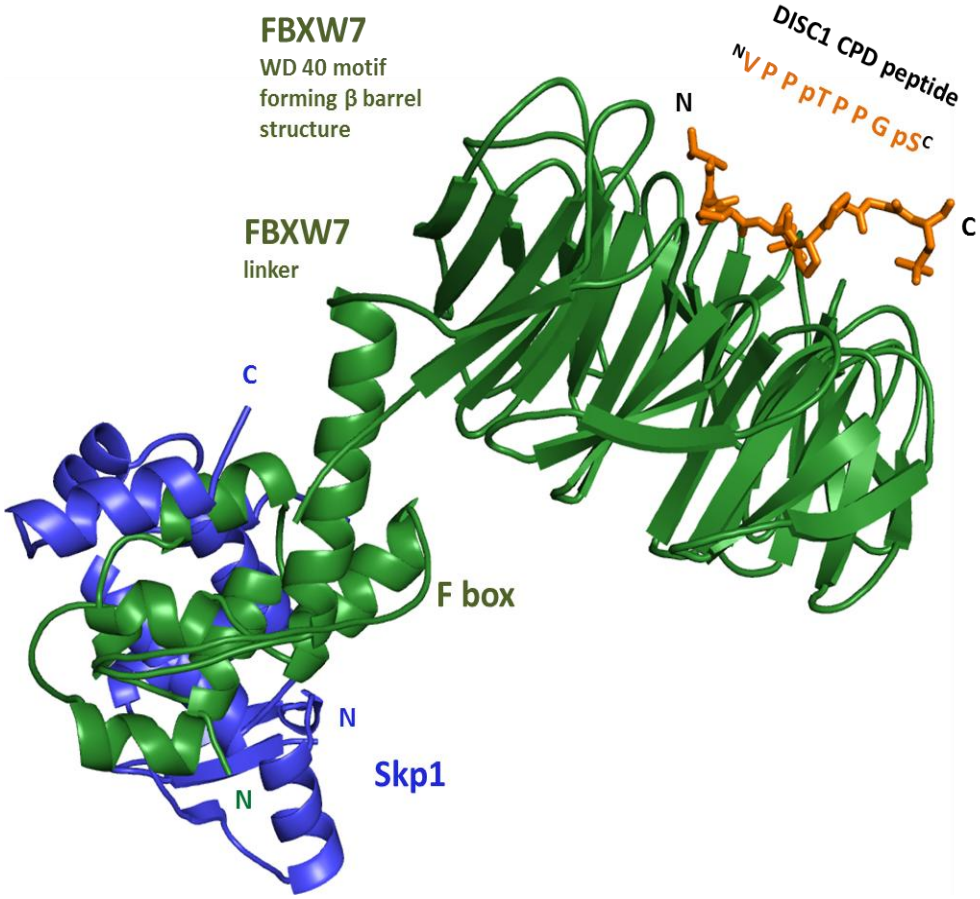


Figure 8.2a Crystal structure of the Skp1-Fbw7-DISC1 CPD peptide complex
General architecture of Skp1-Fbxw7 complex, with the secondary structure elements of Skp1 (blue), F box domain, and linker domains (green) labelled accordingly. The DISC1 CPD peptide (^{N}VPPTpTPPGpS^{C}) can be seen binding to the WD40 domain. The overall structure of Skp1-Fbxw7 complex remains the same as described in (Hao et al., 2007a).

Crytallogrpahy studies were performed by Dr.Yunfeng Li, Prof. Bing Hao's laboratory, University of Connecticut, USA

