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The Role of Cellular Prion Protein in the Development of Schwannomas and other Merlin-Deficient Tumours

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The role of cellular prion protein in the development of schwannomas and other merlin-deficient tumours

by

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“One, remember to look up at the stars and not down at your feet.

Two, never give up work. Work gives you meaning and purpose and life is empty without it.

Three, if you are lucky enough to find love, remember it is there and don't throw it away.”

— Stephen Hawking

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Sub-Committee.

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Abstract - The role of cellular prion protein (PrP^C) in the development of schwannomas and other Merlin-deficient tumours, Lucy Provenzano

Neurofibromatosis type 2 (NF2) is an inherited, multiple tumour disease caused by loss of the tumour suppressor protein, Merlin. There are several tumours associated with NF2 including; ependymomas, meningiomas and schwannomas. Merlin loss can also occur sporadically in all of these tumours and is associated with upregulation of various growth factor receptors and their relevant signalling pathways. At present the only treatment options for NF2 are surgery or radiosurgery, both of which incur serious morbidity and are unable to prevent recurrence of tumours. Either new drug treatments, or re-profiling of other drugs already commercially available, are urgently needed to improve outcome for NF2 patients.

Cellular prion protein (PrP^C), encoded by *PRNP* gene, is involved in tumour development by altering proliferation, adhesion, and survival in some cancers via focal adhesion kinase (FAK) /Src/ NFκB, cyclin D1 and p53 -proteins. Our group previously showed a strong elevation of *PRNP* gene activity in schwannoma. I hypothesise that PrP^C may contribute to schwannoma development.

To study the role of PrP^C in schwannoma development I have used the well-established *in vitro* model of schwannoma that comprises primary human Schwann and schwannoma cells. I show that PrP^C is upregulated in schwannoma as well as in Merlin-deficient meningiomas and human malignant mesotheliomas. In schwannoma PrP^C is released both via exosomes and by α-cleavage which forms biologically active N- and C-terminal portions of the protein. PrP^C contributes to pathological proliferation, adhesion and survival of

schwannoma cells by activating ERK1/2, PI3K/AKT, cyclin D1, FAK, p53 pathways via the 37/67kDa non-integrin laminin receptor (LR/37/67kDa) and CD44.

Furthermore, schwannoma cells appear to be intrinsically drug-resistant due to upregulation of MDR1 protein p-glycoprotein (p-gp) expression. P-gp expression is dependent on PrP^C thus, inhibiting PrP^C may be a good potential new therapeutic option for schwannoma patients, either alone or in combination with Sorafenib and p-gp inhibitor Valspodar (PSC833). An inhibitor of LR/37/67kDa/PrP interaction, NSC47924, or Bortezomib, a proteasome/NFκB inhibitor which has been approved for the treatment of multiple myeloma, could also be of beneficial therapeutic effect and is something to investigate in future work.

I conclude that PrP^C is an interesting new therapeutic target through its involvement with schwannoma patholgenesis and resistance to drug treatments PrP^C may prove to be a good therapeutic target in other NF2-related tumours like meningiomas and schwannomas.

Contents

Author's Declaration 2

Acknowledgements 3

Abstracto 4

Contents 6

List of illustrations, figures and tables 11

1. Introduction 14

1.1 Neurofibromatosis type 2 and associated tumours 14

1.1.1.1 Schwannomas and schwannomatosis 16

1.1.2 Meningiomas 19

1.1.3 Ependymomas and other NF2-associated tumours 21

1.2. *NF2* gene and its protein product, Merlin 22

1.2.1 NF2 gene structure, location and mutations 22

1.2.2 Merlin as a tumour suppressor protein 23

1.2.3 Merlin and integrin-related signalling 26

1.2.4 Receptor tyrosine kinase signalling and Merlin 27

1.3. Cellular prion protein (PrP^{C}) 28

1.3.1 Prion protein and its role in prion diseases 28

1.3.2 Structure and regulation of *PRNP* 30

1.3.3 PrP^{C} Structure, biogenesis and degradation 31

1.3.3.1 PrP^{C} biosynthesis and cleavage 31

1.3.3.2 GPI anchoring and shedding of PrP^{C} 34

1.3.3.3 PrP^{C} glycosylation 36

1.3.3.4 Different topological isoforms of PrP- ^{Ctm}PrP ^{Ntm}PrP 38

1.3.3.5 PrP^{C} degradation 39

1.4 Other members of the prion protein family 41

1.4.1 *PRND* and doppel (Dpl) 41

1.4.2 *PRNT* and PRT 43

1.4.3 *SPRN* and shadoo 44

1.5 Significant biological functions of PrP^{C} 45

1.5.1 PrP^{C} in cell adhesion 45

1.5.2 PrP^{C} in survival and neuroprotection 46

1.5.3 PrP^{C} in cell proliferation and tumorigenesis 47

1.6 In search of a receptor for PrP^{C} 49

1.6.1 $P75^{NTR}$ and the NFkB signalling axis 49

1.6.2 Laminins and their receptors 51

1.6.2.1 37/67kDa non-integrin laminin receptor protein as a 'receptor' for PrP^C ... 53
1.7 Multi-drug resistance ... 54
1.7.1 P-glycoprotein and multi-drug resistance in cancer cells ... 54
1.7.1.1 P-gp isoforms, gene variants and mutations ... 56
1.7.1.2 P-glycoprotein sub-cellular localisation and function ... 56
1.7.2 PrP^C and multi-drug resistance ... 57
1.7.3 Adhesion-mediated drug resistance ... 58
1.8 Cluster of differentiation 44 (CD44) ... 59
1.8.1 CD44 structure and function ... 59
1.8.2 CD44 in cancer ... 62
1.8.3 CD44 in schwannoma and other Merlin-deficient tumours ... 64
1.8.4 CD44 in multi-drug resistance ... 65
1.8.5 CD44 and its interaction with PrP^C ... 67
1.9 Aims and objectives ... 68
1.10 Brief summary of main findings ... 68
Chapter 2: Materials and methods ... 70
2.1 Sample collection and cell culture ... 70
2.1.1 Primary human cells ... 70
2.1.1.1 Clinical sample collection and ethics ... 70
2.1.1.2 Schwann cell collection and culture ... 70
2.1.1.3 Schwannoma cell collection and culture ... 71
2.1.1.4 Meningioma cell collection and culture ... 71
2.1.2 Cell line culture ... 71
2.1.2.1 Human meningeal cell (HMC) culture ... 71
2.1.2.2 Ben-Men-1 benign meningioma cell culture ... 72
2.1.2.3 Human malignant mesothelioma cell (HIB and TRA) culture ... 72
2.1.2.4 HEK293FT cell culture ... 72
2.2 Chemicals, peptides & antibodies ... 72
2.2.1 Chemicals ... 72
2.2.2 Antibodies ... 74
2.3 Immunohistochemistry and immunocytochemistry ... 77
2.3.1 Immunohistochemistry (IHC) ... 77
2.3.2 Immunofluorescence and confocal microscopy ... 77
2.3.2.1 Fluorescent immunocytochemistry (ICC) ... 77
2.3.2.2 Immunofluorescence proliferation and survival assays ... 78
2.3.2.3 Confocal microscopy ... 78

2.4 Quantifying PrP^C release from cells 78
2.4.1 Culture medium collection and concentration 78
2.4.2 Exosome isolation 79
2.4.3 Quantifying PrP^C cleavage by PIPLC and ADAM10 79
2.4.4 Analysis by enzyme-linked immunosorbent assay (ELISA) 79
2.5 Viral infections of Schwann and schwannoma cells in culture 80
2.5.1 Adenoviral re-introduction of Merlin into schwannoma cells 80
2.5.2 Lentiviral knockdown of *PRNP* in schwannoma cells 80
2.5.2.1 Homemade TRC *PRNP* shRNA lentiviral particles 80
2.5.2.2. Pre-made *PRNP* shRNA lentiviral particles 80
2.5.2.3 Lentiviral infection of schwannoma cells with *PRNP* shRNA 81
2.5.3 *PRNP* overexpressing clone 81
2.5.3.1 Production of the *PRNP* overexpressing clone 81
2.5.3.2 Infection of Schwann cells with *PRNP* overexpressing clone 82
2.5.4 Knockdown of 37/67kDa-non-integrin laminin receptor 83
2.5.5 Knockdown of *CD44/HCAM* 83
2.6 Co-immunoprecipitation 83
2.7 Other functional assays 84
2.7.1 Proteinase K sensitivity assay 84
2.7.2 De-glycosylation assay 85
2.7.3 Adhesion assay 85
2.7.4 MTS viability assay 85
2.8 Western blotting 86
2.8.1 Sample lysis 86
2.8.1.1 Cell lysis 86
2.8.1.2 Tissue lysis 86
2.8.2 SDS-PAGE and transfer to PVDF membrane 87
2.8.3 Immunoblotting 87
2.9 Data analysis 88
3. Results 89
3.1 PrP expression and structure 89
3.1.1 Different anti-PrP^C antibodies show different staining patterns in schwannoma cells 89
3.1.2 PrP is overexpressed in schwannoma compared to Schwann cells 91
3.1.3 PrP is also overexpressed in meningioma and in Merlin-deficient mesothelioma cell line, TRA cells 93

3.1.4 The PrP expressed in schwannoma is the same di-glycosylated, protease-sensitive form seen in Schwann cells 95
3.1.5 PrP^C overexpression in schwannoma and TRA is Merlin-dependent but not in meningioma 96
3.1.6 PrP expression and structure – discussion 98
3.2 Control of PrP^C expression 101
3.2.1 PrP^C overexpression in schwannoma is due to increased gene transcription and not due to altered protein degradation 101
3.2.2 PrP^C expression is not regulated by DCAF1 103
3.2.3 PrP^C overexpression in schwannoma may be regulated by NFκB 103
3.2.4 Control of PrP^C expression – Discussion 105
3.3 PrP^C release from schwannoma cells 108
3.3.1 PrP^C is released from schwannoma cells via exosomes 108
3.3.2 Non-exosome PrP^C release is not regulated by GPI anchor cleavage 110
3.3.3 PrP^C is released from schwannoma by α-cleavage by ADAM10 111
3.3.4 PrP^C release from schwannoma cells – Discussion 112
3.4 PrP^C in schwannoma proliferation, adhesion and survival 114
3.4.1 PrP^C contributes to increased schwannoma proliferation and survival 116
3.4.2 PrP^C in schwannoma protects against free radical-induced apoptosis but not Bax-mediated apoptosis 118
3.4.3 PrP^C plays a role is schwannoma pathological cell matrix adhesion 119
3.4.4 PrP^C affects schwannoma proliferation, survival and cell matrix adhesion by signalling via cyclin D1, ERK1/2, PI3K/AKT, FAK and p53 120
3.4.5 PrP^C in schwannoma proliferation, survival and adhesion – Discussion 123
3.5 The effect of PrP treatment and overexpression in Schwann cells 125
3.5.1 Treatment with PrP increases proliferation, expression of c-Jun and alters Schwann cell morphology 125
3.5.2 Overexpression of PrP^C increases expression of cyclin D1 and c-Jun 128
3.5.3 The effect of PrP treatment and overexpression in Schwann cells – discussion 129
3.6 LR/37/67kDa as a receptor for PrP^C in schwannoma 130
3.6.1 LR/37/67kDa is not differentially expressed between Schwann and schwannoma cells 130
3.6.2 LR/37/67kDa co-localises and physically interacts with PrP^C in schwannoma 131
3.6.3 Knockdown of LR/37/67kDa significantly affects the ability of PrP^C to activate several key signalling pathways 132
3.6.4 LR/37/67kDa as a receptor for PrP^C – discussion 133
3.7 P-glycoprotein in schwannoma 135

3.7.1 Different p-gp and CD44 antibodies identify different patterns of p-gp expression in schwannoma cells 135
3.7.2 P-gp is over-expressed and localises to the nucleus of schwannoma cells 138
3.7.3 P-gp overexpression in schwannoma is Merlin-dependent 140
3.7.4 LR/37/67kDa and p-glycoprotein co-localise in schwannoma but not Schwann cells 140
3.7.5 P-gp in schwannoma – Discussion 142
3.8 Control of p-glycoprotein expression by PrP^C 143
3.8.1 PrP^C regulates p-gp expression in schwannoma cells 145
3.8.2 Control of p-glycoprotein expression by PrP^C – discussion 147
3.9 CD44, the magical linker between PrP^C and p-gp? 148
3.9.1 CD44 is overexpressed in schwannoma and meningioma cells in a Merlin-dependent manner 148
3.9.2 CD44 partly co-localises and interacts with serveral p-gp isoforms in schwannoma 151
3.9.3 PrP^C and CD44 physically interact in schwannoma cells 152
3.9.4 Reduction of PrP^C decreases expression of CD44 153
3.9.5 CD44 acts as a cell surface receptor for PrP^C in schwannoma 154
3.9.6 CD44, the magical linker between PrP^C and p-gp? Discussion 155
3.10 Drug resistance in schwannoma 159
3.10.1 MTS assay demonstrates that inhibition of PrP and p-gp can enhance the effects of Sorafenib 159
3.10.2 Treatment with both TCS and PSC833 may also improve inhibition of downstream signalling pathways by Sorafenib in schwannoma 163
3.10.3 Drug resistance in schwannoma – discussion 165
4. Conclusions and future work 168
4.1 Summary of results 168
4.2 PrP^C signalling in schwannoma 169
4.3 Schwannoma resistance to drug treatments 175
5. Bibliography 178
Appendix 205
6. Supplementary data 205
6.1. Upregulation of autophagy-related proteins in schwannoma compared to Schwann cells 205
7. Abbreviations 206
8. Publications 213

List of illustrations, figures and tables

Figure 1. Differences in cell shape between Schwann and schwannoma cells. 17
Figure 2. Merlin loss causes activation of several signalling pathways. 23
Figure 3. PrP^C can undergo α or β-cleavage and be shed from the cell surface by the ADAM family of proteases. 33
Figure 4. Gene and protein structure of the prion protein family PRNP/PrP^C, PRND/Dpl, PRNT and SPRN/Sho. 42
Figure 5. Schematic structure of p-glycoprotein (p-gp). 55
Figure 6. Protein and gene structure of CD44 and its variants. 61
Figure 7. Overview of CD44 signalling pathways and their resulting effects on cells. 65
Figure 8. PRNP overexpressing clone structure. 82
Figure 9. N-terminal PrP is localised throughout the cytoplasm and cell membrane whereas C-terminal PrP is primarily localised to the nucleus. 90
Figure 10. PrP is overexpressed in schwannoma cells and tissue. 92
Figure 11. PrP is overexpressed in Merlin-negative meningiomas and human malignant mesothelioma cells. 94
Figure 12. PrP in schwannoma is the cellular, fully glycosylated form of the protease-sensitive protein. 96
Figure 13. PrP^C overexpression is Merlin-dependent in schwannoma and mesothelioma but not significantly so in meningioma. 97
Figure 14. Loss of Merlin leads to overexpression of the di-glycosylated, cellular form of prion protein. 99
Figure 15. PrP^C in schwannomas is increased due to enhanced gene transcription and is degraded by both proteasomal and lysosomal degradation. 102
Figure 16. Transcriptional regulation of PrP^C occurs via NFκB but not DCAF1. 104
Figure 17. Overexpression of PrP^C in schwannoma is due to increased gene activity, not altered protein degradation and appears to involve NFκB. 106
Figure 18. PrP^C is released from schwannoma cells via exosomes. 109
Figure 19. PrP^C is released from schwannoma cells by cleavage by ADAM10 but not by GPI anchor cleavage by PIPLC. 111
Figure 20. PrP^C is released from schwannoma cells via exosomes and by cleavage by ADAM10. 113
Figure 21. Knockdown of PrP^C using PRNP shRNA and TCS prion inhibitor 13. 115
Figure 22. PrP^C is involved in schwannoma proliferation and resistance to apoptosis. 117

Figure 23. Protection of Bax and free radical-mediated schwannoma apoptosis by PrP. 118
Figure 24. PrP^C plays a role in schwannoma pathological cell matrix adhesion. 119
Figure 25. PrP^C acts via key pathways involved in schwannoma proliferation, survival, adhesion and tumourigenesis. 122
Figure 26. PrP^C acts via several key signalling pathways to promote schwannoma survival, proliferation, cell matrix adhesion and tumorigenesis. 124
Figure 27. Long-term treatment of Schwann cells with PrP peptide increases proliferation and causes a schwannoma-like phenotype. 126
Figure 28. Overexpressing PRNP in Schwann cells leads to increased cyclin D1 and c-Jun expression. 128
Figure 29. PrP^C also acts via c-Jun to increase proliferation and tumourigenesis in schwannoma cells. 130
Figure 30. PrP^C physically interacts with LR/37/67kDa in schwannoma cells. 131
Figure 31. PrP^C signals via LR/37/67kDa to activate key signalling pathways in schwannoma. 133
Figure 32. PrP^C signals via LR/37/67kDa in schwannoma cells to activate AKT, cyclin D1, ERK1/2 and FAK pathways. 134
Figure 33. Different p-gp antibodies show different staining patterns. 136
Figure 34. P-gp is overexpressed and localises to the nucleus of schwannoma cells. 139
Figure 35. P-gp expression in schwannoma is Merlin-dependent. 140
Figure 36. P-gp co-localise with LR/37/67kDa in schwannoma but not Schwann cells. 141
Figure 37. PrP^C and p-gp co-localise in the peri-nuclear region of schwannoma cells but appear not to directly interact. 144
Figure 38. P-gp expression is regulated by PrP^C in schwannoma cells. 146
Figure 39. Merlin and PrP^C modulate p-gp overexpression in schwannoma cells..... 147
Figure 40. CD44 is overexpressed in meningioma as well as schwannoma but its expression is not Merlin-dependent. 149
Figure 41. CD44 interacts with PrP^C and with several p-gp isoforms in schwannoma cells. 151
Figure 42. CD44 expression is controlled by PrP^C in schwannoma. 153
Figure 43. PrP^C signals via CD44 to activate key signalling pathways in schwannoma. 155
Figure 44. PrP^C can act via CD44 to activate AKT, cyclin D1, ERK1/2 and FAK which, in turn, enhance schwannoma survival, proliferation and adhesion. 157

Figure 45. Treatment of schwannoma cells with TCS prion inhibitor or a combination of Sorafenib and PSC833 (Valspodar) is more effective at reducing cell viability than treatment with sorafenib alone. 161
Figure 46. Combined inhibition of p-gp, PrP^C and RTKs in schwannoma leads to a reduced signalling through key pathways involved in tumourigenesis. 164
Figure 47. Combined treatment of schwannoma cells with TCS prion inhibitor, PSC833 and Sorafenib could be more beneficial than a single drug treatment approach. 167
Figure 48. Overview of PrP^C signalling in schwannoma and potential therapeutic targets. 171
Figure 49. Hypothesis of interplay between PrP^C, p-gp and CD44 in schwannoma... 176

Table 1. World Health Organisation (WHO) meningioma classifications ……...20
Table 2. Table of chemicals and peptides ……..72
Table 3. Table of antibodies ……...73
Table 4. Table of PrP antibodies and their staining patterns…….90
Table 5 Table of p-gp and CD44 antibodies and their staining pattern…….136
Table 6. Table of abbreviations……204

1. Introduction

Schwannomas are tumours arising from Schwann cells which occur either spontaneously or as part of the hereditary, multi-tumour disease Neurofibromatosis Type 2 (NF2). NF2-associated tumours show loss of the tumour suppressor protein Merlin, overactivation of key intracellular signalling pathways, ultimately leading to pathological increases in proliferation, cell matrix adhesion and resistance to apoptosis, characteristics of all tumour cells.