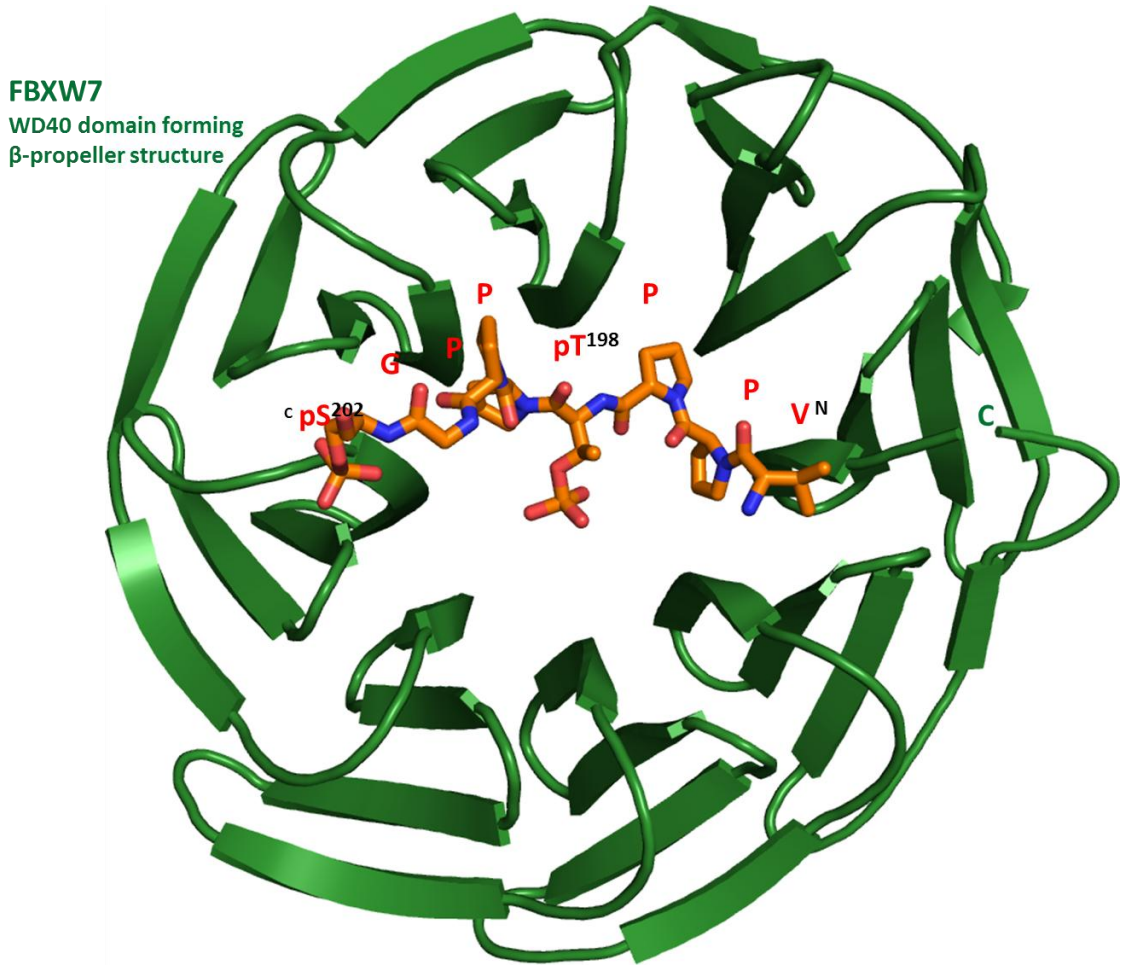


Figure 8.2b Crystal structure of the Skp1-Fbw7-DISC1 CPD peptide complex Fbxw7 WD40 domain (green) can be seen as a β propeller structure forming 8 blades with the bound DISC1 CPD peptide (^{N}VPPTpTPPGpS^{C}).

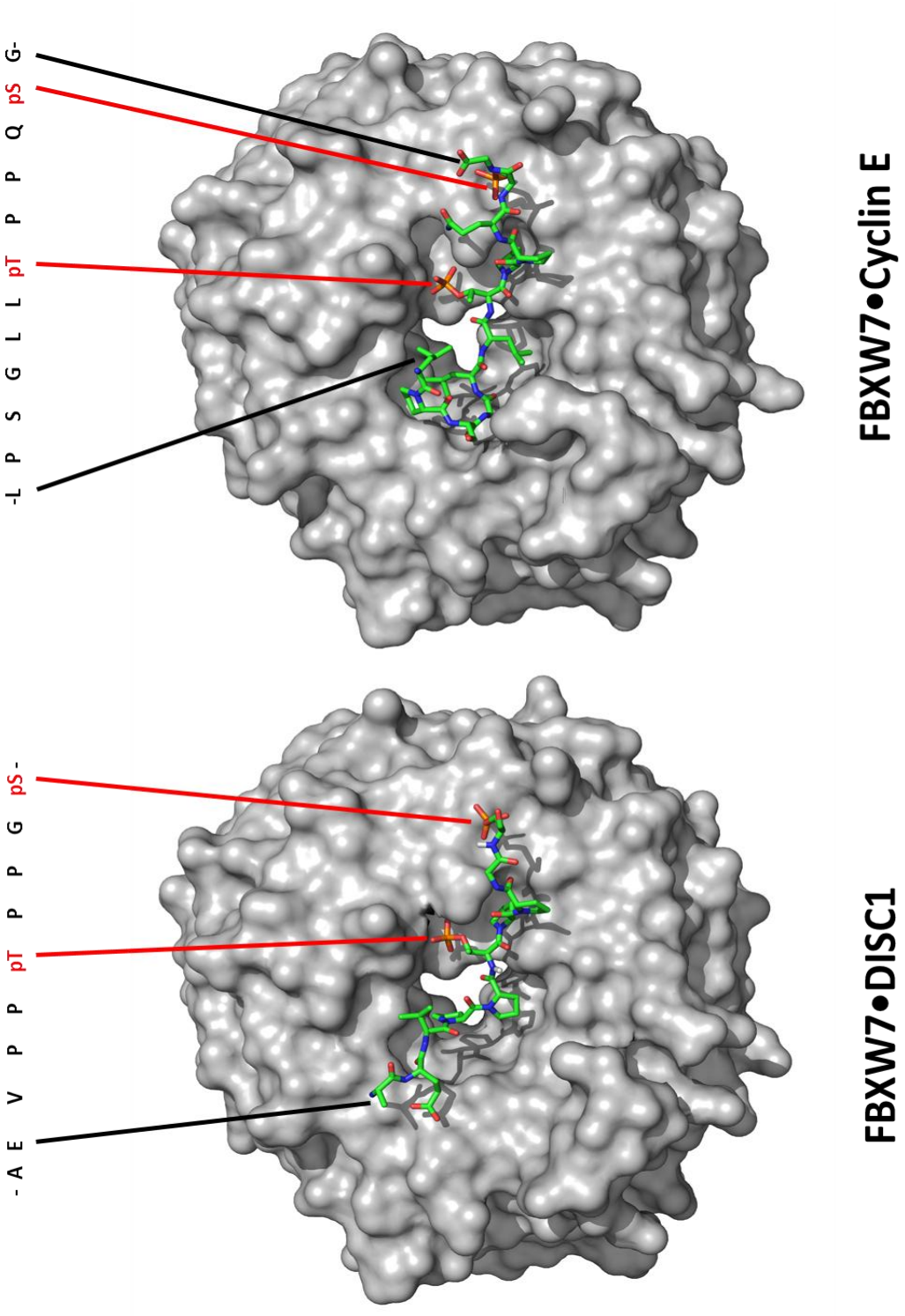


Figure 8.2c Molecular surface representation of WD40 domain bound DISC1 and CyclinE CPD peptides individualy.
DISC1 pT and pS make contacts with Fbxw7 WD40 domain similar to CyclinE paradigm, while Cyclin E has additional interaction (Glycine on the C terminus) which may contribute to its higher affinity.

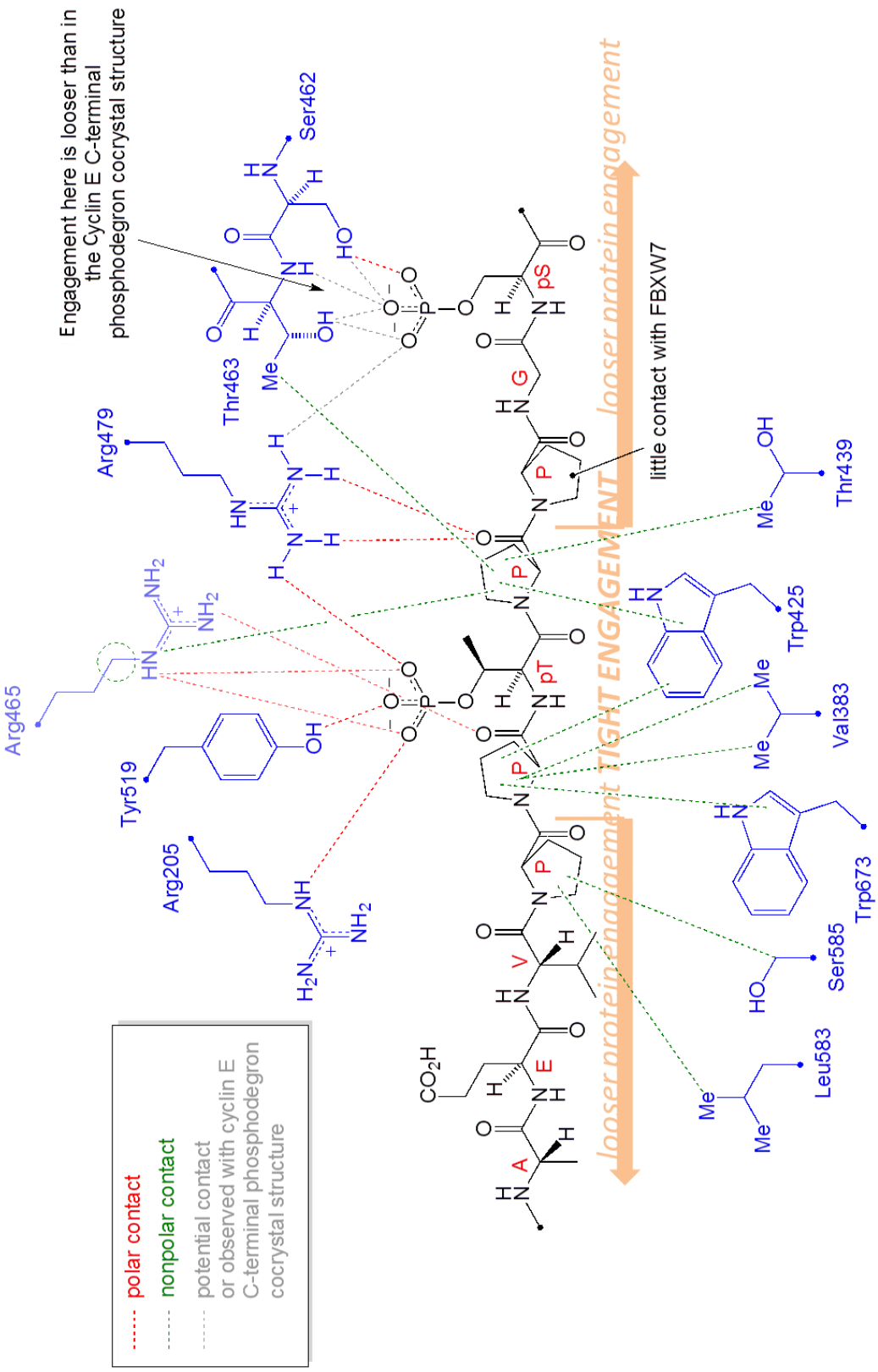


Figure 8.2d Summary of key contacts between FBXW7 and DISC1 CPD
Blue residues represent amino aicds in FBXW7 WD40 and black resdues are DISC1 CPD.