Recently a large subset of tumours from a multitude of different cancers were shown to overexpress cellular prion protein (PrP^C), included in this list are schwannomas. As well as playing a role in development, myelination and prion diseases, PrP^C has been shown to activate several of the key signalling pathways involved in the development of schwannomas and other tumours.

Drug resistance is one of the biggest issues facing modern cancer medicine. Increased PrP^C has also been linked to multi-drug resistance (MDR) of chemotherapy agents in several cancer types via interactions with two key MDR-associated proteins. Firstly, p-glycoprotein, an efflux transporter which pumps drugs out of cells and secondly CD44, a cell surface hyaluronan receptor that is widely expressed on the surface of drug-resistant cells. The knowledge from the literature makes the study of PrP^C in tumours and the search for a potential drug-target within schwannomas a very interesting and important topic.

1.1 Neurofibromatosis type 2 and associated tumours

Neurofibromatosis type 2 (NF2) is an autosomal dominantly inherited tumour predisposition syndrome characterised by multiple tumours of the nervous

system including bilateral vestibular schwannomas (VS). NF2 is caused by biallelic mutations of the *NF2* gene located on the long arm of chromosome 22 at position q12, originally cloned in 1993 (1,2). *NF2* codes for the tumour suppressor protein Merlin, also known as schwannomin, mutations in which lead Merlin to be either absent from or non-functional within cells. Schwannomas often present with symptoms including deafness, tinnitus, dizziness and balance problems due to compression of the vestibulocochlear nerve that transmits sensory signals from the inner ear to the brainstem. As well as vestibular schwannomas, patients often also present with multiple other nervous low-grade system tumours such as subcutaneous and spinal schwannomas, meningiomas and ependymomas which may lead to paralysis, seizure, sensory problems and compression of the brainstem which can be fatal. Symptoms often begin to become noticeable around adolescence although they can appear at any age. The clinical diagnostic criteria for NF2 from the National Institute of Health is evidence of bilateral VS, OR a family history of NF2, plus any two of the following; schwannoma, meningioma, ependymoma, glioma neurofibroma or posterior subscapular lenticular opacities (Reviewed in (3)). In the UK, birth incidence of NF2 at present is estimated to be approximately 1 in 33,000 (4).

These NF2-associated tumours are mostly benign (WHO Grade I) so therefore currently existing treatment options are limited to surgery or radiotherapy, depending on tumour site and complexity (5). However, these techniques often require invasive procedures (surgery), incur serious morbidity and resection of the entire tumour is often incomplete, leading to recurrence. Standard chemotherapy is not a viable treatment option as the tumours are too slow-

growing and therefore do not respond. NF2 patients also often present with a huge tumour load, with multiple Merlin-deficient tumours in different locations, therefore, for NF2 patients, a systemic treatment option such as a drug would be highly beneficial.

As well as occurring as part of NF2, spontaneous Merlin-loss can also lead to the sporadic development of the same types of tumour; 50-60% of meningiomas, 70-80% schwannomas and 33% of ependymomas. Unlike NF2-associated tumours, these spontaneous Merlin-deficient tumours often affect patients later in life (40 years+) rather than during adolescence with some patients often developing asymptomatic tumours which are only discovered during post mortem (6). Other non-brain tumour or NF2-related cancers have also been shown to harbour Merlin mutations including human malignant mesothelioma, as well as a portion of melanoma, prostate and breast cancers (7-10).

1.1.1.1 Schwannomas and schwannomatosis

Schwannomas are benign, encapsulated proliferations of the Schwann cells that myelinate peripheral nervous system axons and cranial nerves contributing to successful nerve conduction. They are slow-growing tumours and, although they do not invade the nerve, they are able to compress it. Thus, vestibular schwannomas often cause severe complications including tinnitus, deafness, balance problems and even death (reviewed in (11)). Peripheral neuropathies may also occur and can lead to altered pain and tactile sensitivity or progressive distal muscle atrophy (12). Unlike healthy Schwann cells which have a thin, elongated, bi-polar structure, schwannomas often show increased cell

spreading, membrane ruffling, proliferation, survival and adhesion to the cell matrix (Fig. 1, reviewed in (11)).

Schwannomas are often thought of as the hallmark tumour for NF2 as it was originally thought that 100% of schwannoma tumours display loss of functional Merlin. Clinical diagnosis of NF2 requires the presence of bilateral vestibular schwannomas or unilateral vestibular schwannoma presenting with another NF2-related tumour as a minimum criteria (reviewed in (13,14)). This also made schwannoma the ideal *in vitro* model for studying NF2 with results that are able to be extrapolated for other Merlin-deficient tumours. However, a recent publication has shown that around 30% of non-NF2 (sporadic) schwannomas may actually be Merlin-positive (15), completely changing the face of both sporadic and NF2-related schwannoma research.

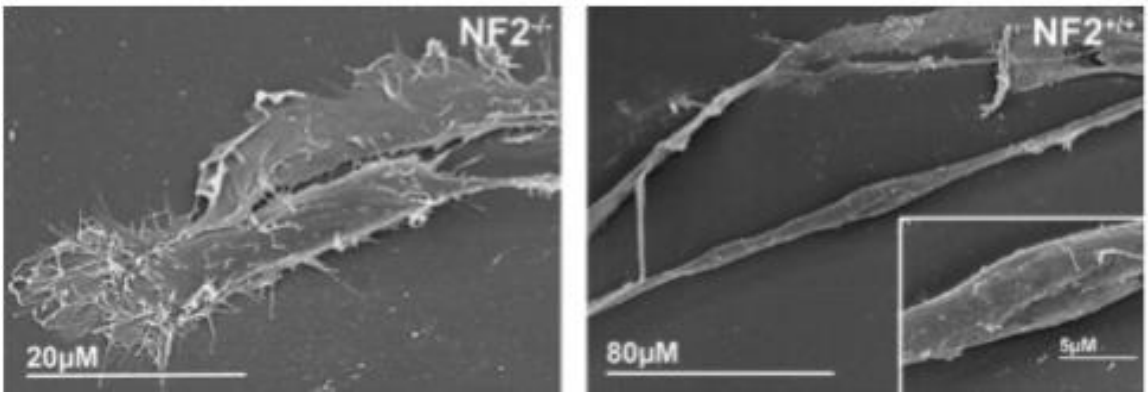

Figure 1. Differences in cell shape between Schwann and schwannoma cells.

Schwann cells (NF2+/+) are bipolar with a thin, elongated structure (right panel) whereas schwannoma cells (NF2-/-) show cell spreading, loss of contact inhibition, membrane ruffling and increased cell matrix adhesion through focal adhesions. Image taken from (11).

As well as being part of NF2, schwannomas may also occur in a condition known as schwannomatosis. Schwannomatosis shows large clinical overlap with NF2 and spontaneous schwannomas but these schwannoma tumours arise due to germline mutations in either the SW1/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily B, member 1 (SMARCB1) gene, which contributes to ~7% of sporadic schwannomatosis cases (16) and ~45% of familial cases (17) or in the leucine zipper-like transcriptional regulator-1 (LZTR1) tumour suppressor gene, germline mutations of which contributes around 9.8% of sporadic cases and 48% of familial cases of schwannomatosis (18). *NF2* is, however, also frequently inactivated in these tumours (19) suggesting an incredibly complex and highly variable genetic pattern. It was originally thought that the schwannomatosis occurred entirely spontaneously, but since the familial role of SMARCB1 muttions had been outlined, studies have also shown that a variant form of the *NF2* gene may be present in these patients which has around a 50% chance of being passed on genetically (20).

Schwannomatosis is characterised by the presence of two or more schwannomas in the absence of the bilateral vestibular schwannomas that are so often seen as part of NF2 (21). Reported incidence of schwannomatosis varies, with some reports suggesting that it affects only one person in 1.8 million, but the majority of reports suggest an incidence similar to that of NF2 at one in 40,000 (22). Patients with schwannomatosis often have multiple schwannomas on peripheral nerves around the body with symptoms being dictated by the location of each tumour, although chronic pain is experienced by most schwannomatosis patients.

1.1.2 Meningiomas

Meningiomas are the most common primary brain tumours, affecting around five in every 100,000 individuals (23) and found incidentally in around 2-3% of the general population (24). Meningiomas originate from arachnoidal cap cells which help form part of the protective meninges that covers the brain and spinal cord (25) and are two-fold more common in females compared to males (26) with some studies linking tumour size and growth to progesterone levels (27). The symptoms associated with these tumours depend entirely on their location but common symptoms to all brain tumours include headaches and potential seizures. The genetics of meningiomas is a lot more complicated than other tumours associated with NF2. The majority of meningiomas are spontaneous although only a small proportion are associated with NF2 (~15%), around 50-60% of all spontaneous meningiomas and all NF2-associated meningiomas have mutations in the *NF2* gene that leads to its inactivation (28). Meningiomas not associated with NF2 have mutations involving one of the following genes; KLF4, AKT1, SMO, TRAF7, Plk3CA, POLR2A or PRKAR1A (29). Each of these different mutations activates different signalling pathways associated with tumour development; SMO activates sonic hedgehog signalling and is seen in around 5% of meningioma cases, TRAF7 is an E3 ubiquitin ligase and TNF receptor associated factor 7 whose signalling affects nuclear factor κappa B (NFκB activation/inactivation), KLF4 mutations affect pluripotency and AKT1 mutations alter signalling through phosphatidylinositol-3 kinase (PI3K) (reviewed in (29)), PI3CA, the catalytic subunit of PI3K, is able to auto-activate PI3K and its downstream signalling pathways (30), POLR2A is a DNA polymerase and PRKAR1A codes for the protein kinase cAMP-dependent regulatory subunit 1 (reviewd in (29)). These mutations occur mutually

exclusively from *NF2* mutations and it is thought that there may still be more mutations in other genes still to be discovered as a cause of meningiomas.

Currently there are 15 distinct histopathological subtypes of meningiomas that span three WHO gradings from benign, atypical to malignant. Approximately 80-90% of meningiomas are WHO type I and benign (reviewed in (31)). They are the most diverse class of NF2-associated tumours and are often difficult to accurately diagnose without histopathological tools. To go into the various meningioma subtypes and their properties is beyond the scope of this thesis but a basic overview can be found in Table 1 which shows the various histopathological subtypes of meningioma and their associated grade. As with schwannomas, the primary treatment option for the majority of meningiomas is surgery or radiosurgery.

Table 1. World Health Organisation (WHO) meningioma classifications

WHO Grade I - Benign	WHO Grade II - Atypical	WHO Grade III - Malignant
Meningiothelial	Choroid	Papillary
Fibrous	Clear cell	Rhabdoid
Transitional (mixed)	Atypical	Anaplastic
Psammomatous		
Angiomatous		
Microcystic		
Secretory		
Lymphoplasmacyte-rich		
Metaplastic		

Although this current grading system provides prognostic value, it is based solely on histology and ignores biomarkers. A recent comprehensive characterisation of meningiomas using DNA methylation status (32) has been shown to produce more homologous groups of meningiomas compared to the WHO grading system above and appear to help stratify patients for more personalised treatments in the future. Furthermore, the presence of *TERT* promoter mutations in these tumours has been associated with a faster progression rate (32) and therefore would be useful to combine with both histological and genetic data in order to provide a fully comprehensive classification of meningioma subtypes and provide more personalised therapies in the future.

1.1.3 Ependymomas and other NF2-associated tumours

Ependymomas are much rarer than either meningiomas or schwannomas, accounting for only 2-5% of adult intracranial neoplasms [10]. Although originally, as their name suggests, ependymomas were thought to arise from the ependymal cells lining the spinal canal, more recent work has postulated that they are in fact glial in origin, arising from radial glial cells (reviewed in (33)). Approximately 33% of ependymomas show mutations in the *NF2* gene leading to loss of Merlin expression (11). Merlin loss appears to be more common in spinal ependymomas although mutations in genes other than *NF2* also cause a significant proportion of ependymomas, including some genes coding for other members of the 4.1 superfamily of proteins (34).

There are nine different ependymoma subgroups that have been described recently based on DNA methylation profiling and tumour location (35). This

method appears to be far superior to histological subtyping for assessing and stratifying risk for ependymoma development and grade in patients.

1.2. *NF2* gene and its protein product, Merlin

1.2.1 NF2 gene structure, location and mutations

The *NF2* gene is 110KB in length and consists of 17 coding exons, although only 15 of which have been identified to harbour pathological mutations (2,36). Transcription of the *NF2* gene can be initiated from several start points creating up to eight alternatively spliced isoforms, two of which are predominantly expressed; isoform II, the full-length gene product and isoform I which lacks exon 16 (37). It is thought that the diversity of the *NF2* isoforms could be down to the presence of multiple polyadenylation sites (37).

Normally a defect in one allele of the *NF2* gene is either inherited as a germline mutation or occurs sporadically, before a spontaneous mutation in the other allele leads to the development of NF2. Approximately 50% of NF2 patients have *de novo*, spontaneous *NF2* germline mutations and around one third of these are mosaic for the underlying disease-causing mutation, enabling production of the disease phenotype. The most common mutations are truncating mutations (including nonsense and frameshift mutations) which also happen to cause the most severe disease. The most frequent and well described *NF2* mutation is a single nucleotide polymorphism (SNP) C>T that leads to a nonsense mutation. However, single and/or multiple exon deletions within the *NF2* gene have also been documented (reviewed in (3)).

1.2.2 Merlin as a tumour suppressor protein

Merlin, as previously mentioned, is the tumour suppressor protein product of the *NF2* gene. It is a 586 amino acid (aa), Moesin Ezrin Radixin-like (ERM) protein that belongs to the superfamily of band 4.1 proteins (1,2). The ERM family proteins link the cytoskeleton to the cell membrane via integral membrane proteins or indirectly via membrane-associated proteins (38). Merlin consists of a C-terminal domain which lacks the canonical actin-binding motif of other ERM proteins, a coil segment and an N-terminal FERM domain that contains an actin-binding domain through which Merlin is able to interact with the cytoskeleton (39).

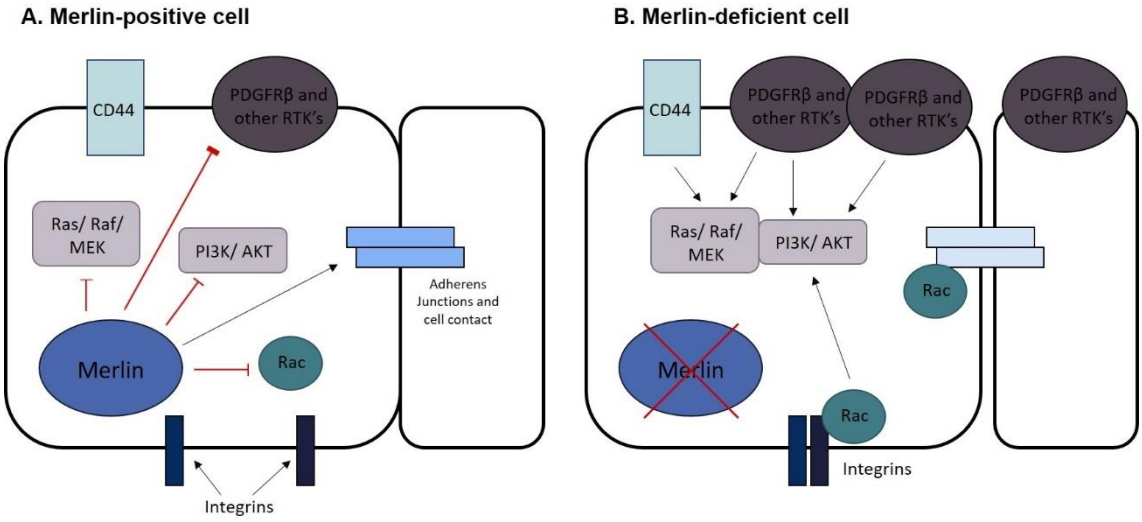

Figure 2. Merlin loss causes activation of several signalling pathways.

Merlin acts as a tumour suppressor by preventing activation of the Ras/Raf/MEK, PI3K/AKT and Rac/PAK pathways, regulating cell-cell contact at adherens junctions, regulating growth factor receptor, CD44 and integrin expression (A). Loss of Merlin causes dysregulation of this, leading to altered cell adhesion properties, increased proliferation and survival (B). Image adapted from Hanemann et.al. (11).

Merlin, when present, exists in one of two states; either an open state whereby it is able to control growth or a less-active, more-closed state. Conversion between these two states is mediated by p21-activated kinase (PAK). PAK is activated by integrin-dependent cell matrix adhesion and receptor tyrosine kinase activation and phosphorylates the C-terminus of Merlin at Serine 518 (S518), causing an increase in the C-terminal-FERM interaction, maintaining the protein in a more closed conformation and decreasing its ability to control growth (40).

There appears to be an auto-regulatory loop between Merlin and PAK as Merlin itself is able to inhibit activation of PAK via its binding to the PAK protein binding domain and also by inhibiting PAK recruitment to focal adhesions. Concurrent with this theory, loss of Merlin has been shown to lead to hyperactivation of PAK (41). De-phosphorylation of Merlin occurs via myosin phosphatase MYPT1-PP1δ (42), promoting growth inhibition when activated by cell-cell adhesion at high cell density or upon CD44-hyaluronan interaction (43,44). Merlin loss has been observed in a proportion of several different tumours including mesothelioma, glioblastoma multiforme, melanoma, breast, colorectal, hepatic, and prostate cancers. Merlin loss promotes oncogenic RAS-induced tumour formation via YAP-dependent transactivation of RAS proteins (reviewed in (15,45)).

Merlin acts as a tumour suppressor through its roles in several key pathways, firstly via its role as a cytoskeletal linker. Although Merlin lacks the actin-binding site that most ERM proteins have, it does have its own unique, N-terminal actin binding motif, meaning Merlin still interacts with actin although in a weaker and more dynamic way than other ERM proteins (46,47). Merlin indirectly links F-

actin with transmembrane receptors and effectors including receptor tyrosine kinases, small GTPases, mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K)/AKT pathway and HIPPO pathway, enabling it to act as a tumour suppressor protein by controlling cell proliferation and survival (48) (Fig. 2). Merlin has been shown to regulate activity of ERK1/2 and c-Jun-NH2-kinase (JNK) as Merlin-loss leads to a maintained elevated activation via of both ERK1/2 and JNK pathways, providing evidence of Merlin's role both as an effector of Rac and a sensor of Rac signalling (49) (Fig. 2).

A major tumour suppressor role of Merlin is thought to involve the role of $CRL4^{(DCAF1)}$, a nuclear cullin-ring E3 ubiquitin ligase. The more-open, growth inhibitory form of Merlin translocates to the nucleus where it binds and suppresses activity of the E3 ubiquitin ligase $CRL4^{(DCAF1)}$ (50), preventing excessive growth and proliferation of cells (51).

Research using D*rosophila Melanogaster* have shown that Merlin functions together with other band 4.1 proteins to block the endocytosis of several signalling receptors, including receptor tyrosine kinases, causing their accumulation at the plasma membrane (52), this was also demonstrated in mammalian cells (reviewed in (53)). Merlin is activated to suppress cell proliferation and growth upon formation of cell-cell contacts in a process known as 'contact inhibition', loss of which is a hallmark of invasive cancers (54). When performing normally upon contact inhibition Merlin down-regulates several key growth factor receptors (55), suppresses Rac recruitment to cell matrix adhesion points at the cell membrane to inhibit mitogenesis (56) (Fig. 2).

1.2.3 Merlin and integrin-related signalling

An interaction between Merlin and integrin β1 was first demonstrated in Schwann cells in 1998 (57). Loss of Merlin is associated with an upregulation in the expression of integrins, including integrin β1 at both the protein (58) and mRNA level (59). Integrin activation and increased cell-matrix adhesion has been shown to activate small membrane GTPases Rac1 and Cdc42 which, in turn, activate PAK. As well as its role in phosphorylating Merlin, PAK is also able to activate stress protein kinases SAPK and JNK, which translocates to the nucleus and leads to alterations in inflammation, proliferation and cell survival (reviewed in (60)) and regulates activity of the p53 tumour suppressor protein and transcription factor pathway (61). Thus, Merlin loss in schwannoma has been shown to hyperactivate this integrin/Rac/PAK/JNK axis and suppress p53 levels (62-64).

As well as activating the small molecule GTPases and the Rac/PAK/JNK axis, integrin activation also regulates focal adhesion kinase (FAK). FAK is a non-integrin receptor tyrosine kinase with key roles in adhesion and migration (65). FAK has been shown to have key roles in cancer initiation and progression and is upregulated in several cancer forms and in schwannoma where it signals via both PI3K/AKT and Raf/MEK/ERK pathways to promote schwannoma proliferation, adhesion and survival (66-68). FAK is commonly phosphorylated at two distinct tyrosine phosphorylation sites; the autophosphorylation tyrosine Y397 site and Src phosphorylation site tyrosine Y925, activation at both of which are increased in schwannoma compared to Schwann cells (67,68). In schwannoma cells FAK is able to localise to the nucleus, suggesting that it may be able to regulate cell proliferation without requiring the presence of Merlin (68).

Integrins also promote activation of cyclin D1, a cell cycle marker protein important in the transition from G1 to S phase, that is upregulated in ~50% of human breast cancers (69), marking a potentiation of the cell cycle and a subsequent increase in proliferation. Although the most well-known role for cyclins is in cell cycle progression, they also play a role in migration, metastasis and gene transcription (69). Cyclin D1 expression control via integrins appears to occur via mTOR, activation of which has been linked to increased cell survival and resistance to apoptosis (70). mTOR expression is Merlin-dependent in both meningioma and schwannoma cells (71).

1.2.4 Receptor tyrosine kinase signalling and Merlin

Merlin normally functions to promote clearance and recycling of growth factor receptors and other receptor tyrosine kinases (RTKs) from the cell surface, regulating receptor accumulation and signalling in Schwann and schwannoma cells, inhibiting the export of epidermal growth factor receptor (EGFR) and other RTKs to the plasma membrane (55). It has also been suggested that Merlin negatively regulates the transcription and thus the expression of RTKs via $CRL4^{DCAF1}$ (72). Unsurprisingly, loss of Merlin both in these cells and in other cell types has been shown to lead to an accumulation of EGFR, (73), ErbB2, ErbB3, insulin-like growth factor receptor (IGF1-R), vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor β (PDGFRβ) (55). Merlin also functions independently of the actual RTKs at the plasma membrane to regulate growth signals by inhibiting activation of their downstream signalling molecules such as Ras and Rac (74). All of these mechanisms contribute to Merlin's function as a tumour suppressor protein, regulating growth factor signalling.

1.3. Cellular prion protein (PrP^C)

In 2006, a gene microarray comparison between schwannoma and healthy Schwann cells highlighted a significantly increase in expression in schwannoma compared to Schwann cells of *PRNP*, the gene located at 20p13 that codes for cellular prion protein (PrP^C) (59,75).

1.3.1 Prion protein and its role in prion diseases

Prion protein (PrP) is a membrane-bound glycoprotein most commonly known for its involvement in the development of prion diseases such as Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker disease (76), kuru and fatal familial insomnia (FFI (77)). Prion diseases, also known as transmissible spongiform encephalopathies, are a family of rare, progressive neurodegenerative disorders characterised by fast-onset dementia (as little as six weeks) although, for a small subset of patients, symptoms can appear over a number of years. Unlike a number of other neurodegenerative disorders such as Alzheimer's disease or Parkinson's disease, prion diseases are highly heterogeneous and affect both humans and animals. These disease are among a small subset of recently-defined proteinopathies - diseases caused by proteinaceous particles that contain amino acids but not nucleic acids, RNA or DNA (78). Prion diseases can occur sporadically through point mutations, be acquired (external inoculation) or be inherited. For example, a familial form of CJD accounts for 10-15% of all human prion diseases and is associated with around 40 different missense mutations in the *PRNP* gene (reviewed in (79)).

In healthy adults, the normal, cellular form of prion protein (PrP^C) is particularly highly expressed in the nervous system in both neurons and glial cells, although levels of PrP^C in the peripheral nervous system (PNS) are lower than in the

CNS (reviewed in (80)). In the CNS PrP^{C} is thought to play a role in neuroprotection, signalling functions and regulation of circadian rhythm (81). This normal cellular, protease-sensitive form of PrP^{C} that is seen throughout the CNS in healthy patients is not the same form of PrP^{C} that causes prion diseases (82). In prion disease conformational change of PrP^{C} to PrP^{Sc} occurs, either via infection with the disease-associated scrapie form of the protein (PrP^{Sc}) or through conversion of PrP^{C} to PrP^{Sc}, which can arise either by a dominant point mutation in the *PRNP* gene, creating PrP^{C} that is more readily converted to PrP^{Sc}, or sporadically as a result of a random event that causes a PrP^{C} to PrP^{Sc} conversion (83). PrP^{res} is another term used to describe misfolded PrP that is resistant to degradation by proteinase K, although this encompasses PrP^{Sc} form of the protein it has been suggested that not all PrP^{res} are infectious, therefore producing a confusing gap in the nomenclature.

Although the 253 amino acid primary amino acid structure of PrP^{C} and PrP^{Sc}/ PrP^{res} are identical, the secondary structure of PrP^{C} is mainly made up of alpha helices whereas for PrP^{Sc} it is beta sheets (84). It is this increased proportion of beta sheets that makes PrP more stable and form aggregates which, in turn are capable of forming peptide aggregates known as amyloid fibrils. Parts of these fibrils are thought to be able to break off and act as a template for the recruitment of other PrP^{C} molecules which can be converted to PrP^{Sc} (85). The exact mechanism of conversion of PrP^{C} to PrP^{Sc} through inoculation is not fully understood but there are two main competing theories as to how prion propagation occurs. Firstly Prusiner (86) describes the template-directed replication of prions in which PrP^{Sc} induces the conformation change in PrP^{C}, secondly Lansbury and Caughey (87) suggest that prion propagation occurs

when an oligomeric form of PrP acts as a nucleus or seed from which polymerisation of PrP^{C} occurs, as opposed to a single-molecule conformational change. This theory may help to explain why not all exposed cells take up PrP^{Sc} and become infected (88).

To further confuse things, PrP^{C} has now been shown to exist in healthy human brains in insoluble dimeric and oligomeric forms (termed insoluble PrP^{C} or $iPrP^{C}$) as well as in the traditional monomeric form and accounts for between 5-25% of the PrP^{C} expressed in the CNS. At high levels these proteins, like PrP^{Sc}, can also be resistant to degradation by proteinase K and $iPrP^{C}$ associates with gene 5 protein (g5p) and sodium phosphotungstate, something that PrP^{Sc} but not monomeric PrP^{C} does. This suggests that these aggregates may function as dormant 'silent prions' which could be activated and lead to prion diseases (89).

1.3.2 Structure and regulation of *PRNP*

The *PRNP* gene that codes for PrP^{C} has been mapped to the short arm of chromosome 20 in position p12 in humans (75). *PRNP* comprises a 134bp untranslated exon 1, followed by a 12,696bp intron 1 and a 2.35kb exon 2 which contains the entire 759bp open reading frame (ORF) that encodes the 253 amino acid (aa) PrP^{C} protein (90). A novel 99bp 'exon-like' sequence was also found beginning 2303bp downstream of exon I (91).

PRNP has previously been described as a "housekeeping gene" due to the absence of a TATA box, presence of CpG islands and the presence of several Sp1 binding sites (92). However, the fact that transcription of *PRNP* depends on chromatin structure (93) and that the promoter region contains binding sites for several transcription factors that are yet to be identified (94), suggests that *PRNP* expression is likely to depend on several cellular factors. Other aspects

of the *PRNP* gene also appear to regulate promoter activity; exon II has been shown to play a role in regulation of PrP^C expression and intron I is required for full activity of the bovine *PRNP* promoter (95). Regulation of PrP^C expression is complex and appears to be able to be modified in several different ways.

As well as *PRNP*, there are three other members of the prion protein gene family that include *PRND*, *PRNT* and *SPRN.* Two of these genes, *PRND* and *PRNT*, share the same chromosome 20 gene locus as *PRNP* but are located further downstream, *SPRN*, however is located completely separately on chromosome 10 (Figure 4). A brief overview of the structure and function of these proteins can be found in the section entitled 'Other members of the prion protein family' but to fully and extensively review these proteins would be outside of the scope of this thesis.

1.3.3 PrP^C Structure, biogenesis and degradation

1.3.3.1 PrP^C biosynthesis and cleavage

The mammalian *PRNP* gene encodes a protein approximately 250aa in length that contains several distinct domains; an N-terminal signalling peptide, 5 proline-rich octapeptide repeats, a central, highly-conserved hydrophobic region and a C-terminal hydrophobic region which is the site of glycosylation and to which the glycosylphosphotidylinositol (GPI) anchor is attached (reviewed in (96), see Fig. 3). PrP^C is initially synthesised in the nucleus as a pre-pro-PrP which contains a leader peptide of 22aa which resides at the N-terminal and a C-terminal GPI anchor signalling peptide (termed GPI-PSS) (reviewed in (97)).

The N-terminal lead peptide translocates the PrP into the endoplasmic reticulum (ER) where it is co-translationally transported and synthesised. In the ER, PrP^C

associates with lipid rafts and undergoes cleavage of the N-terminal leader signal peptide to generate pro-PrP. PrP also undergoes glycosylation with the addition of two oligosaccharides (discussed further in chapter 1.3.3.3), formation of a single disulphide bond between two cysteine molecules within the C-terminal (reviewed in (98)).

Healthy pro-PrP is then trafficked to the Golgi apparatus where it undergoes cleavage of the GPI signal molecule (GPI-PSS) and addition of the GPI anchor, modification of the oligosaccharide attachments and addition of a sialic acid molecule to both the carbohydrate attachments and to the core of the GPI anchor, the latter making the PrP^C GPI anchor unusual compared to other proteins (99). The majority of mature PrP^C is then trafficked either directly or within endosomes (reviewed in (100)) to the cholesterol-rich domains of the outer leaflet of the cell membrane, where it binds via the GPI anchor. PrP^C does not remain on the cell surface for very long, undergoing recycling between the plasma membrane and endocytic compartment of cells (101).

In neuroblastoma cells in culture, PrP^C molecules take approximately 60 minutes to cycle from the cell membrane through the endosomes and back to the membrane again, each time this occurs, 1-5% of the PrP^C undergoes α-proteolytic cleavage directly or indirectly by ADAM8, ADAM10, ADAM17 and ADAM9 9 (reviewed in (102), see Fig. 3), within a segment of 24 hydrophobic amino acids between residues 110/111 or 111/112 (103). This α-cleavage of PrP^C, also known as 'normal' PrP^C cleavage (104) and can be stimulated by agonist of the protein kinase C pathway (105) and forms a bioactive, C1 fragment which has been linked to apoptotic signalling (106), that has been shown to accumulate at the plasma membrane via its GPI anchor (103).

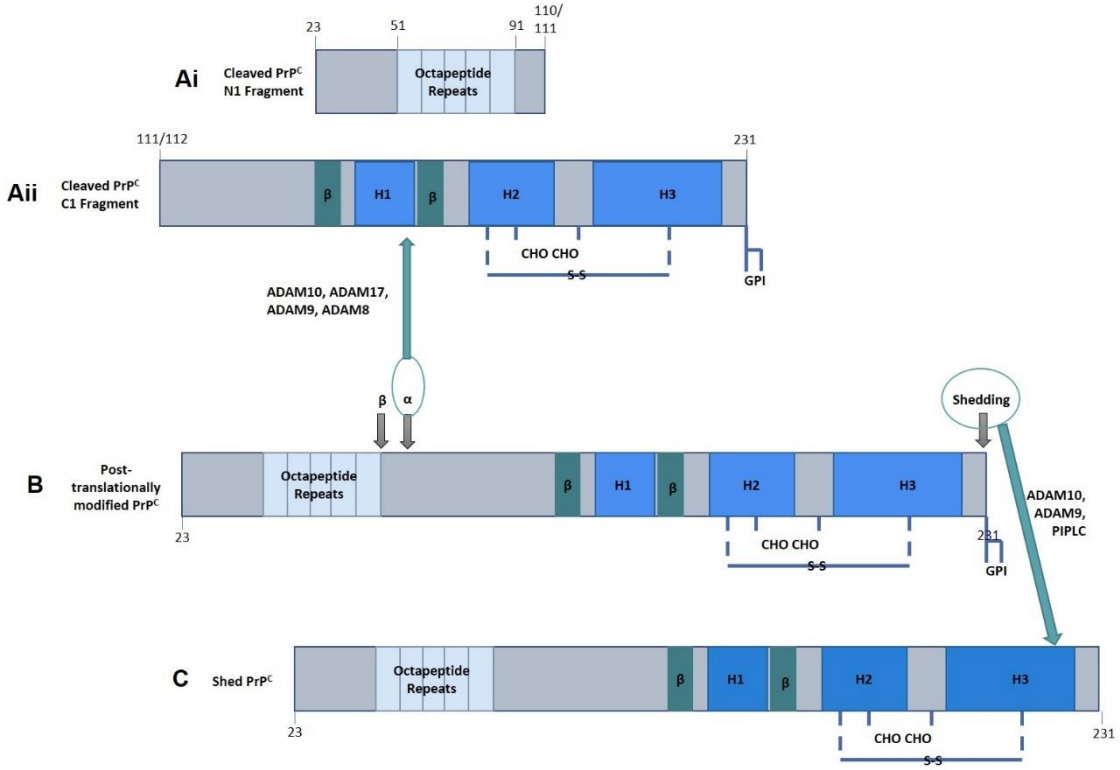

Figure 3. PrP^C can undergo α or β-cleavage and be shed from the cell surface by the ADAM family of proteases.

The post-translationally modified PrP^C (B) undergoes two significant types of cleavage at the cell membrane. Firstly, the whole PrP molecule can be shed completely via cleavage of the GPI anchor (C), this can occur via ADAM's 9 and 10 or, in vitro, with the use of phosphatidylinositol phospholipase C (PIPLC). Alternatively, PrP^C can undergo α-cleavage which cleaves PrP molecule just after the octapeptide repeat region and produces two PrP^C fragments (Ai and Aii); N-terminal fragment which contains the octapeptide repeat region and a C-terminal fragment that contains the hydrophobic segments, glycosylation sites and GPI anchor. PrP^C can also undergo β-cleavage which occurs just downstream of alpha cleavage. β-cleavage of PrP^C (not shown) is less common and little is known about the mechanisms surrounding it.

A-cleavage also produces a 10kDa N-terminal fragment (N1) of PrP that spans amino acids 23-110, which may serve as a biologically active ligand with a neuroprotective function (107). It has been demonstrated that it is within this endocytic recycling pathway that PrP^{Sc} acquires its protease resistance as the

region cleaved, between aa109-122, is thought to be involved in key conformational changes underlying the conversion of PrP^{C} to PrP^{Sc} (108).

There is debate as to whether this α-cleavage occurs entirely within the endosomes (96) or at the plasma membrane. Initially it was hypothesised that α cleavage of PrP^{C} occurred within lipid rafts residing in the plasma membrane (109) but more recent data suggests that α-cleavage in fact occurs within the late compartment of the endocytic pathway (110). Other research suggests that cleavage to produce C1 likely occurs in an acidified environment such as within lysosomes as α-cleavage can be blocked by inhibitors of lysosomal degradation (111).

PrP^{C} can also be cleaved by an event mediated by reactive oxygen species (ROS) known as β-cleavage (not shown in Fig. 3). This cleavage event occurs adjacent to the octapeptide repeat region to produce a 19kDa GPI-anchored C-terminal portion termed C2 and a 7kDa N-terminal fragment known as N2 ((112), reviewed in (110).) and has been shown to be essential in the resistance of neuroblastoma cells against oxidative stress (113) thus making β-cleavage a potentially important stage of PrP^{C} processing in cancer cells.