8.3 Disruptor peptide development and optimisation

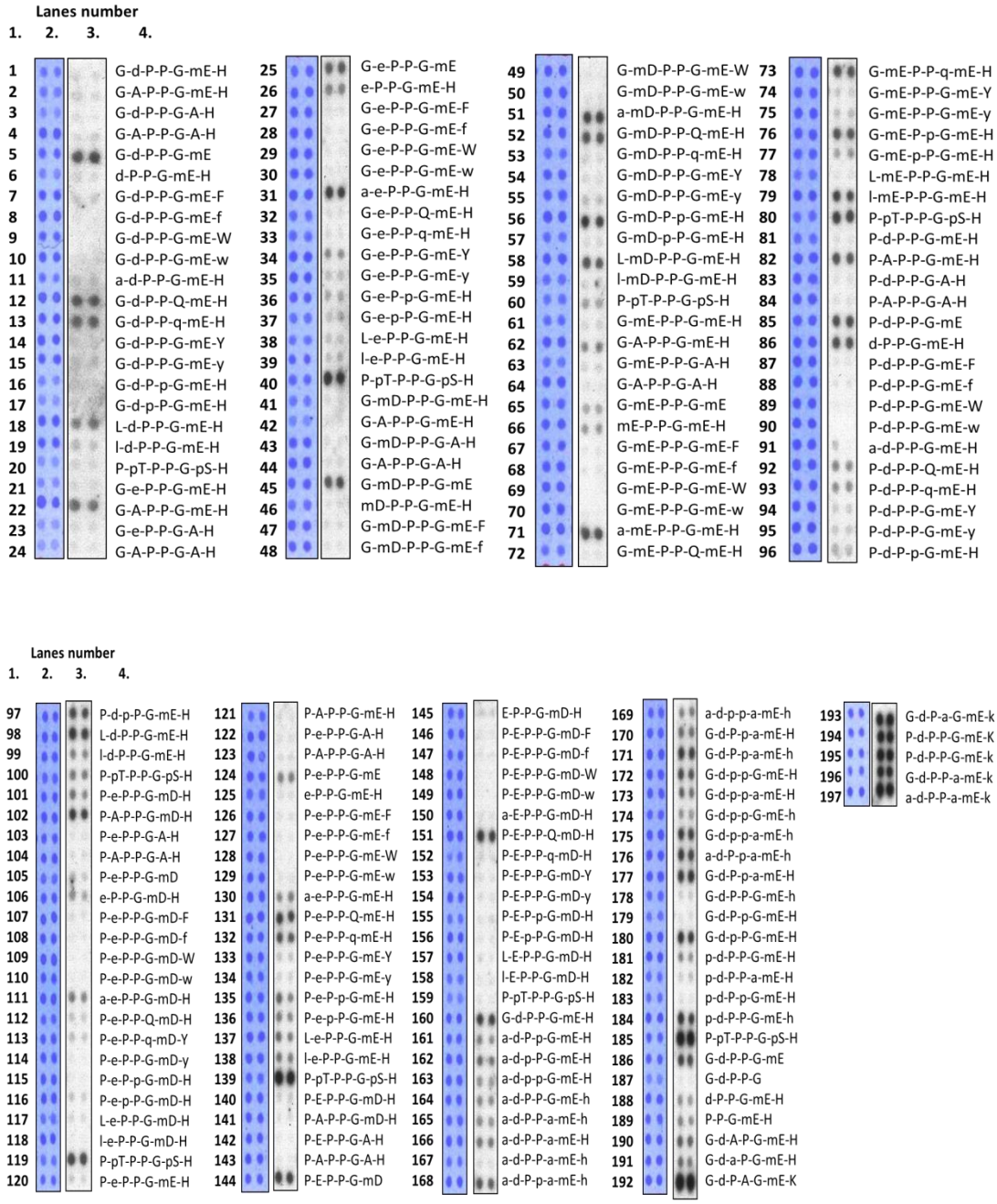


Figure 8.3a Peptide array studies to optimise and develop disruptor peptide to inhibit DISC1- [Skp1-Fbxw7] interaction. Optimisation part 1.
Lane 1- Spot number, Lane 2-Coomassie stained array, Lane 3- Test array, Lane 4- Peptide sequence