1.3.3.2 GPI anchoring and shedding of PrP^{C}

A portion of PrP^{C} is localised and tethered to the cell membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor (114). The GPI anchor of PrP^{C} has been shown to be highly flexible and is key to maintaining the protein a set distance (9-13 Ångström (Å)) from the membrane surface (115). Although a large portion of the membrane-bound PrP^{C} undergoes endocytic recycling, some of the membrane bound PrP^{C} also undergoes a second cleavage within the GPI anchor by a cell surface phospholipase (116) (Fig. 3). This cleavage

releases an almost full-length PrP^C into the extracellular medium in a process known as ‘shedding’. Shed PrP^C has been demonstrated in supernatant of cultured cells as well as in human cerebrospinal fluid (117-119). A disintegrin and metalloprotease-containing protein (ADAM)s 9 and 10 have been demonstrated as functionally relevant enzymes in PrP^C shedding *in vivo* (120). ADAMs 9 and 10 cleave between residues Gly228 and Arg229 (121), both of the full length PrP peptide and the C1 fragment produced by α-cleavage (122). This study supports the idea that metalloproteases, such as members of the ADAM family, cleave membrane bound PrP^C as metalloprotease inhibitors were able to reduce shedding of PrP^C. Phosphatidylinositol phospholipase C (PIPLC) has been shown to evoke PrP^C shedding *in vitro* (117). In some studies, full-length soluble PrP^C released from cells which is then able to be converted to PrP^{Sc} has been proposed as one of the mechanisms responsible for the intercellular transfer of prions and thus the dissemination of prion diseases (119). However, others have reported that PrP^C shedding is concurrent with a reduction in PrP^{Sc} formation (123) thus the physiological consequences of PrP^C shedding from the cell membrane remain elusive.

The importance of the GPI anchor can further be questioned as studies in human neurons show that anchorless PrP^C is present in small quantities in healthy human brains (124). In healthy brains it is thought that there is still a signalling role for GPI-anchorless PrP^C as studies have shown that PrP^C does not require the GPI membrane anchor in order to protect cells against apoptosis (125,126).

The fact that PrP^C is cycled to and from the membrane and undergoes endocytosis via clathrin-coated pits suggests that one function of PrP^C is to

serve as a receptor for one or more extracellular ligands. However, as a GPI-anchored membrane protein PrP^C lacks a cytoplasmic domain that is able to directly interact with signalling adapter proteins and clathrin (98) it is, as most GPI-anchored molecules, excluded from coated pits and instead internalised via caveolae (127). The proposed mechanism by which the GPI-anchored PrP^C may still be internalised by clathrin coated pits is via the several PrP^C interacting partners or putative PrP^C 'receptors' able to recruit clathrin which have been hypothesised, including 37/67kDa non-integrin laminin receptor (LR/37/67kDa) (128), stress-inducible protein 1 (STI-1) (129) and copper ions (130).

1.3.3.3 PrP^C glycosylation

Glycosylation is a post-transcriptional modification process for many proteins. It has many different important functions within cells; regulating protein-protein interactions as the sugars can act as ligands for various receptors, regulating attachments (131) and even used to monitor protein folding and affect the solubility of proteins (132) making the glycosylation process key for determining the activity of certain proteins. PrP^C is a sialo-glycoprotein which undergoes glycosylation in the Golgi apparatus by the addition of N-linked high-mannose-type oligosaccharide chains to a portion of the C-terminal of the protein, as well as undergoing glycosylation of the GPI anchor, an event that is uncommon in mammalian GPI anchors (133). N-glycans have been shown to play a critical role in quality control and folding of proteins in the endoplasmic reticulum, particularly in the unfolded protein response (UPR) and ER associated proteasomal degradation (ERAD) (134). The attachment of these N-linked sugar chains also modulates the function of cell surface proteins involved in migration, adhesion and myelination, all important in schwannoma development,

they also affect transport of proteins through the Golgi apparatus and sorting of secreted proteins such as prions (reviewed in (135). This makes glycosylation of PrP^C interesting to look at with regards to schwannoma development.

To the newly synthesised PrP^C, oligosaccharide chains are added at two C-terminal asparagine residues at Asn181IleThr, located in one of the α-helix regions and Asn197PheThr, located in a loop region of PrP^C in humans, during post translational modification in the ER and before addition of the GPI anchor (115,136), leading to the ability of three glycoisoforms of PrP^C to be produced – di-, mono- and un-glycosylated. It appears that glycosylation of PrP^C is dependent on membrane topology and tethering, as only PrP with a C-terminus anchored by a GPI anchor or transmembrane section and not soluble PrP was glycosylated in the ER, even though both proteins have a structurally identical C-terminal (137). More than 50 sugar chains have been identified as able to glycosylate PrP (138) and some studies have shown a shift in the proportion of glycans between the normal cellular prion protein and PrP^{Sc}; with PrP^{Sc} containing more complex tri- and tetra-antennary glycans compared to PrP^C (139). Upon translocation to the Golgi apparatus, the N-linked oligosaccharides undergo further processing into more complex sugar types, which have had a sialic acid residue attached and become resistant to digestion by endoglycosidase H (140).

Glycosylation appears to be an important step in the formation of the protease resistant forms of PrP too; inhibiting glycosylation of PrP^C using tunicamycin has been shown to predispose PrP^C to convert to PrP^{Sc} and made PrP more resistant to protease degradation (141,142). Non-glycosylated 26kDa PrP has been detected in N2a neuroblastoma cells expressing PrP^C which may be the

result of a failure to N-glycosylate the protein in the first place or suggests that the PrP^{C} has been deglycosylated in the cytosol of the cells by N-glycosidase (143).

1.3.3.4 Different topological isoforms of PrP- ^{Ctm}PrP ^{Ntm}PrP

As well as the cleaved C1/2 and N1/2 PrP^{C} fragments generated by endoproteolytic α- and β-cleavage (described above), several other topological isoforms of PrP^{C} have also been described that correspond to specific secreted and transmembrane forms of the prion protein ^{Sec}PrP, ^{Ctm}PrP and ^{Ntm}PrP (144). Upon synthesis, PrP^{C} is translocated, via the N-terminal signal sequence, and inserts itself into the ER membrane in order to be able to undergo the first stage of post-translational modifications. Is has been shown, however, that the C-terminal hydrophobic region (TM2) can also function as a signal sequence, yielding a C-terminal transmembrane form of the prion protein (^{Ctm}PrP) of which the C-terminus is directed towards the ER lumen (145)). ^{Ctm}PrP spans the membrane once via the hydrophobic region between residues 111 and 134 and undergoes the addition of the GPI anchor and N-glycosylation in the same way as full length PrP^{C} (137). ^{Ctm}PrP is able to accumulate in the ER and can contribute to neurotoxicity and cell death via mechanisms involving ER stress (146,147). In fact, it has been suggested that ^{Ctm}PrP is the main effector of neurodegeneration in prion diseases and that PrP^{Sc} only functions to increase levels of ^{Ctm}PrP although this is still not widely accepted (148).

The other topological isoform ^{Ntm}PrP which also traverses the cell membrane between residues 111-134 has the N-terminal directed towards the ER lumen (Reviewed in (144)). ^{Ntm}PrP lacks the GPI anchor and N-linked glycosylatation seen in both PrP^{C} and ^{Ctm}PrP (137), it has been shown to have no role in any of

the prion diseases and has only been demonstrated in transgenic animals and cell culture models (reviewed in (144). Finally, a third topological isoform of secreted PrP (^{Sec}PrP), not associated with the lipid bilayer, is the predominant form of PrP^{C} produced. From the lumen of the ER ^{Sec}PrP can then be translocated to the secretory pathway where it is able to travel via the Golgi apparatus and endosomal vesicles (149). It is suggested that relative proportions of the three topological isoforms of PrP are determined by currently unknown accessory proteins which are present during translation or a region of nine hydrophilic acids adjacent to the transmembrane domain of PrP (reviewed in (150)), although more work is required to understand the precise mechanism by which the PrP transmembrane variants arise.

1.3.3.5 PrP^{C} degradation

During PrP^{C} synthesis in the ER, several forms of misfolded or mutated PrP are recognised, ubiquitinated and sent for ER-associated protein degradation (ERAD). This has been demonstrated in cell cultures where proteasome inhibitors such as MG132 have been shown to increase levels of PrP^{C}. It was initially thought that ERAD degradation was specific to PrP^{Sc} although further research has shown that approximately 10% of all newly generated PrP^{C} is diverted to degradation via ERAD (151) .

There is evidence of PrP degradation by a different pathway – lysosomal degradation. Lysosomal degradation is a degradation process carried out by acidified spherical vesicles known as lysosomes that contain >60 different hydrolytic enzymes, as well as maintaining a low pH, to break down their contents. Lysosomal degradation can occur alone or via autophagy which initially involves the material to be degraded being engulfed in an

autophagasome before being targeted to lysosomes. A lot of the evidence that PrP is degraded via lysosomal degradation comes from studies looking into the abnormal form of PrP - PrP^{Sc} (152-154), suggesting at the very least that lysosomal degradation is a key pathway via which levels of PrP^{Sc} are regulated in infected brains.

Knockdown and pharmacological blockade of key components of the autophagy pathway, including Rapamycin, an inhibitor of the mTOR pathway, increase PrP^{Sc} levels reviewed in (155), whereas chemical stimulation of autophagy in infected cells has been shown to decrease levels of PrP^{Sc} (156). One rapalogue (an analogue of Rapamycin known as Tacrolimus) was even shown to decrease levels of PrP^{C} in an anti-prion screen (157). This suggests that autophagy is the main route by which PrP^{Sc} and, potentially also PrP^{C}, are targeted and transported for lysosomal degradation in chronically infected cells (156). Although, other data has shown that PrP^{Sc} can also be a substrate within the Golgi quality control (QC) pathway - a pathway involved in non-autophagy dependent lysosomal degradation (155). This points to the fact that lysosomal degradation of PrP^{Sc} is a key process in regulating PrP^{Sc} levels but this process can occur either with, or independently of, autophagy. Thus, two major clearance pathways – both proteasomal and lysosomal degradation- appear to be involved in PrP clearance, this complicated any findings that can be drawn from PrP intracellular levels, making it impossible to deduce information about degradation rate of PrP solely based on intracellular levels but making both proteasomal and lysosomal degradation important therapeutic targets to reduce PrP expression, both of PrP^{Sc} and PrP^{C}.

1.4 Other members of the prion protein family

1.4.1 *PRND* and doppel (Dpl)

PRND was the first discovered of three other genes related to *PRNP*. Discovery of the *PRND* gene was rather indirect. Upon creation of Npu and Zrch 1 $PRNP^{0/0}$ mice which are homologous for the inactivated *PRNP* gene due to disruptions in the open reading frame (ORF) of *PRNP*, animals to show no overt phenotypical differences to their wild-type counterparts aside from a slight alteration in circadian rhythm. Upon generation of the next generation of $PRNP^{0/0}$ mice (Ngs and Zrch II), not only was the ORF disrupted but also the flanking regions, including the splice acceptor site of the third exon (reviewed in (158)). This unexpectedly allowed expression of *PRND*, located just 16kB downstream of *PRNP*, to be put under the control of the *PRNP* promoter when, normally, its expression in the CNS would be blocked and expression would be restricted to the testes (159). This led the Nagasaki mice to demonstrate cerebellar Purkinje cell loss leading to late onset cerebellar ataxia.

PRND encodes a 179-aa long prion-like protein called Doppel (Dpl). *PRND* shares 24% coding sequence homology with *PRNP* and, like *PRNP* is highly expressed during embryogenesis (Fig. 4). During embryogenesis *PRND* has a role in development of both the testes and ovaries and plays a role in male fertility (160) but, due to its location in the gonads of both sexes and the fact that it lacks the N-terminal octapeptide repeat domain, key for $PrP^{C} \rightarrow PrP^{Sc}$ conversion, Dpl has never been linked with prion diseases (reviewed in (161)).

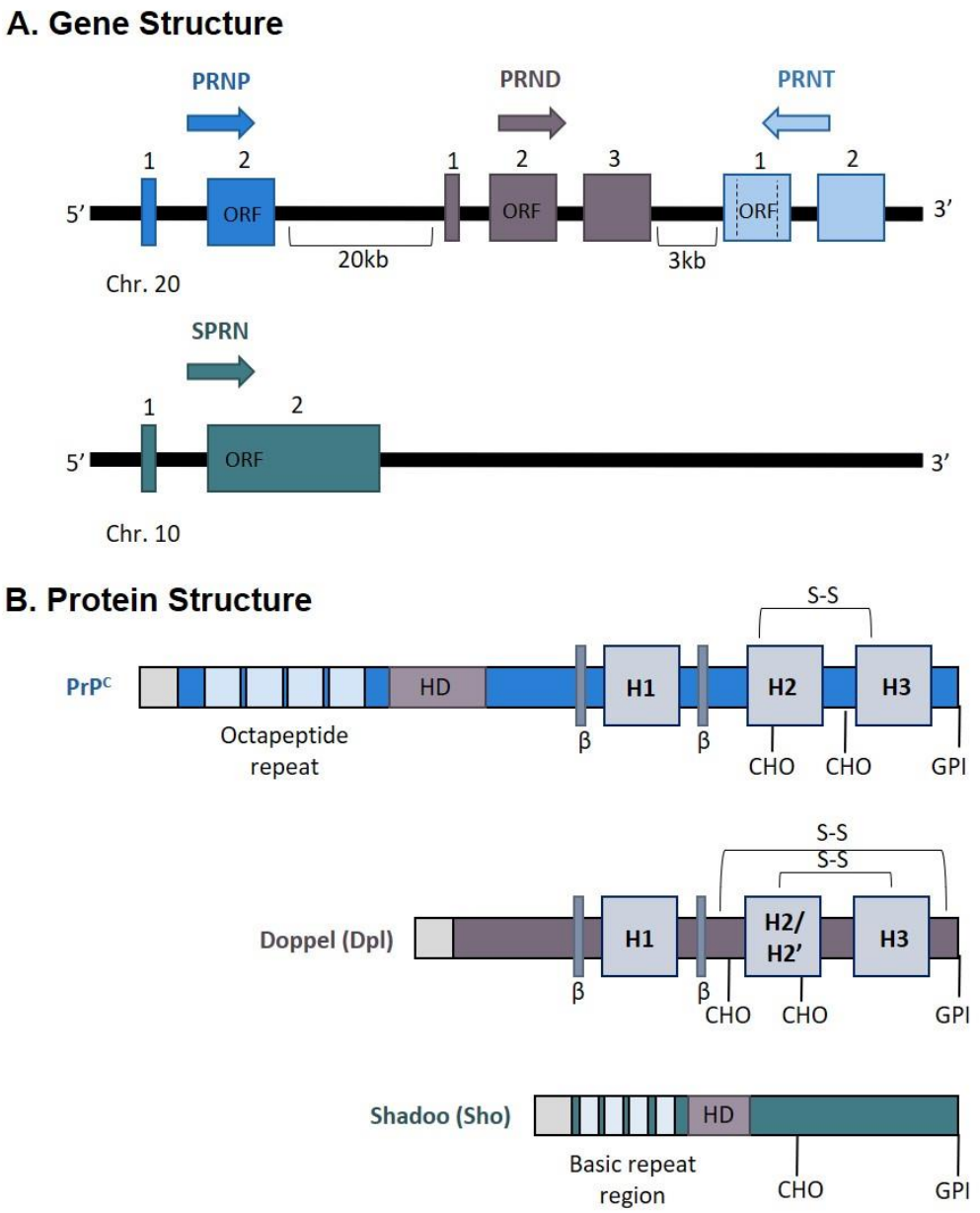

Figure 4. Gene and protein structure of the prion protein family *PRNP*/PrP^{C}, *PRND*/Dpl, *PRNT* and *SPRN*/Sho.

PRNP, *PRND* and *PRNT* are all located within the same gene cluster on chromosome 20. *PRNP* consists of just two exons. Located just downstream of *PRNP* are *PRND* and *PRNT* which are under control of the *PRNP* promoter (A). The final Prion Protein member, *SPRN*, is located elsewhere on chromosome 10 but, like the majority of other prion protein members, only consists of two exons (A). All four genes have the open reading frame contained within a single exon. All three of the major prion protein family are GPI-anchored glycoproteins. Protein structures of PrP^{C} and Dpl are similar whereas Sho only shares a loosely similar repeat region, hydrophobic domain and only has one glycosylation site (B). Figure modified from (160).

The structure of PrP^C and Dpl are similar with both containing three α-helices, Dpl also undergoes the same post-translational modifications as PrP^C leading to the production of a GPI-anchored membrane glycoprotein with two N-glycosylation sites (162) and a disulphide bond (Fig. 4B). However, the *PRND* gene does not appear to contain a classical GPI recognition sequence (159) and there is evidence that more than one disulphide bond exists (162).

There is also data to suggest an antagonistic interaction between the two proteins. PrP^C has been shown to rescue neuroblastoma cells and astrocytes from Dpl-induced apoptosis via a direct interaction with Dpl in detergent-resistant domains of the cell membrane (163) and a physical interaction has been demonstrated by way of immunoprecipitation in Fischer rat thyroid (FRT) epithelial thyroid cells (164).

1.4.2 *PRNT* and PRT

PRNT is the hypothetical third and most recently described member of the prion protein superfamily, it belongs to the same gene cluster as *PRNP* and *PRND* and is located approximately 3kB downstream of *PRND* ((165) see Fig. 4), appearing to be specifically expressed only in primate populations (166). The *PRNT* gene consists of two exons and has three distinct alternatively spliced transcripts, none of which have any homology to PrP^C or Dpl. The protein product of *PRNT*, PRT, is expressed exclusively in adult testes, the major site of Dpl expression (165) suggesting that, although not homologous, PRT and Dpl are functionally related (166). The open reading frame of *PRNT* is predicted to encode a 94 residue protein (Fig. 4B), although no evidence of this protein has

yet been found and some are unsure whether *PRNT* represents a genuine human gene at all (167).

1.4.3 *SPRN* and shadoo

Shadow of Prion Protein (*SPRN*) is a 3.9kB gene located on chromosome 10q26.3 that encodes the prion-like protein Shadoo (Sho) (Fig. 4A, bottom panel). Sho is a 150aa, 14kDa highly-conserved protein seen in many organisms from mammals to fish (168). The protein sequence of Sho is closely related to PrP and Dpl, it contains a highly conserved N-terminal signal sequence, an Arginine-rich basic region, a 20-residue hydrophobic region highly homologous to PrP, a less well conserved C-terminal domain containing a conserved glycosylation motif and a C-terminal signal sequence peptide for GPI-anchor attachment (168) (Fig. 4B). Unlike PrP, Sho is both a glycoprotein and a lipoprotein as it undergoes both glycosylation and lipidation and there are mixed results about whether Sho expression is essential for completion of embryogenesis; some research suggests that Sho is not essential for embryonic development and others suggesting Sho knockout leads to embryonic lethality (169,170). Bioinformatic data suggests that a very primitive, early form of prion protein was related to *SPRN*, with *PRNP* and *PRND* evolving later (168). Sho has been shown to have neuroprotective functions similar to PrP^{C} and has been shown to rescue $PRNP^{0/0}$ cerebellar granular neurons from the cytotoxic effects of Dpl (171). Reduced expression of Sho in prion diseases has been described (171) and polymorphisms in the *SPRN* signal peptide have been linked to development of sporadic CJD (172).

1.5 Significant biological functions of PrP^C

Unlike PrP^{res}, function of the 'normal' PrP^C was, unknown for many years as *PRNP*-null mice initially appeared phenotypically normal [10-12], aside from a few defects in their circadian rhythm. Conservation of parts of the PrP^C sequence throughout evolution and the presence of PrP^C in species from yeast to mammals suggest it does, however, have an important biological role [13]. Global PrP^C expression begins during embryogenesis but in the adult it is predominantly expressed in the central nervous system (CNS) and has been linked to several key roles that may be relevant in tumourigenesis which are discussed in more detail in the following subchapters.

1.5.1 PrP^C in cell adhesion

PrP^C has been implicated in adhesion in both healthy and tumour cells [17]. In melanoma, pro-prion protein (an incompletely processed form of PrP^C) binds to filamin A, facilitating the interaction with integrin β1 (173). Binding of PrP^C to filamin A disrupts its normal function leading to altered adhesion of cells but also to changes in cytoskeletal organisation and signal transduction pathways, allowing cells to grow more aggressively (174). PrP^C has also been shown to control β1 integrin-mediated adhesion in human monocytes, mediated by RhoA, cofilin and ERM-mediated membrane cytoskeletal linkage (175) in which Merlin is known to play an important role.

The N-terminus of PrP^C has a neuronal cell adhesion molecule (NCAM) binding site (176) providing further evidence for PrP^C as a mediator of cell adhesion. Other reports suggest that NCAM acts as a neuron-specific receptor for PrP^C (177) to not only affect cell adhesion but also regulate downstream signalling pathways involved in development and tumourigenesis as NCAM itself is

expressed in an array of cancers from brain tumours to acute myeloid leukaemia (AML (178)).

Finally, PrP^C has been shown to interact with another cell adhesion molecule E-cadherin. During development PrP^C regulates the stability of adherens junctions and, thus, cell adhesion by modulation of E-cadherin transport to and from the plasma membrane (179,180) and that downregulation of PrP^C actually modifies and impairs the formation of adherens junctions (181). PrP^C may also play a role in modulating focal adhesions (FA) as PrP^C overexpression leads to the formation of new FA-like structures, whereas downregulation of PrP^C leads to reduced FA numbers as well as activation of Src and FAK (182) which is known to be upregulated and over-activated in schwannoma and contribute to schwannoma increased adhesion (183).

1.5.2 PrP^C in survival and neuroprotection

It is well known that the aggregated and misfolded forms of PrP^C, PrP^{Sc} and PrP^{res} lead to neuronal cell death on a huge scale, as seen in the prion diseases. However, in more recent years a role for the cellular form of PrP in neuroprotection and cell survival has been established, with PrP^C protecting cells from oxidative stress [15], from the pro-apoptotic effects of another form of cellular prion protein, Dpl [16] and protecting cells from Bax-mediated apoptosis [14]. This is thought to occur through Bcl-2-dependent apoptotic pathways where Bcl-2 was upregulated and, consequently, p53 and Bax downregulated in gastric cancer cells (184).

Resistance to apoptosis provided by PrP^C is regulated by its glycosylation state as inhibition of N-linked glycosylation of PrP^C eliminated the ability of both oral squamous cell carcinoma (HSC-2) and colon adenocarcinoma (LS 174T) cell

lines to resist oxidative stress, leading to a decrease in viability of these cells (185). In the same colon adenocarcinoma cell line, PrP^C also helped resist against doxorubicin-induced apoptosis (186), further suggesting a protective role for PrP^C.

In serum deprived neuronal cells, PrP^C was able to prevent apoptotic neuronal cell death and the mitochondrial dysfunction that often leads to oxidative stress (187) and depletion of PrP^C led cells to have increased levels of programmed cell death as well as increased levels of apoptotic proteins such as p53, BAX, caspase-3, Poly(ADP-ribose) polymerase (PARP) and cytochrome C upon starvation, again suggesting a role of neuroprotection for PrP^C that may occur via the aforementioned pathways.

1.5.3 PrP^C in cell proliferation and tumorigenesis

Transcription of *PRNP* is upregulated in several cancer cell lines (188) and PrP^C itself has been shown to play a role in cell proliferation both in cancer cells and in other cell populations. In gastric cancer patients PrP^C was shown to promote G1/S transition, in part via the activation of the PI3K/AKT, as well as by activating levels of cyclin D1 both at the mRNA and the protein level (189). PrP^C has also been demonstrated in the nucleus of proliferating intestinal epithelial cells, where it interacts with the Wnt signalling pathway including alternative downstream pathway members of the HIPPO family such as Yap (190), a pathway that regulates organ size, proliferation and survival and which have been shown to be involved in tumourigenesis of many different types of cancers including breast, pancreatic and colorectal cancers (191).

Under normal development, PrP^C plays a role in the CNS, regulating neuronal cell and glial cell precursor cell proliferation (192,193) suggesting a role for PrP^C

in proliferation way before it becomes involved in tumourigenesis and that increased proliferation of tumour cells may, in some cases be caused by a dysregulated expression of PrP^C.

Previous work has shown PrP^C to be involved in the promotion of tumourigenesis and metastasis in several different cancer types including gastric, colorectal and breast cancers (194-196). One of the main phenotypes of cancer cells is their ability to resist apoptosis, this contributes not only to tumour growth but also the metastatic and drug-resistance potential of these cells. Resistance to apoptosis can be the result of several different cellular events including overexpression of anti-apoptotic proteins or a downregulation of pro-apoptotic proteins which may be caused by the expression of oncogenes, epigenetic modification or loss of tumour-suppressor proteins such as in the case of NF2 (reviewed in (197)).

PrP^C expression may also increase the invasiveness of certain tumour types such as gastric cancer (198) where PrP^C was able to promote the invasive, adhesive and metastatic capabilities of these cells in culture via activation of the MEK/ERK pathway and matrix metalloprotease 11 (MMP11). It has been reported that PrP^C activates several of the key signalling pathways known to be involved in schwannoma development including p53 [24], AKT [25], [26] and phospho-ERK1/2 (pERK1/2) [27].

1.6 In search of a receptor for PrP^C

1.6.1 $P75^{NTR}$ and the NFkB signalling axis

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a transcription factor with roles in inflammation (reviewed in (199)), immunity and cell survival (reviewed in (200)). NFκB belongs to a class of transcription factors known as 'Rapid-Acting Transcription Factors' which are present in an inactive form within cells and are subsequently activated, rather than relying on new protein synthesis to have their effect. NFκB activity is regulated by a set of inhibitory cytoplasmic factors known as IκB. IκBα binding to NFκB inhibits its translocation to the nucleus thus forcing it to remain inactive. Activation of kinases IKKα and IKKβ phosphorylate IκBα causing it to undergo proteasomal degradation, freeing NFκB and allowing its translocation to the nucleus. In the nucleus NFκB acts to trigger transcription of various target genes, including inhibitory factor IκBα, in turn temporarily suppressing the NFκB response (reviewed in (201)).

NFκB has been linked to both tumour initiation as well as cancer progression by regulating proliferation, resistance to apoptosis, epithelial to mesenchymal transition (EMT) and metastasis (202-205). Recently, increases in NFκB levels in cancer patients undergoing chemotherapy or radiotherapy have been shown to cause resistance of tumour cells to apoptosis, giving NFκB a role not only in tumour development but also in drug and treatment resistance through its control of transcription of the MDR1 gene (206), making it an interesting potential therapeutic target in schwannoma and other tumours. Similarly, NFκB has been shown to regulate transcription of other key genes relevant to schwannoma including; CD44 (207), p53 (208) and cyclin D1 (203).

NFκB is essential in Schwann cell developmental biology and injury repair (209,210). Dilwali et.al. (211) recently showed using pathway analysis that aberrant NFκB activation appears to be one of the major causes of increased proliferation in VS and demonstrated that use of NFκB inhibitors such as BAY11-7082 can decrease schwannoma proliferation and survival *in vitro* (reviewed in (212)). Previous work by myself and others in our research group showed that NFκB is also activated downstream of the AXL/Gas6 growth factor axis in schwannoma and that NFκB overexpression seen in schwannoma contributes to increased expression of survivin, cyclin D1 and FAK, leading to increased schwannoma proliferation, adhesion and survival (213).

One of the major modulators of NFκB expression is the cell-surface growth factor receptor p75 neurotrophin receptor ($p75^{NTR}$) (201). $P75^{NTR}$ can act both independently or in conjunction with tropomyosin-related kinase (Trk) receptors modulating Trk-ligand interactions, augmenting their binding capabilities to neurotrophins and pro-neurotrophins as well as altering their signalling capabilities either to promote cell survival or cell death depending on the co-receptor that its coupled with (reviewed in (214)). $p75^{NTR}$ is known to play an important role in both Schwann cell migration during development and in Schwann cell pathology after injury (215,216) and was in the list of upregulated genes found in schwannoma compared to Schwann cells (59).

Signalling of $p75^{NTR}$ has been linked to activation of c-Jun transcription, a master regulator of Schwann cell regeneration and proliferation that is upregulated in schwannoma, leading to increased tumour proliferation and survival (217,218). $P75^{NTR}$ activates NFκB via JNK-independent pathways in VS to provide a pro-survival response which likely contributes to their ability to

survive even in the absence of axons (219). Merlin is a key mediator of $p75^{NTR}$ expression and signalling in Schwann cells, this expression is highly upregulated in VS due to inactivation or loss of Merlin (220) (reviewed in (212)). Research in a mouse neuroblastoma cell line has implicated $p75^{NTR}$ activation of NFκB as the cause of cell death mediated by the prion protein fragment 106-126, suggesting that $p75^{NTR}$ acts, either alone or in complex with other Trk receptors, to act as a receptor for PrP (221). Other research has shown that $p75^{NTR}$ is upregulated in schwannoma and produces a pro-survival response due to activation of NFκB (220) and PrP^{C} has also been shown to have a neuroprotective role and contribute to cell survival.

1.6.2 Laminins and their receptors

Interaction between tumour cells and the extracellular matrix and basement membrane is a key step in tumourigenesis as most tumour cells show increased cell matrix adhesion, adhesion to the basement membrane also plays a role in tumour invasion and metastasis (222). Laminins (LAs), a group of approximately 11 isoforms of large, non-collagenous glycoproteins, formed from two polypeptide chains joined by disulphide bonds. LAs were found to be one of the key components of the basement membrane and thus, a key component in mediating tumour invasion and metastasis (223). Further studies have shown the fragments of LA are able to exert opposing effects on tumour cells; some fragments enhance the metastatic activity of tumours (224) whereas others inhibit the metastatic activity of some malignant cells (225,226); this may be due to actions at different laminin receptors on the cell surface. Two families of laminin receptors have been discovered; the integrins and the non-integrin laminin receptors.

Integrins are transmembrane, cell surface receptors heavily involved in cell adhesion that are composed of an alpha and beta subunit. So far 19 alpha and 8 beta subunits have been described (227) of which several heterodimers serve as receptors for LA, these include alpha 1 beta 1, alpha 2 beta 1, alpha 3 beta 1, alpha 6 beta 1, alpha 7 beta 1 and alpha 6 beta 4 (228) . Integrins regulate a wide range of cellular functions critical for the initiation, progression and metastasis of solid tumours. They are key to several integrin-mediated signal transduction processes, transducing signals across the membrane upon ligation with ligands such as laminin and fibronectin (reviewed in (229)), pathways known to play important roles in tumourigenesis and are known to be involved in schwannoma development as previously described. Overexpression of several key integrins (alpha 6 beta 1 and alpha 6 beta 4) has previously been demonstrated in schwannoma and been attributed to the increased pathological adhesion seen in these cells (58).

The first of the non-integrin laminin receptors to be discovered was the 67kDa non-integrin laminin receptor (230-232) which, later on, was found to arise from a 37kDa laminin receptor precursor (LRP). Evidence of this precursor laminin receptor protein led to the protein being named as 37/67kDa non-integrin laminin receptor (LR/37/67kDa) (233,234). However, this precursor was shown not to be a homodimer of 37kDa and that instead may be formed by heparin sulphate proteoglycan (HSPG) binding to the 37kDa LR (235). A separate group from a different field of research also identified the same protein and named it p40. This 37kDa protein was found to bind to the 40S ribosome where, although it does not function here as a laminin receptor, it instead plays a role in gene transcription (236), hence why LR/37/67kDa is also known as ribosomal protein

SA (RPSA). LR/37/67kDa is formed by transcription of the 5.8Kbp *RPSA* gene on chromosome 3p22.2 which consists of seven exons and six introns (237).

Many tumours show increased expression of LR/37/67kDa including colorectal and breast carcinomas, melanoma and acute myeloid leukaemia (238-241). LR/37/67kDa expression has been demonstrated to be important in tumour cell proliferation, survival, adhesion, migration, invasion, angiogenesis and intracellular signalling (242-246). Furthermore, LR/37/67kDa may be a novel potential therapeutic target for treating tumours as studies have shown that using siRNA to reduce LR/37/67kDa expression leads to cell cycle arrest by reducing levels of cyclins, cyclin-dependent kinases and resistance to apoptosis by increasing levels of survivin and p21 (245). No studies have, thus far, commented on the expression and role of LR/37/67kDa in schwannoma.

1.6.2.1 37/67kDa non-integrin laminin receptor protein as a 'receptor' for PrP^C

In 1997, a group first identified the existence of a prion protein receptor of around 66kDa by complementary hydropathy -a practice that uses the hydrophobic properties of amino acid side chains to determine protein properties and predict protein interactions (247). After this, another group (248) showed in a yeast two-hybrid system that the 37kDa LRP is able to interact with PrP^C and further verified this interaction in mammalian COS-7 cells suggesting that LR/37/56kDa could act as a receptor for PrP^C. In 2001 LR/37/67kDa was shown to act as a cell surface receptor for PrP^C in neuroblastoma cell lines (128) via PrP^C/LRP binding domain 1 (amino acids (aa) 144–179) as a direct and PrP^C/LRP binding domain 2 (aa 53–93) as an indirect, HSPG-dependent LR binding site on PrP^C, as both LR and PrP^C have been shown to bind HSPG's (249,250). A second direct PrP-binding domain on LRP was also found

between aa 161 and 179 (235). LR/37/67kDa appears to be an interesting potential receptor, upregulated in many tumour types, via which PrP^C acts to contribute to pathological proliferation, invasion, adhesion and survival of tumour cells and may prove to be a novel therapeutic target in schwannoma cells.

1.7 Multi-drug resistance

1.7.1 P-glycoprotein and multi-drug resistance in cancer cells

In recent years, PrP^C has been shown to be upregulated in multi-drug resistant cancer cells compared to parent cell lines [176]. Multi-drug resistance (MDR) is the most common reason why cancer patients stop responding to chemotherapy regimens as cancer cells become resistant to the cytotoxic effect of chemotherapy drugs over time. This can occur through several mechanisms: Decreased drug uptake, increased drug export, decreased drug activation, increased drug de-activation, decreased formation of drug-target complexes and increased repair of drug damage (251). MDR cell lines were first isolated in 1968, but it was almost another decade later until high expression of phospho-glycoprotein (p-gp) in such cell lines was demonstrated to be the cause of the MDR phenotype (252,253). Since its initial discovery in drug-resistant Chinese hamster ovary cells and its purification, p-gp expression has been shown to confer an MDR phenotype in several human cancer cell lines and is a well-established cause of drug-resistance in many tumours today.

P-gp, also known as ATP binding cassette sub-family B member 1 (ATPB1), is a transmembrane ATP-dependent efflux pump which functions to actively extrude toxins, xenobiotics and chemotherapeutic agents as well as a wide variety of other small molecules out of cells and plays a significant role in

regulating drug absorption and deposition. Although 48 ABC-binding cassette transporter subfamily genes exist, it appears that only three are associated with chemoresistance *in vivo*, these are; P-gp/MDR1, ABCG2 also known as Breast Cancer Resistance Protein (BCRP) and ABCC1/ Multi-drug resistance protein-1 (MDRP-1) (Reviewed in (254)). Of these, p-gp is most well-known and thus the most researched.

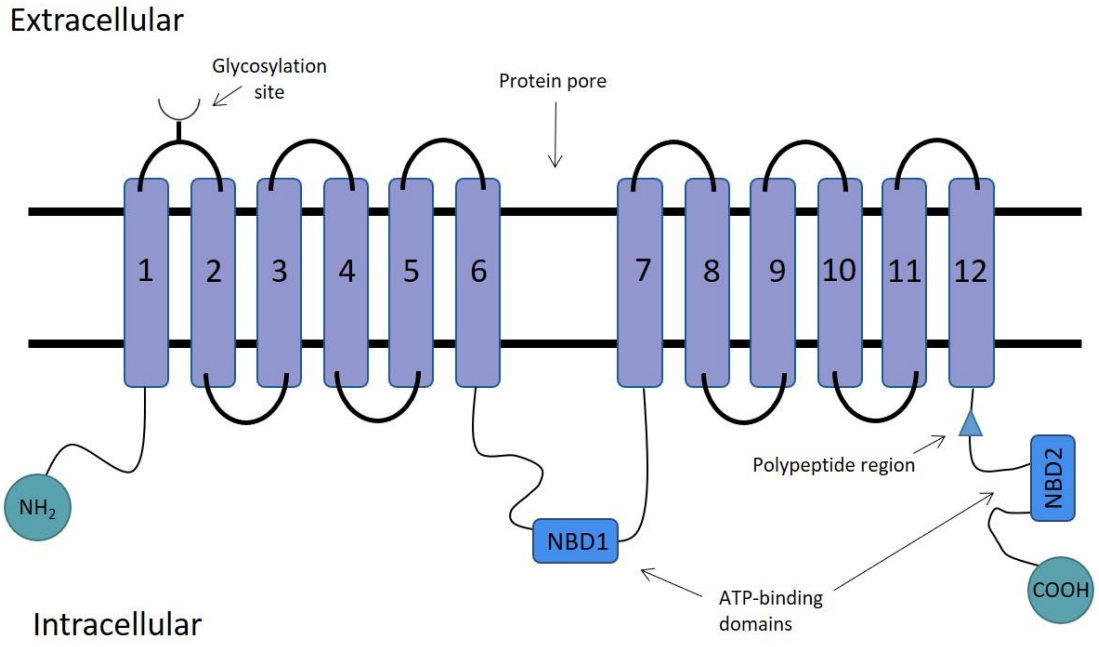

Figure 5. Schematic structure of p-glycoprotein (p-gp).