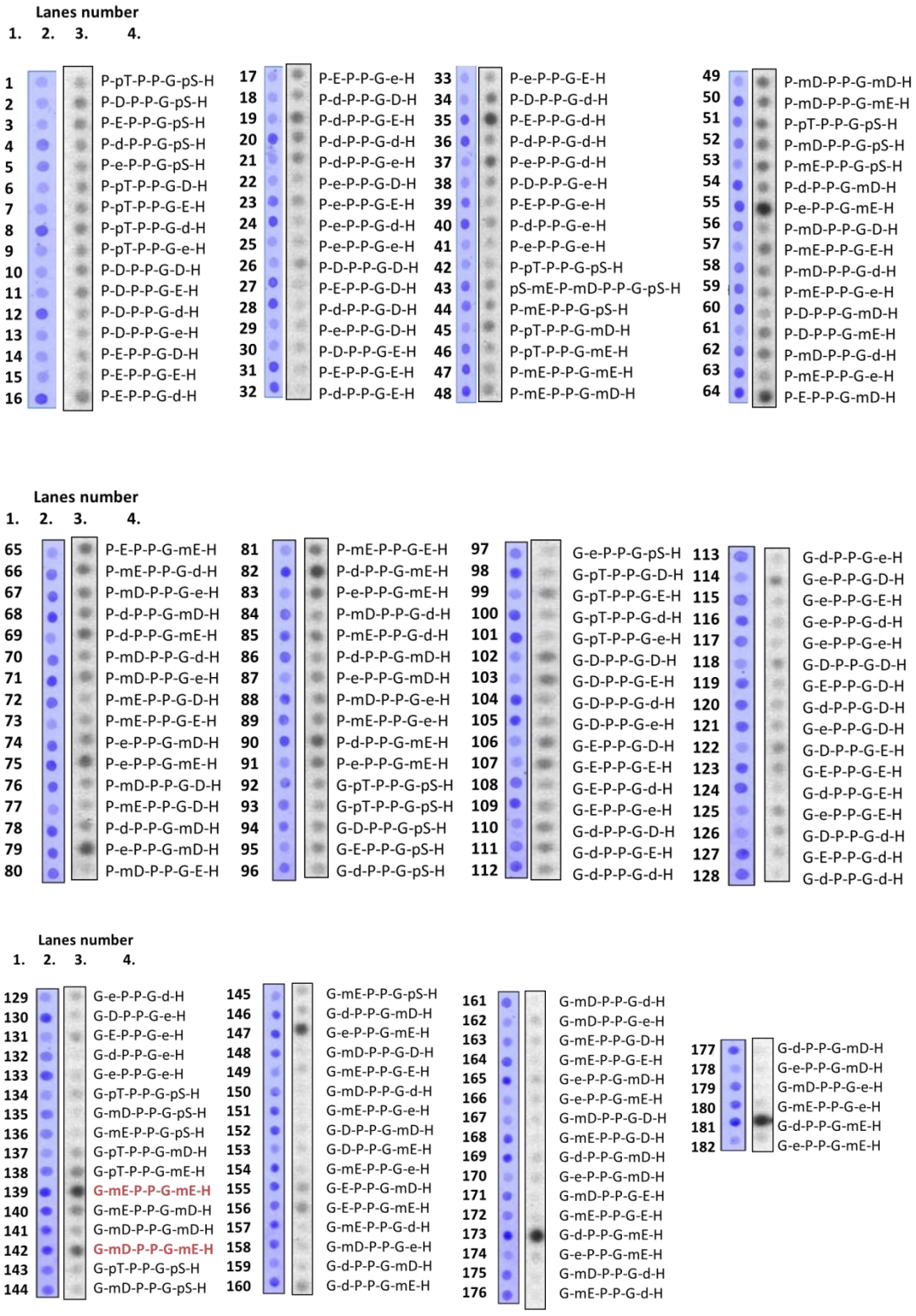


Figure 8.3b Peptide array studies to optimise and develop disruptor peptide to inhibit DISC1- [Skp1-Fbxw7] interaction. Optimsiation part 2.
Lane 1- Spot number, Lane 2-Coomassie stained array, Lane 3- Test array, Lane 4- Peptide sequence