P-gp consists of 12 transmembrane domains split into two similar halves. The two halves form together to make a transmembrane pore through which substrates are extruded from the cell upon binding of ATP to the ATP-binding domains. These two ATP-binding domains present in p-gp also double up as the p-gp nucleotide binding domains (NBD1 and NBD2). The glycosylation site of p-gp contains three potential glycosylation points, the overall result is a large, highly complex transmembrane protein which forms a pore through which drugs are extruded in an energy-dependent manner. Image adapted from (255).

P-gp has a molecular weight of around 170kDa and is thought to contain two similar transmembrane segments with 63% homology to each other (256), each

containing six transmembrane domains and an intracellular loop containing an ATP-binding site, which form a channel through which drugs can be exported (257) (Fig. 5). P-gp is initially transcribed as a 140kDa precursor but undergoes glycosylation at its three N-linked glycosylation sites at amino acids 91, 94 and 99 to form the 170kDa mature p-gp. Complete glycosylation of p-gp is key to formation of the cellular MDR phenotype, after glycosylation p-gp must then undergo phosphorylation to enable it to form an effective efflux pump (258).

1.7.1.1 P-gp isoforms, gene variants and mutations

The *MDR1* gene is located on chromosome 7q21.12 and consists of 209,691bp, forming 28 exons, although many different polymorphisms have been reported (259). One of p-gp's most important physiological roles is cytoprotective -to limit cellular uptake of drugs and other toxic moleules from the circulation through the blood brain barrier into the CNS. However, this significantly limits the bioavailability of anti-cancer (257) and anti-retroviral drugs (260). As well as being present at high levels in the brain, p-gp is also present in several other tissues including adrenal cortical cells, pancreas excretory ducts, testicles and placenta which may explain why these organs are particularly resistant to uptake of cytotoxic, chemotherapy drugs. Some tumours deriving from tissues with high base levels of p-gp are more likely to have an intrinsic MDR phenotype (251) although, most often, MDR phonotype is acquired, arising after long-term exposure to anti-cancer agents.

1.7.1.2 P-glycoprotein sub-cellular localisation and function

P-gp is known as an energy-dependent plasma-membrane-based efflux transporter, where it resides within the plasma membrane, forming a pore through with substrates can be transported. A substrate binds to the binding site

on the inner leaflet of the plasma membrane, upon ATP binding and being reduced to ADP, the substrate is expelled from the cell and the process can begin again (Fig. 5).

There has recently been evidence suggesting that p-gp is also located in subcellular organelles. Molinari et.al. (261) show that, in MDR MCF-7 breast cancer cell line, there are significant levels of p-gp in the Golgi apparatus, suggesting a role for p-gp in the sequestration and vesicular transportation of drugs out of the cells, another mechanism by which p-gp is able to produce an MDR phenotype. Another group showed, in doxorubicin-resistant MCF-7 cells, that p-gp is expressed in the mitochondria where it has an efflux function within these organelles (262), this suggests a role for p-gp in protecting the mitochondria from the effects of harmful molecules.

As well as being located on the plasma membrane to export drugs out of cells and within cytoplasmic organelles, there is strong evidence that p-gp is also located in the nuclear envelope of MDR cells. This would act as an extra defence mechanism of these cells against cytotoxic agents such as chemotherapy drugs (263), protecting the cell's DNA and preventing them from inhibiting cell replication.

1.7.2 PrP^C and multi-drug resistance

PrP^C has been reported to be upregulated in several drug-resistant cancer cell lines from breast, gastric and colon cancers. This includes increased PrP^C expression in adriamycin-resistant gastric cancer (SGC7901/ADR) cells compared to the parental cell line (SGC7901) where PrP^C is not only ubiquitously expressed but confers resistance to both phospho-glycoprotein (p-gp) related and non-p-gp related drugs on these cells, preventing apoptosis

(184). The group also show that inhibition of PrP^C in these cells using RNAi led to a reduction in MDR phenotype and that overexpression of PrP^C led to an increase in p-gp expression.

Finally, another group (264) show a novel interaction of p-gp and PrP^C in Paclitaxel-treated breast cancer cells, with paclitaxel facilitating p-gp/PrP^C cluster formation in caveolar domains at the cell surface and again inhibiting apoptosis of these cells. This also, for the first time, provides evidence for a physical interaction between PrP^C and p-gp. Contrarily, in a different breast cancer cell line, oestrogen receptor-negative MDA-MB-435, silencing of PrP^C was shown to enhance doxorubicin resistance, partially via the ERK1/2 pathway (265).

These studies suggest an important role of PrP^C overexpression in p-gp-related drug resistance so several of the key chemotherapeutic agents (including adriamycin, paclitaxel and doxorubicin) in cancer cell lines. There has, however, been little research into PrP^C expression and its contribution to MDR in primary tumour samples.

1.7.3 Adhesion-mediated drug resistance

Adhesion of tumour cells to extracellular matrix (ECM) components such as fibronectin (FN) via β1 integrin has previously been shown to contribute to resistance to a host of chemotherapy agents (266) in what has been termed cell adhesion-mediated drug resistance (CAM-DR), a novel form of drug resistance. The interaction between FN and β1 integrin is already known to influence survival, cell growth and proliferation of schwannoma cells. This is particularly important as β1 integrin has been previously shown to be upregulated in schwannoma compared to healthy Schwann cells (58).

MDR-associated protein MGr1-Ag has been shown to be upregulated in drug-resistant gastric cancer cells compared to the parental cell line (267). The same group later found this MDR-associated MGr1-Ag to be identical to the 37Kda laminin receptor precursor (37LRP) (268). It is thought that this MGr1-Ag/37LRP is also capable of inducing CAM-DR via signalling through FAK/PI3K and MAPK pathways (269). As previously described, PrP^C has been shown to interact with the 37kDa LRP in the N2a neuroblastoma cell line (248), more recently, overexpression of both PrP^C and MGr1-Ag/37LRP has been shown to be a marker of poor prognosis and even resistance to chemotherapeutic interventions in gastric cancer (270). These experiments make the PrP^C/37LRP interaction interesting to examine with regards to potential cell adhesion-mediated drug-resistance in schwannoma cells.

1.8 Cluster of differentiation 44 (CD44)

1.8.1 CD44 structure and function

There have been several studies that link expression of both CD44 and PrP^C in different tumours. CD44 expression has been associated with various tumour types, cancer stem cells (CSCs) and drug resistance although these aspects will be discussed fully in chapters 8.1.2-4. CD44 is encoded by a single copy of the CD44 gene located on the short arm of chromosome 11 that spans approximately 50kb of DNA (reviewed in (271)). It is an extremely complex gene made up of 21 exons with at least 12 alternatively spliced exons (272). Out of the 21 exons, 10 are ubiquitously expressed and produce a heavily glycosylated 85–90 kDa isoform known as standard form CD44 (CD44s) where exon 5 is spliced directly to exon 16 (Fig. 6A). The other exons are able to be alternatively

spliced in various combinations, these splice variants incorporated into the polypeptide backbone encoded by the standard form exons to produce a huge range of CD44 isoforms (CD44v, Fig. 6A) which are differentially expressed in various tissues (273-275).

The encoded CD44 protein forms a single-chain, cell surface glycoprotein also known as homing cell adhesion molecule (HCAM) involved in cell-cell interaction, signalling, cell adhesion and migration. The CD44 molecule consists of an N-terminal extracellular domain, made up of exons 1-5, that is related to several hyaluronate binding proteins (276,277) and contains docking sites for various ligands (Fig. 6B).

The primary ligand for CD44 is hyaluronan (HA), although binding of CD44 to β1 integrin, laminin, growth factors, metalloproteases and cytokines also occurs. CD44 contains a small, variable, membrane proximal stem region, encoded by part of exon 5, 16 and 17 (plus any included variable exons), with binding sites for metalloproteases ADAM10, ADAM17 and disintegrin. This proximal stem region separates the extracellular loop from the plasma membrane, through which the transmembrane section, encoded by part of exon 18, runs (reviewed in (278)). On the other side of the transmembrane domain, at the inner surface of the plasma membrane, there is a gamma secretase cleavage site. This cleavage site allows the cytoplasmic tail of CD44 to be cleaved and translocated to the nucleus where it is able to act as a transcriptional regulator (279).

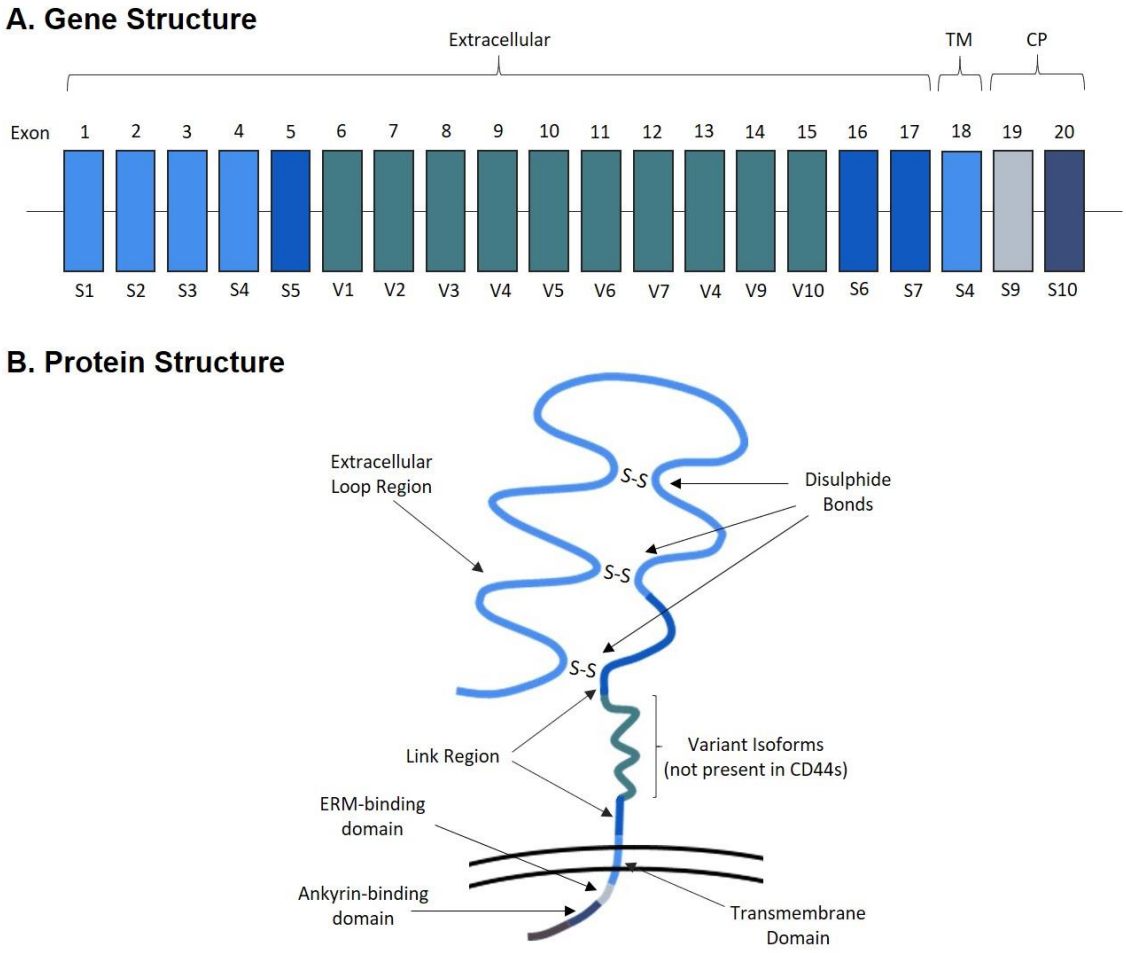

Figure 6. Protein and gene structure of CD44 and its variants.

CD44 is a complex cell surface receptor which consists of between 10 and 20 exons that are differentially spliced depending on the CD44 variant (A). The standard CD44 isoform (CD44s) is coded by 10 exons (A, S1-10), seven of which form the complex extracellular loop region, one forms the transmembrane region and two form the C-terminal cytoplasmic portion (CP) that contains the ERM- and ankyrin-binding domains (B). All the isoforms of CD44 share this similar pattern but also contain up to 10 extra, variant isoforms (A, V1-10) inserted at the end of the extracellular loop (B, teal region), just before the transmembrane region. Image adapted from (283).

The C-terminal cytoplasmic domain is subject to alternative splicing via different variant of exons 19 and 20 generating either a short, 3 amino acid or long, 70 amino acid version (reviewed in (274)). The C-terminal domain plays an important role in intracellular signal transduction with binding sites for signalling

molecules including; Rho GTPases (280) and members of the Src family kinases including Src, Lck, Fyn and Lyn (281). There are also binding sites for key cytoskeletal elements such as Ankyrin and the ERM family of proteins (282), making CD44 a key molecule in cytoskeletal organisation and intracellular signalling to affect pathways involved in cell adhesion, survival, proliferation (Fig. 7).

The structural variability of CD44 is further complicated by post-translational modifications; N- linked and O-linked glycosylations as well as glycosaminoglycan (GAG) attachments which contribute to a doubling of the molecular weight of CD44 from 37kDa to a heavily-glycosylated form at 90kDa (these glycosylation sites are shown in Fig. 7B). There is conflicting evidence on the effects of glycosylation on HA binding to CD44, however. Inhibiting N-linked glycosylation has been shown to both enhance (284,285) and inhibit (286) HA binding, whereas inhibiting O-linked glycosylation can either enhance (287) or have no effect (285) on HA binding.

Various CD44 isoforms are expressed in a wide range of tissues; including the skin, lung, stomach, intestine, oesophagus, bladder and cervical epithelium, glandular epithelial cells including that of the pancreas, sweat glands, bile ducts, prostate gland and mammary glands and endothelial cells, fibroblasts and white matter of the brain (in particular perivascular astrocytes), as well as in tumours arising from these tissues (288).

1.8.2 CD44 in cancer

CD44 has been shown to be expressed and involved in enhanced tumourigenicity in several cancer types including melanoma (289), colorectal carcinoma (290), non-Hodgkin's lymphoma (291) and some gliomas (292).

Often CD44 expression level correlates with the grade of the tumour and poorer prognosis. Some tumours (such as glioma) express only the standard CD44s variant which lacks the entire variable region and is also expressed in normal cells, whereas other cancers, including gastric, bladder, cervical and breast cancers express variants of CD44 (CD44v1-10) as well as CD44s. It appears that not all CD44 isoforms act in the same way within cancer cells; involvement of variants CD44v4-v7 and CD44v6-v9 in tumour progression have been confirmed for various different tumour types in multiple clinical studies. In contrast, downregulation of the standard CD44 isoform (CD44s) has been shown to increase tumourigenesis in colon cancer cells. The mechanism for this is thought to be due to stromal-derived hepatocyte growth factor (HGF) production and release stimulating synthesis of MT1MMP, a matrix metalloprotease that is able to induce shedding of CD44 variants which, in turn, promotes invasion in both colon and prostate cancer cells (reviewed in (293)). Silencing of the appropriate CD44v (depending on cancer type) inhibits tumour cell adhesion to the cell matrix *in vitro* and prevents *in vivo* tumour cell invasion, blocking the HA/CD44/PI3K signalling axis that induces invasiveness (294).

As well as CD44 itself, hyaluronan (HA), the primary ligand of CD44 which is a key component of the extracellular matrix and a glycosaminoglycan that, like PrP^{C}, is more abundant during development compared to in mature adults (295) may play an important role in tumour formation and progression. CD44 (296) and HA (297) are both known to be aberrantly expressed in schwannoma (296).

HA expression is increased in a variety of cancers including breast, prostate, colorectal and gastric (298). Its interaction with CD44 can promote migration, invasion, inflammation, angiogenesis and cell proliferation (293,299); all key

features of tumourigenesis (300). Further to this, animal models have shown that interfering with CD44-ligand binding with the use of specific antibodies is able to inhibit growth a metastatic spread of the tumours (reviewed in (274)) making CD44, HA and their interaction a set of promising therapeutic targets in various neoplasms.

1.8.3 CD44 in schwannoma and other Merlin-deficient tumours

As previously stated, expression of both CD44 (standard isoform as well as CD44 variants, specifically CD44v6) and its primary binding partner HA are known to be aberrantly expressed in schwannoma.Oligodendrocyte and Schwann cell-specific CD44-overexpressing mice show widespread CNS dysregulated myelination and progressive demyelination (301), thus, CD44 overexpressed by these myelinating cells, such as in the case of schwannoma (302), may contribute to de-differentiation of Schwann cells and thus demyelination. The CD44 ligand, HA, has also been shown to accumulate in glial cells of CD44 overexpressing mice (303).

Immunoprecipitation of the CD44 cytoplasmic tail in embryonic hamster kidney cells showed that CD44 is associated with ERM family proteins, which include Merlin, that link cell surface proteins with the actin cytoskeleton (305). Merlin has been shown to co-localise and co-immunoprecipitate with CD44 in cultured cells (306) and has been shown to mediate contact inhibition and growth via interactions with CD44, ezrin and moesin by acting as a molecular switch to arrest growth at high cell density (307). Furthermore, knockdown of Merlin, using siRNA, from healthy primary Schwann cells in culture caused an upregulation in levels of CD44 (308). As well as its role in mediating contact inhibition, CD44 is essential to mediate many other elements of tumourigenesis

including cell proliferation, cytoskeletal reorganisation, migration, angiogenesis and cell survival/resistance to apoptosis by several pathways known to be involved in schwannoma development, these are outlined in Figure 7.

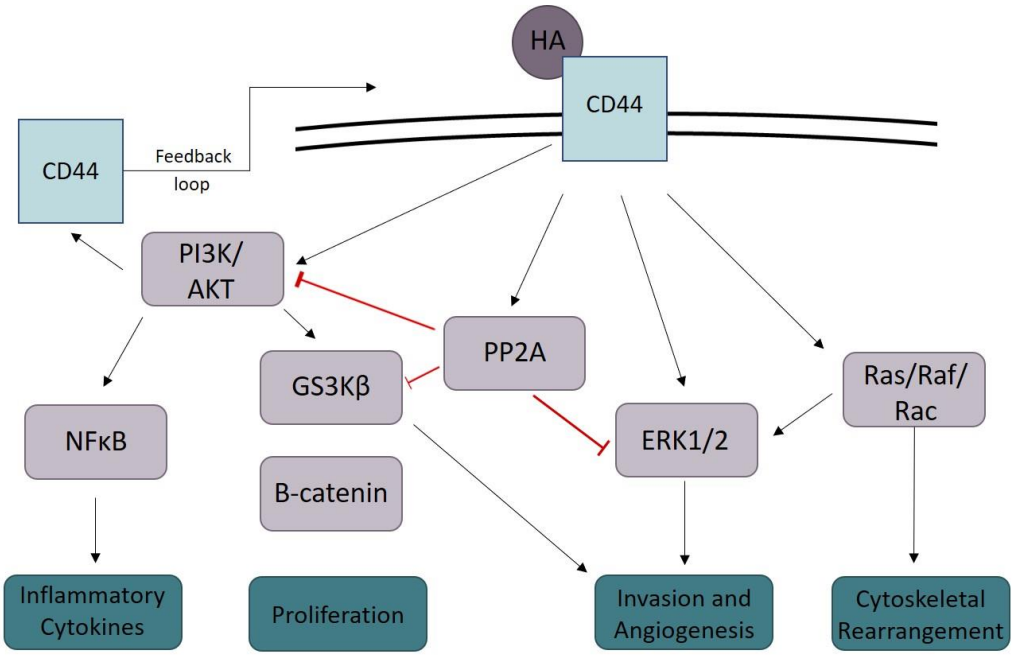

Figure 7. Overview of CD44 signalling pathways and their resulting effects on cells.

Signalling via CD44, either alone or via interaction with a co-receptor, activates several key pathways already previously described to be involved both in schwannoma development and the development of other tumours. This includes activation of PI3K/AKT, NFκB, β-catenin and Ras/Rac/ERK1/2 pathways which, overall, lead to the activation of the inflammatory response, increased cell proliferation, invasion and angiogenesis as well as cytoskeletal rearrangement. Image adapted from (304).

1.8.4 CD44 in multi-drug resistance

CD44 is a cell surface glycoprotein with major roles in cell adhesion, proliferation, angiogenesis and migration and recently has been linked to roles in drug-resistance (271). Cancer stem cells (CSCs) are subpopulations of

tumour cells which are more resistant to treatment that their surrounding counterparts, therefore successful treatment is dependent on removal of these specific subpopulations as well as the main tumour mass, failure to do so leads to relapse in patients. CSCs have very similar properties to tumour initiating cells which have previously been described in the brain (309)– they have the capacity for self-renewal, the potential to develop into any cell in the overall tumour population, have proliferative abilities to drive expansion of malignant cells and are a major cause of cancer aggressiveness (310). CSCs also express high levels of proteins associated with efflux of anti-cancer drugs, protecting the cancer cells from the chemotherapy agents. These proteins involved in the efflux of anti-cancer drugs include members of the ATP Binding Cassette (ABC) family of transporters, to which p-gp (MDR1) belongs (311).

CD44, in particular CD44v isoforms, have been described as the most common cell surface markers of CSCs either alone or alongside other markers such as CD24, CD133 and CD34 (reviewed in (310)) where it integrates signalling between normal stem cell, CSC's and pre-metastatic niches (312). Furthermore, CD44 plays a large role in tumour initiation and progression as discussed earlier, making them not only a good therapeutic target but also a potential biomarker to identify aggressive forms of cancer. The role of CD44 on these cells is thought to involve communication of CSCs with the microenvironment and surrounding stromal cells as well as regulating 'stemness' properties (310). CD44 binding regulates CSC survival, self-renewal, maintenance, and chemoresistance, which helps explains the importance of CD44 for disseminated cancer cells to adapt to new environments (313) and why CD44 is required for metastatic colonisation (314).

As well as traditional MDR-related proteins, it has been suggested that CD44 can induce drug resistance independently of p-gp (315) Interactions between p-gp and CD44 (both standard and variant isoforms, depending on the cancer type) have been well documented in cancer cells (316) and appear to promote migration and invasion as well as mediating MDR (317). This makes CD44 protein that requires a much further investigation in order to fully appreciate the interplay between LR/37/67kDa, PrP^C, p-gp and MDR in schwannoma.

1.8.5 CD44 and its interaction with PrP^C

CD44 has been associated with CSCs; CD44-positive CSCs that express PrP^C contribute to colon cancer metastasis which was inhbited by using RNAi to knockdown PrP^C (318) suggesting that both CD44 and PrP^C may act as biomarkers on CSCs to identify particularly aggressive or metastatic tumours and suggesting a previously untapped role for PrP^C in CSCs and tumour initiation.

A more direct interaction between PrP^C and CD44 has been demonstrated by Cheng et.al. (2014) who show that PrP^C and CD44 both physically and functionally interact within an adriamycin-resistant breast cancer cell line (319) and that knockdown of either PrP^C or CD44 was able to abolish cell migration, proliferation and invasion. The group concluded that the interaction between PrP^C and CD44 affects the response to neoadjuvant chemotherapy and enhances malignancy in breast cancer. There is some evidence that the interaction between PrP^C and CD44 could also involve a third member – MDR-associated protein p-gp which has also been shown to interact with PrP^C in drug-resistant breast cancer cell lines. Thus, the interaction between these

three proteins appears to be a novel and interesting point of study in order to gain insight both within the realms of MDR and CSCs.

1.9 Aims and objectives

The aims of this research project are: 1) to investigate the expression patterns of PrP^C in schwannoma and other Merlin-deficient tumours in comparison to normal tissue, 2) to look at the role of PrP^C in schwannoma pathological proliferation, adhesion and survival via the key pathways already well-known to be involved in schwannoma development, 3) to investigate whether altered glycosylation, degradation or aberrant protein folding of PrP^C may contribute to schwannoma pathology, 4) to check whether PrP^C is released from schwannoma cells and is able to act in an autocrine or paracrine manner to further trigger downstream signalling pathways, 5) to investigate previously identified potential receptors, including LR/37/67kDa, $P75^{NTR}$ and CD44, via which PrP^C may act in schwannoma cells and 6) to check whether LR/37/67kDa, $P75^{NTR}$ and CD44 receptors or other PrP^C-interacting partners could be involved in schwannoma drug resistance or whether intrinsic drug resistance may play a role instead.

The findings of this study should begin to elucidate whether PrP^C is a good novel potential therapeutic target for the treatment schwannoma and other Merlin-deficient tumours and also whether it may be able to function as a biomarker to aid diagnosis and help evaluate tumour load of patients.

1.10 Brief summary of main findings

I show that upregulation of glycosylated PrP^C occurs in schwannomas in a Merlin-dependent manner and contributes to the increased pathological

adhesion, survival and proliferation seen in these cells. This occurs via activation of key signalling pathways ERK1/2, P13K/AKT, FAK and cyclin D1 downstream of both LR/37/67kDa and CD44. Finally, I show that p-gp is upregulated in schwannoma downstream of both Merlin and PrP^C, making PrP^C a potential new therapeutic target, not only for schwannomas, but also other Merlin-deficient tumours.

Chapter 2: Materials and methods

2.1 Sample collection and cell culture

2.1.1 Primary human cells

Primary human tumour samples were used for the majority of the experiments in this project to reduce the limitations of using a cell culture model and to bridge the gap between the bench and the bedside, allowing the work done within this project to be more clinically translatable.

2.1.1.1 Clinical sample collection and ethics

Schwann cells were isolated from post-mortem donors at Derriford Hospital with informed consent from next of kin and after ethical approval under the Research Ethics Committee (REC) number REC6/Q2103/123. Schwannoma samples were collected after informed consent from patients at both Derriford Hospital, Plymouth and Bristol Southmead Hospital under local R&D approval Plymouth Hospitals NHS Trust: R&D No: 14/P/056 and North Bristol NHS Trust: R&D No: 3458. A subset of the schwannomas and all the meningiomas used were collected under the Molecular Targets (MOT) project again involving both Derriford and Southmead Hospitals. The project was granted full ethics approval by the South West research ethics committee (REC number 14/SW/0119). All tumours used in this project were classified as WHO grade 1.

2.1.1.2 Schwann cell collection and culture

Human nerve samples were isolated from the sural nerve in the lower leg, samples were dissected and the fascicles removed from the nerve sheaths. Individual fascicles were then digested with a mixture of type IIA collagenase and dispase as well as undergoing mechanical digestion by pipetting. The

individual Schwann cells were then cultured in a growth factor medium (GFM): DMEM; 100U/ml pen/strep; 2.5 µg/ml Insulin (Thermofisher Scientific, MA, USA); 0.5µM forskolin (Tocris, Bristol, UK); 10% FBS, 2.5µg/ml amphotericin (Sigma, MO, USA); 10nM β1 heregulin; 0.5mM 3-isobutyl-1-methylxanthine (IBMX, Bio-Techne, MN, USA) and maintained in a humidified atmosphere of 10% CO_2 at 37ºC, on plates coated with both poly-L lysine (Sigma) and laminin (Thermofisher Scientific). Immunofluorescent staining with S100 marker was routine to ensure a pure Schwann cell population devoid of fibroblasts, as previously described ((320) and (321)).

2.1.1.3 Schwannoma cell collection and culture

Cells were isolated by digestion using a mixture of collagenase, dispase and mechanical digestion by pipetting. Once digested cells were cultured in growth factor medium (GFM): DMEM, 10% FBS, 100U/ml pen/strep; 0.5µM forskolin; 2.5µg/ml amphotericin; 2.5 µg/ml Insulin; 10nM β1 heregulin, maintained in a humidified atmosphere of 10% CO_2 at 37ºC on plates coated with both poly-L lysine and laminin.

2.1.1.4 Meningioma cell collection and culture

Meningioma cells were again isolated using a mixture of collagenase, dispase and mechanical digestion by pipetting. Once digested cells were cultured in a simple medium of DMEM, 10% FBS and 100U/ml pen/strep.

2.1.2 Cell line culture

2.1.2.1 Human meningeal cell (HMC) culture

Foetal-derived, human meningeal cells from ScienCell™ were maintained in the manufacturer's recommended media (meningeal cell media, ScienCell™) at 37^0C, 5% CO_2. Cells were split when confluent and plated on uncoated plates.

2.1.2.2 Ben-Men-1 benign meningioma cell culture

BenMen-1 (abbreviated to BenMen in this report) are a benign meningioma cell line originally created by Püttmann et.al. in 2005 (322) via a telomerase reverse transcriptase (hTERT)-mediated immortalisation of benign meningioma cells. Cells were routinely cultured in DMEM with the addition of 10% FBS and 100U/ml pen/strep at 37^0C at 5% CO_2. BenMen cells were used in this project as a control for grade 1 meningioma results and also as a tool for directly comparing between meningioma samples and HMC without use of primary cells.

2.1.2.3 Human malignant mesothelioma cell (HIB and TRA) culture

The characteristics of the HMM cell lines HIB (Merlin-positive) and TRA (Merlin-deficient), reported previously (323). Cells were seeded on uncoated cell culture dishes at a density of 10,000 cells/cm^2 and maintained in medium consisting of RPMI, 10% FBS, 100 U/ml pen/strep, in a humidified atmosphere of 5% CO_2 at 37 °C.

2.1.2.4 HEK293FT cell culture

Human Embryonic Kidney HEK293FT cells (Thermofisher Scientific) were maintained on uncoated cell culture dishes in a simple medium of DMEM, 10% FBS, 100U/ml Pen/Strep at 37ºC, 5% CO_2 and passaged when confluent. 293FT cells were used for the production of lentiviral particles, see section 2.5.2.1 for further information.

2.2 Chemicals, peptides & antibodies

2.2.1 Chemicals

Table 2: Table of chemicals and peptides used

Chemical	Company	Catalogue Number	Use	Treatment Protocol
TCS Prion Inhibitor 13 (TCS)	Insight Biotechnology, Wembley, UK	3238	Inhibition and reduction of PrP^C	20, 100 and 500µM for 72h, 37ºC
Cycloheximide (CHX)	Sigma-Aldrich, Gillingham, UK	C7698	Inhibitor of protein synthesis	1µM, 24h, 37ºC
Actinomycin D (Act D)	Sigma-Aldrich, Gillingham, UK	A9415	Inhibitor of transcription	10nM, 24h, 37ºC
Proteinase K (PK)	Sigma-Aldrich, Gillingham, UK	P2308	Protease enzyme	5% in lysis buffer, 2h, 37ºC
PMSF	Sigma-Aldrich, Gillingham, UK	P7626	Inhibitor of proteinase K	10ug/ml, 10', RT
MG132	Sigma-Aldrich, Gillingham, UK	M8699	Proteasomal degradation inhibitor	1µM, 24h, 37ºC 10' then RT 1hr
DAPI	Sigma-Aldrich, Gillingham, UK	D9542	Nuclear stain	1:500, 30'
H_2O_2 (30% w/w solution)	Sigma-Aldrich, Gillingham, UK	H1009	Apoptosis inducer	500µM, 12h, 37ºC
GI254023X	Sigma-Aldrich, Gillingham, UK	SML0789	ADAM10 Inhibitor	10µM, 3h. 37^0C
Choloroquine (CQ)	Sigma-Aldrich, Gillingham, UK	C6628	Lysosomal degradation inhibitor	12.5µM, 24h, 37ºC
PIPLC	Sigma-Aldrich, Gillingham, UK	P5542	Cleaves GPI-membrane-bound proteins	0.2U/ml, 3h, 37^0C
PNGaseF	Promega, Southampton, UK	V4831	De-glycosylase enzyme	As per manufacturer protocol.
P-glycoprotein blocking peptide	Insight Biotechnology, Wembley, UK	BS-0563P	Show unspecific binding of the P-gp antibody	As per manufacturer protocol.
Protamine Sulfate	Sigma-Aldrich, Gillingham, UK	P3369	Facilitates gene/DNA transfection.	8µg/ml

Chemical	Company	Catalogue Number	Use	Treatment Protocol
PSC833 (Valspodar)	Biotechne, Abingdon, UK	#4042	P-gp inhibitor	4µg/ml, 20 minute pre-treatment
Puromycin	Sigma-Aldrich, Gillingham, UK	P9620	Antibiotic used for selection.	4µg/ml
Blasticidin	Sigma-Aldrich, Gillingham, UK	15205	Antibiotic used for selection.	10µg/ml
SN50	Merck Millipore, Feltham, UK	41480	Prevents nuclear translocation of NFκB	8µg/ml, 24h, 37ºC
PrP Peptide (Central Portion)	BioRad, Watford, UK	7672-5509	KTNMKHMAG AAAAGAC AA105-120	4µM, 1-72h, 37ºC

2.2.2 Antibodies

Table 3: Table of antibodies used and their application

Antibody	Type	Company	Cat. Number	Dilution
PrP 12F10	Mouse Monoclonal Antibody	Sigma-Aldrich, Gillingham, UK	No longer available	1:1000 (IHC)
PrP 6D11	Mouse Monoclonal Antibody	Insight Biotechnology, Wembley, UK	Sc-58581	1:500 (WB) 1:100 (ICC)
PrP [EP1802Y]	Rabbit Monoclonal Antibody	Abcam, Cambridge, UK	ab52604	1:500 (WB)
PrP 8H4	Mouse Monoclonal	Sigma-Aldrich, Gillingham, UK	P0110	1µg in 1mg lysate (IP)
GAPDH	Mouse Monoclonal Antibody	Merck Millipore, Feltham, UK	MAB374	1:10,000 (WB)
Merlin D1D8	Rabbit Monoclonal Antibody	New England Biolabs, Hitchin, UK	#6995	1:500 (WB)
Phospho Merlin^{S518}	Rabbit Polyclonal Antibody	New England Biolabs, Hitchin, UK	#9163	1:500 (WB)

Antibody	Type	Company	Cat. Number	Dilution
CD63 (RFAC4)	Mouse Monoclonal	Merck Millipore, Feltham, UK	CBL553	1:500 (WB)
Ki67 (MIB-1)	Mouse Monoclonal Antibody	Agilent Technologies Ltd., Stockport, UK	M7240	1:100 (ICC)
c-Jun (60A8)	Rabbit Monoclonal Antibody	New England Biolabs, Hitchin, UK	#9165	1:100 (ICC) 1:500 (WB)
Cleaved caspase-3 (Asp175)	Rabbit Polyclonal Antibody	New England Biolabs, Hitchin, UK	#9661	1:100 (ICC) 1:200 (WB)
NFκB p65	Rabbit Polyclonal Antibody	Abcam, Cambridge, UK	ab16502	1:100 (ICC) 1:500 (WB)
IκB	Rabbit Monoclonal Antibody	Abcam, Cambridge, UK	ab32518	1:100 (ICC) 1:500 (WB)
VPBRP (C-8) (DCAF1)	Mouse Monoclonal Antibody	Insight Biotechnology, Wembley, UK	sc-376850	1:250 (WB)
Cyclin D1	Rabbit Polyclonal Antibody	New England Biolabs, Hitchin, UK	#2922	1:1000 (WB)
P44/42 MAPK (137F5) (Total ERK1/2)	Rabbit Monoclonal Antibody	New England Biolabs, Hitchin, UK	#4695	1:2000 (WB)
Active MAPK (pTEpY) (pERK1/2)	Rabbit Polyclonal Antibody	Promega, Southampton, UK	#V803A	1:1000 (WB)
Pan AKT (C67E7)	Rabbit Monoclonal Antibody	New England Biolabs, Hitchin, UK	#4691	1:500 (WB)
Phospho AKT^{S473}	Rabbit Monoclonal Antibody	New England Biolabs, Hitchin, UK	#9271	1:500 (WB)
Total FAK	Rabbit Polyclonal Antibody	New England Biolabs, Hitchin, UK	#3285	1:500 (WB)
Phospho FAK^{Y397}	Rabbit Polyclonal Antibody	New England Biolabs, Hitchin, UK	#3283	1:500 (WB)

Antibody	Type	Company	Cat. Number	Dilution
p53 (B-P3)	Mouse Monoclonal Antibody	Insight Biotechnology, Wembley, UK	sc-65334	1:500 (WB)
LR/37/67kDa	Rabbit Polyclonal Serum	Biotechne, Abingdon, UK	Nb110-74761	1:500 (WB) 1µg in 1mg lysate (IP)
Anti 67kDa Laminin Receptor	Rabbit Polyclonal Antibody	Abcam, Cambridge, UK	ab137388	1:500 (WB) 1µg in 1mg lysate (IP)
P-glycoprotein (F4)	Mouse Monoclonal Antibody	Sigma-Aldrich, Gillingham, UK	P7965	1:1000 (IHC) 1µg in 1mg lysate (IP)
P-glycoprotein	Rabbit Polyclonal Antibody	Abcam, Cambridge, UK	ab129450	1:500 (WB)
HCAM (DF1485) (CD44)	Mouse Monoclonal Antibody	Insight Biotechnology, Wembley, UK	sc-7297	1:500 (WB) 1:100 (ICC)
CD44s	Rabbit Polyclonal Serum	Biotechne, Abingdon, UK	NBP1-31488	1:1000 (WB)
Phalloidin	Alexafluor 488	New England Biolabs, Hitchin, UK	#8878	1:500 (ICC)
Goat anti-mouse	488	Thermofisher Scientific, Paisley, UK	A11001	1:500 (ICC)
Goat anti-mouse	594	Thermofisher Scientific, Paisley, UK	A11005	1:500 (ICC)
Goat anti-rabbit	488	Thermofisher Scientific, Paisley, UK	A11008	1:500 (ICC)
Goat anti-rabbit	568	Thermofisher Scientific, Paisley, UK	A11011	1:500 (ICC)
Goat anti-mouse	HRP-conjugated	BioRad, Watford, UK	#170-6516	1:20,000 (WB)
Goat anti-rabbit	HRP-conjugated	BioRad, Watford, UK	#172-1019	1:20,000 (WB)

2.3 Immunohistochemistry and immunocytochemistry

2.3.1 Immunohistochemistry (IHC)

Formalin-fixed, paraffin embedded, thin tissue sections (4µm) were taken from samples collected during surgery or post mortem with patient or family informed consent. Sections were then baked onto slides at 60^0C for 10 minutes. Slides underwent de-waxing and rehydration through xylene (Thermofisher Scientific) and 100% ethanol (VWR) respectively before undergoing a methanol and hydrogen peroxide (3%) block to quench excess peroxidase activity. This was followed by antigen retrieval using a pre-treatment with citrate buffer pH6.0 (during p-gp staining) or EDTA buffer for 30 minutes under heat (for PrP^C staining). Slides were incubated overnight with anti-PrP^C 12F10 (Bioquote, York, UK) at a dilution of 1:1000 or anti-p-gp (Sigma) at 1:500. Detection was performed with the Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK). 3'3'-Diaminobenzidine (DAB (Sigma)) was used to visualise with Mayer's haematoxylin (Sigma) used as a nuclear counterstain. Slides were de-hydrated through ethanol and xylene before being mounted using DPX (Sigma). Slides were imaged on a bright field light microscope and images taken using an attached camera.