8.4 High Throughput Screen 2 (HTS2) data

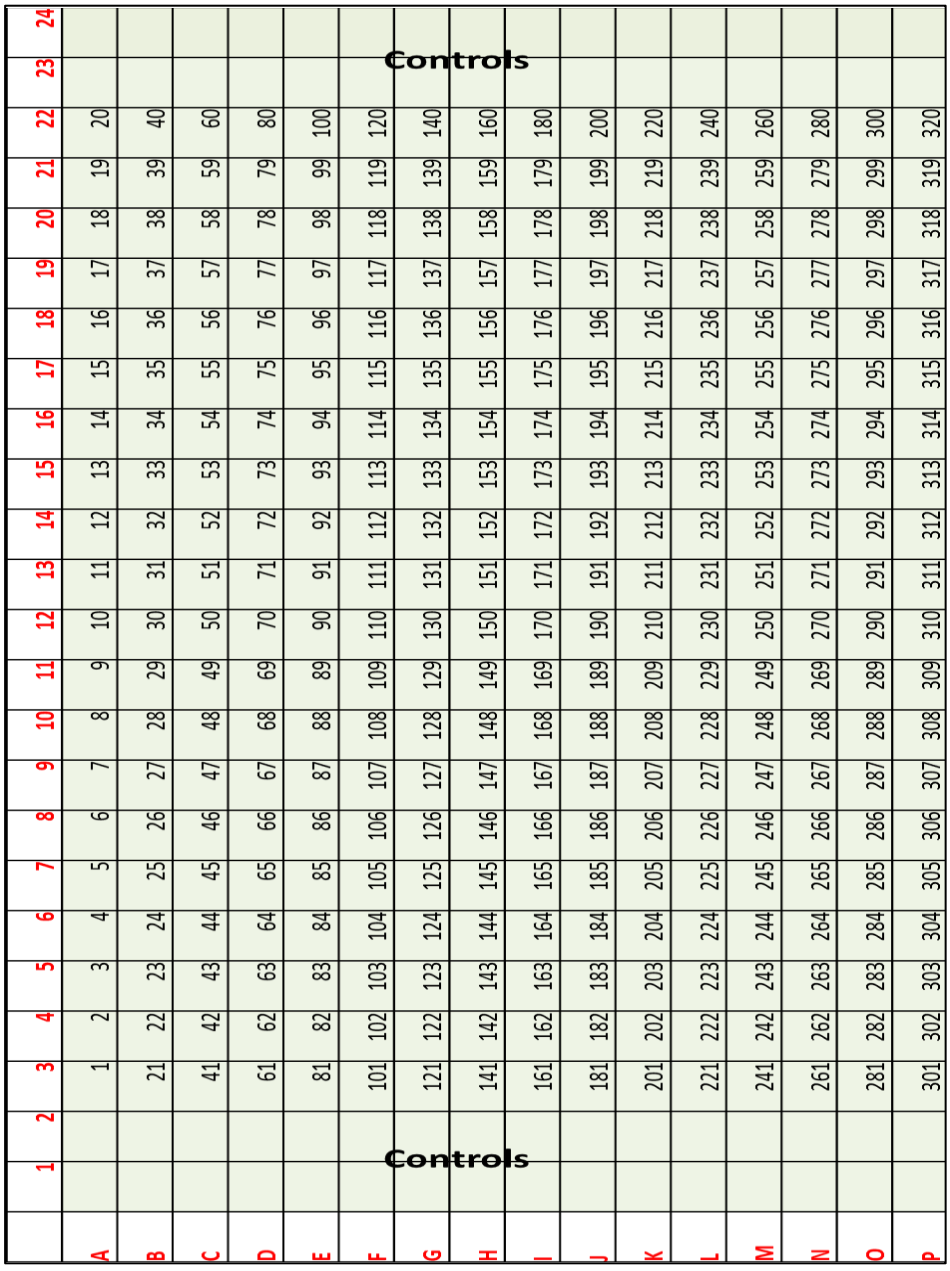
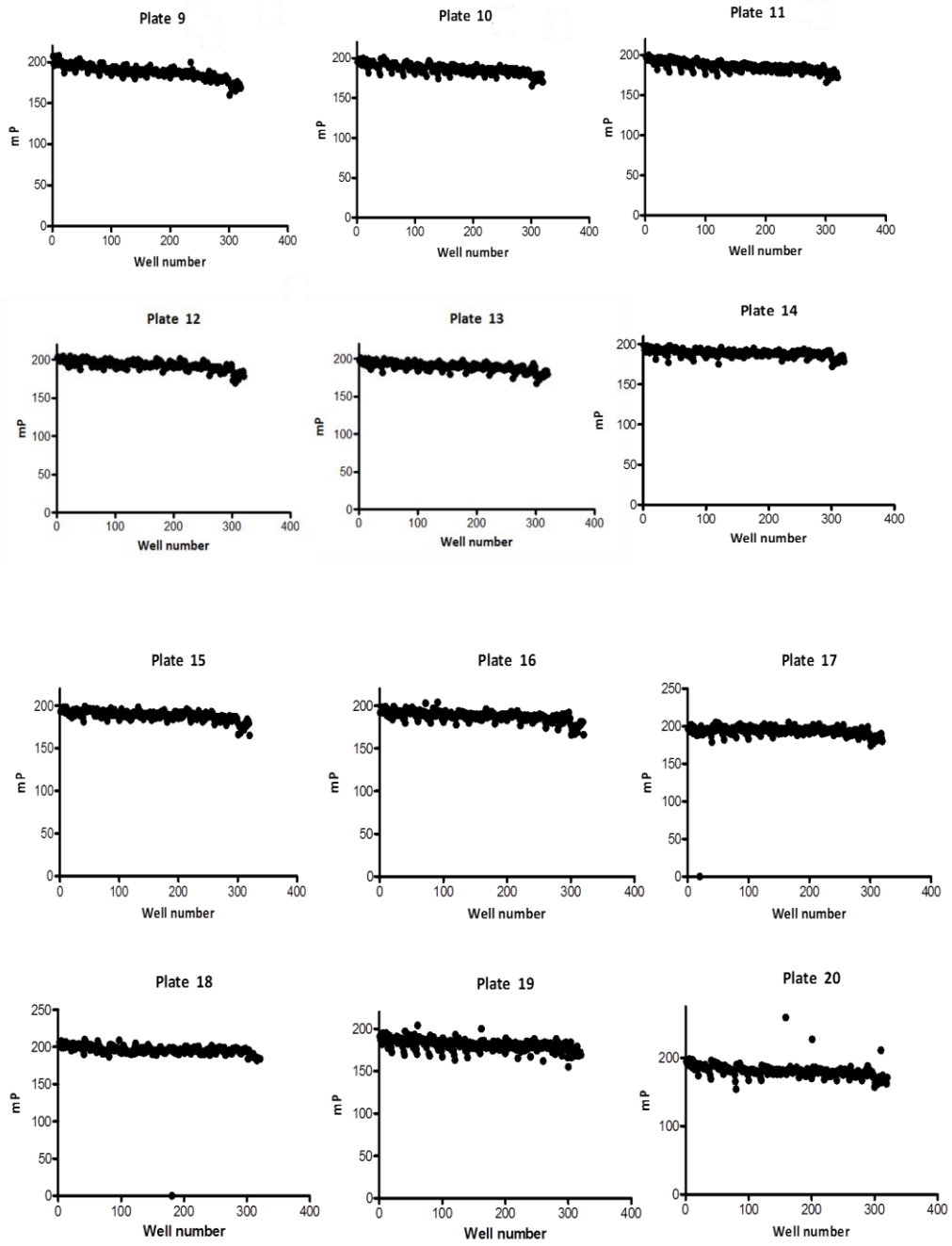


Figure 8.4a 384 well plate layouts for the High Throughput Screens
Green area represents the 384well plate. Controls include positive control (with 100% inhibition) and negative control (with inhibition of protein-protein interaction) described in chapter 6, Section 6.3.1.



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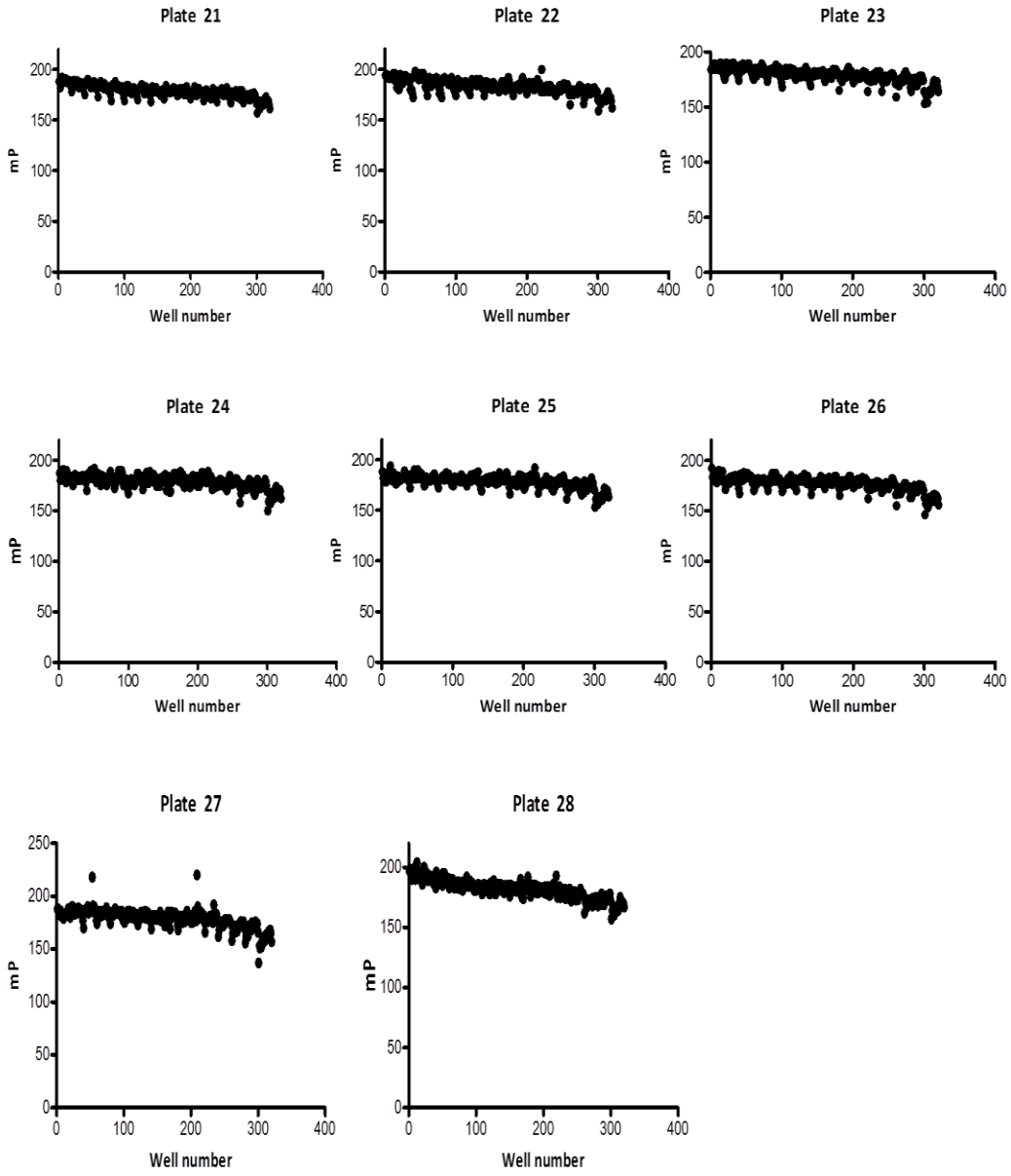
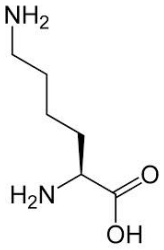


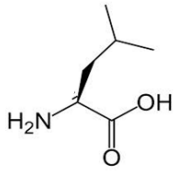
Figure 8.4 High Throughput Screen data (HTS Screen 2)
mP values were plotted against the well number for each plate. Described in Chapter 6, Section 6.3.6.

8.5 Amino acid structures, three letter and single letter representation

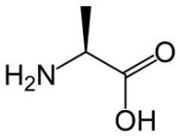
Lysine, Lys, K



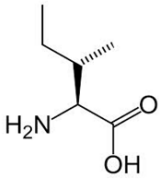
Leucine, Leu, L



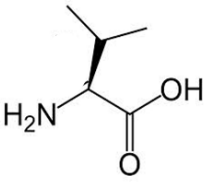
Alanine, Ala, A



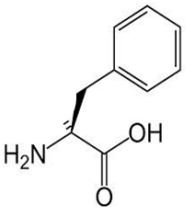
Isoleucine, Ile, I



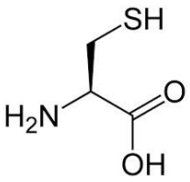
Valine, Val, V



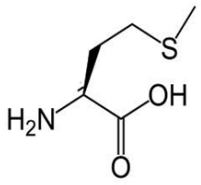
Phenyl alanine, Phe, F



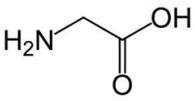
Cysteine, Cys, C



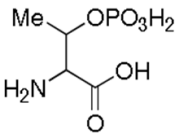
Methionine, Met, M



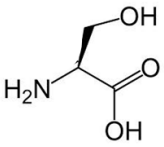
Threonine, Thr, T



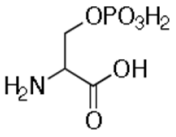
Phospho Threonine, pT



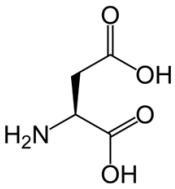
Serine, Ser, S



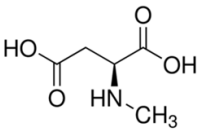
Phospho Serine, pS



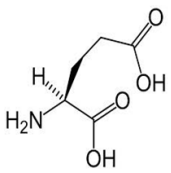
Aspartic acid, Asp, D



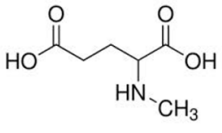
N-methyl aspartic acid, mD



Glutamic acid, Glu, E

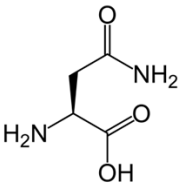


N-Methyl glutamic acid, mE

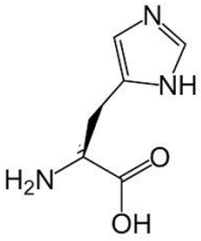


Tyrosine, Tyr, Y

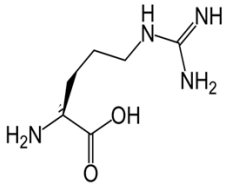
Asparagine, Asn, N



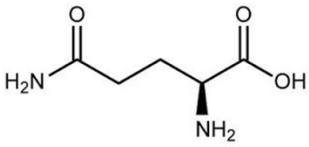
Histidine, His, H



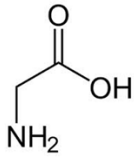
Arginine, Arg, R



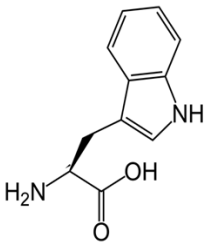
Glutamine, Gln, Q



Glycine, Gly, G



Tryptophan, Trp, W



Proline, Pro, P

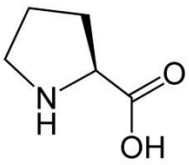


Figure 8.5a Amino acids structures, used in the peptide array studies

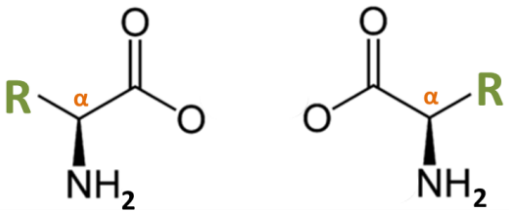


Figure 8.5b Schematic of L and D isomers of an amino acid with R side chain

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