2.3.2 Immunofluorescence and confocal microscopy

2.3.2.1 Fluorescent immunocytochemistry (ICC)

Pre-treated cells were fixed using 4% paraformaldehyde and permeablised using 0.2% Triton X-100 before being blocked using 10% normal goat serum. Cells were then incubated with primary antibodies overnight at a concentration of 1:100. Localization various proteins was performed using anti-human-PrP^C, anti- 37/67kDa non-integrin laminin receptor, anti-p-glycoprotein, anti-CD44

antibodies. Secondary antibodies goat anti-Mouse 594, goat anti-Mouse 488, goat anti- rabbit 594 and goat anti- Rabbit 488 (Invitrogen) were used and Alexa Fluor 488-labeled phalloidin was used to visualize actin filaments (1:100; Molecular Probes, Eugene, OR). DAPI was used to visualise cell nuclei.

2.3.2.2 Immunofluorescence proliferation and survival assays

Cells were treated as required then fixing agent (4% PFA) applied directly into the cell culture medium rather than washing cells first. All other elements of the protocol were carried out as described above. Anti-Ki67 was used as a marker or proliferating cells (324) and anti-cleaved caspase-3 was used as a marker or cells undergoing apoptosis. Again, AlexaFluor^{TM} secondary antibodies goat-anti mouse 594 and goat-anti rabbit 488 were used and DAPI applied as a nuclear stain.

2.3.2.3 Confocal microscopy

Multitrack imaging was performed using a Zeiss Confocal LSM510. Co-localisation experiments were all carried out using z-stack techniques. Apart from proliferation and survival assays which were carried out using a 20x air objective, all other experiments were imaged using a 40x oil pH2 objective. The appropriate Zeiss image manipulation software (ZEN) was used for editing.

2.4 Quantifying PrP^{C} release from cells

2.4.1 Culture medium collection and concentration

Both Schwann and schwannoma cells were cultured for a minimum of seven days in growth factor medium containing exosome-free FBS (Gibco). Medium from cells was collected before being concentrated 10x using Amicon Ultra

centrifugal filters (Millipore). Concentrated media samples were then run using ELISA (see below).

2.4.2 Exosome isolation

Cell culture medium was collected and incubated overnight at 4^0C with Total Exosome Isolation Kit (cell culture medium version, Invitrogen). The next day exosomes were pelleted by centrifugation at 13,000rpm, 4^0C for 30 minutes before being re-suspended in 50µl PBS. Samples were either subject to ELISA (see section 2.4.4) or Western blotting for quantification where anti-CD63 was used as a marker for exosomes.

2.4.3 Quantifying PrP^C cleavage by PIPLC and ADAM10

Schwannoma cells were cultured in normal cell growth medium (GFM) until confluent before being treated either with phosphatidylinositol phospholipase C (PIPLC) 0.2U/ml or ADAM10 inhibitor GI254023X 5µM for 24h at 37^0C. Cell medium was collected for ELISA and cells were lysed as described below (see chapter 2.4.4), before being subjected to Western blotting (refer to chapter 2.4.1). Untreated cells in GFM or GFM cell culture medium were used as a control.

2.4.4 Analysis by enzyme-linked immunosorbent assay (ELISA)

Samples were collected as described above (section and 100µl sample loaded into each well of a Prion Protein (*PRNP*) ELISA kit (CUSABIO from Antibodies-online, ABIN821046) for an overnight incubation at 4^0C to detect PrP^C release from cells. The remainder of the protocol was carried out according to the manufacturer's manual and the plate was read on a microplate reader (Genios) at 450nm to quantify the results. Results were adjusted based on the fold-concentration of the medium where required.

2.5 Viral infections of Schwann and schwannoma cells in culture

2.5.1 Adenoviral re-introduction of Merlin into schwannoma cells

Merlin (NF2) wild type (recombinant adenovirus AdNF2) and control GFP-containing vector adenoviruses were a kind gift from J. Testa [39]. Confluent (70%) schwannoma cells were incubated with virus for 48 hours and then incubated with fresh GFM for additional 24 hours prior to lysis with RIPA buffer. Successful viral infection was determined by the presence of GFP within the cells and backed up with quantification of Merlin levels using Western blotting.

2.5.2 Lentiviral knockdown of *PRNP* in schwannoma cells

2.5.2.1 Homemade TRC *PRNP* shRNA lentiviral particles

TRCN0000083488 hairpin-pLKO.1 construct, part of a TRC (The RNAi Consortium) *PRNP* shRNA, glycerol set (GE Dharmacon, IL, USA) was used to produce lentiviral particles. The plasmid was amplified using DH5α *E.Coli* cells, plasmid DNA was then transfected into 293FT cells in a low-antibiotic, high-serum medium along with packaging plasmid dR8.2 and envelope plasmid VSV-G using Fugene6 (Promega). Medium was collected and spun down to remove cellular debris before being applied to cells (see below for full protocol). Viral titre and multiplicity of infection (MOI) was unable to be determined for the homemade viruses, through a series of different dilutions I found that sufficient knockdown of a well of 70,000 schwannoma cells could only be achieved with 2ml viral particles (unconcentrated) with 1ml GFM.

2.5.2.2. Pre-made *PRNP* shRNA lentiviral particles

Alongside the homemade *PRNP* shRNA the following commercially available shRNA *PRNP* shRNA lentiviral particles and control shRNA particles (Santa Cruz) that consist of a pool of three to five expression constructs each encoding

a different, target-specific 19-25nt region of PrP^C plus a hairpin shRNA were used to knock down *PRNP* expression. The pre-made tubes contain $1.0x10^6$ infectious units of virus (IFU) in DMEM with 25mM HEPES pH7.3. The manufacturer recommends using 10-20µl of virus per 70,000 cells, I found that 10µl of pre-made viral particles per well of a 6 well plate was sufficient for a significant knockdown.

2.5.2.3 Lentiviral infection of schwannoma cells with *PRNP* shRNA

Schwannoma cells were seeded at a high density (minimum 70% confluency) before being incubated with medium containing lentiviral particles or a scramble control (sc-108080, Insight Biotechnology, Wembley, UK) plus protamine sulphate (8µg/ml) and incubated at 37°C for 72 hours. Infected cells were selected with puromycin for a minimum of 72 hours before being lysed or fixed and subjected to Western blotting or immunocytochemistry respectively.

2.5.3 *PRNP* overexpressing clone

2.5.3.1 Production of the *PRNP* overexpressing clone

The coding region of *PRNP* (NCBI gene ID: 5621) was synthesised on request by Life Technologies, it was then PCR cloned and ligated into pENTR11 entry vector before being gateway cloned into the pLenti6.2/V5-DEST vector (Fig. 9). The pLenti6.2/V5-DEST-PRNP DNA was transfected into 293FT cells alongside a viral packaging mix (Life Technologies) and virus-containing medium was collected from cells after 48 hours, spun down and frozen at -80°C. A pLenti6.2/V5-GFP containing lentivirus was also made at the same time to act as a control.

2.5.3.2 Infection of Schwann cells with *PRNP* overexpressing clone

Schwann cells were cultured until confluent then split at a high density. The following day lentiviral particles containing pLenti6.2/V5-DEST-PRNP (Fig.8) or pLenti6.2/V5-DEST-GFP were applied to the Schwann cells with the addition of protamine sulphate ((8µg/ml) Sigma) and incubated at 37°C for 48 hours.

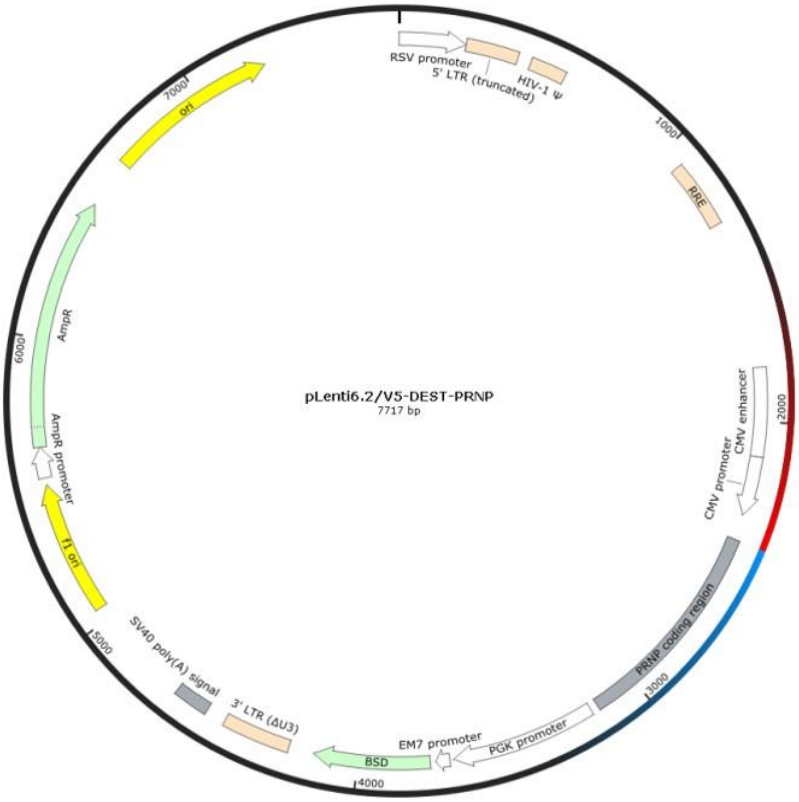

Figure 8. *PRNP* overexpressing clone structure.

The *PRNP* coding region was PCR cloned into a pLenti6.2/V5-DEST vector that uses a CMV promoter to drive overexpression of the target gene. The above vector was transfected into HEK293FT cells alongside the appropriate packaging vectors and the cell culture medium containing the viral particles was collected. Viral titre was determined by a series of dilution experiments before the virus was used to infect Schwann cells. Image courtesy of SnapGene®.

Virus-containing medium was removed and replaced with GFM containing the selective antibiotic blasticidin and incubated for 72 hours or until all uninfected

cells in a control culture were killed. Cells were then lysed then subjected to Western blotting or immunofluorescence as described.

2.5.4 Knockdown of 37/67kDa-non-integrin laminin receptor

Pre-made *RPSA* (the gene coding for LR/37/67kDa) shRNA lentiviral particles was purchased (Santa Cruz). Schwannoma cells were seeded at a minimum of 700,000 cells per well and, the day after splitting were infected with 20µl virus per well with the addition of protamine sulphate (8µg/ml). Cells were incubated with the viral particles for 72 hours before the medium was replaced with selection medium containing GFM and puromycin for a further 48 hours. After this, cells were starved by changing to medium containing only DMEM and puromycin before being subject to treatment with PrP peptide for either 1 hour or 24 hours and then lysed. Cell lysate was used for Western blotting and scramble control shRNA viral particles were used as a control (Insight Biotechnology, Wembley, UK).

2.5.5 Knockdown of *CD44/HCAM*

Pre-made *CD44* shRNA lentiviral particles were purchased (Santa Cruz). Schwannoma cells were cultured, infected, selected and treated with PrP peptide exactly the same as described above for *LR/37/67kDa* shRNA, except with the addition of the *CD44* shRNA lentiviral particles instead. Cell lysate was used for Western blotting and scramble control shRNA viral particles were used as a control (Santa Cruz).

2.6 Co-immunoprecipitation

Tumour cells were lysed in a low-salt Triton X-100 buffer (50mM Tris-HCl pH8, 30mM NaCl, 10% glycerol, 1% Triton X-100 in d.H_2O) with 5% complete protease inhibitor and 1% phosphatase inhibitors. Cell debris was removed by

centrifugation. 1mg cell lysate was incubated overnight at 4°C with pre-washed Upstate fast flow Protein G beads (GE Healthcare) along with either 1µg primary antibody (PrP^C, CD44 (Santa Cruz), 37/67kDa laminin receptor (Novus Biologicals), p-gp (Abcam)), or 1µg normal IgG of the same species (Santa Cruz) as a negative control. Beads were thoroughly washed before adding twice concentrated reducing sample buffer and boiling. Samples were then subject to Western blotting on a 15% acrylamide gel for SDS-PAGE (see chapter 2.8) where schwannoma cell lysate from the same patient was used as an input control. Where noted, frozen schwannoma tumour samples were used instead of cultured cells, tumour samples were homogenised in the same buffer and then subject to the same treatment as cell lysate.

2.7 Other functional assays

2.7.1 Proteinase K sensitivity assay

In order to check whether the PrP^C present in the cell samples was the cellular form of the protease-resistant form, Schwann and schwannoma cells were lysed either as normal (see below) but either with the addition of 5% complete protease inhibitor or without the protease inhibitor and instead with the addition of 5% proteinase K (Sigma). Lysates were incubated at 37°C for 10 minutes then left to stand at room temperature for 1 hour. Proteinase K was inactivated using PMSF for 10 minutes at room temperature before lysates were spun down and run as normal on SDS-PAGE (see Chapter 2.8).

2.7.2 De-glycosylation assay

Both Schwann and schwannoma cells were lysed in radioimmunoprecipitation (RIPA) buffer then treated with 5% sodium dodecyl sulphate (SDS), 1M Dithiothreitol (DTT), 0.5M Sodium phosphate buffer (pH7.5), 10% Triton X-100 and either PNGase F (Promega) or distilled milliQ water as a control. Samples were incubated at 37ºC for 1-3 hours before being run on SDS-PAGE.

2.7.3 Adhesion assay

Suspended schwannoma cells were seeded (2000 cells/well) onto pre-coated 96-well plates (325) and incubated at 37°C in 10% CO_2 for three hours using different conditions: DMEM and 20µM, 100µM and 500µM TCS prion inhibitor 13. For knockdown adhesion assays *PRNP* shRNA and their controls were treated, selected and counted prior to being plated and incubated only in GFM. After 3 hours cells were washed to remove any un-adhered cells, fixed and stained with DAPI before total cell number was counted using an Olympus fluorescent microscope.

2.7.4 MTS viability assay

Schwann or schwannoma cells were seeded onto pre-coated 96-well plates and grown until confluent. Cells were treated (only with treatments lasting >12 hours) before adding the recommended mix of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and PMS (phenazine methosulfate) from the kit (Promega) to each well (1:10 final concentration). Cells were incubated for 3-6 hours, in the dark, checking periodically before being read on a microplate reader at a wavelength of 490nm. MTS is a good fast way of getting a preliminary idea of cell viability but it is

difficult to determine whether changes in readings are due to a reduction in proliferation or an increase in apoptosis, thus more detailed assays are required for a better understanding (e.g. immunocytochemical proliferation and survival assays (Chapter 2.3.2.2)).

2.8 Western blotting

2.8.1 Sample lysis

2.8.1.1 Cell lysis

Cells were cultured on pre-coated plates to at least 70% confluency before being lysed on ice by both chemical and mechanical lysis by cell scraping. RIPA buffer was used as the chemical lysis agent (50mM Tris/HCl, 0.1% SDS, 1% NP-40, 150mM NaCl, 1mM EDTA, 0.5% sodium deoxycholate) with added 5% complete protease inhibitor (Roche) 1% phosphatase inhibitors B and C (Santa Cruz Biotechnologies). Lysates were centrifuged at 4ºC, 13,000rpm for 15 minutes to remove cell debris and supernatant stored at -80ºC. Protein estimation was carried out using a colorimetric Pierce BCA Protein Assay Kit (Thermofisher Scientific).

2.8.1.2 Tissue lysis

Frozen schwannoma samples were manually homogenised in RIPA buffer on ice, the volume used was dependent on the size of the tumour piece. Lysate was stored at -20ºC to improve protein extraction before being centrifuged at 4ºC, 13,000rpm for 15 minutes to remove cell debris. Collected supernatant was then stored at -80ºC. Protein estimation was carried out using a colorimetric Pierce BCA Protein Assay Kit (Thermofisher Scientific).

2.8.2 SDS-PAGE and transfer to PVDF membrane

10% polyacrylamide resolving gels topped with a 4% polyacrylamide stacking gel were made according to the protocol supplied with the 40% acrylamide solution (BioRad). A 15% polyacrylamide gel was used for running co-immunoprecipitation samples. Cell lysate was loaded on the gel at a concentration of 20µg where possible. Before loading, samples were mixed with 4x concentrated reducing buffer (250mM Tris-HCL [pH6.8], 8% SDS, 40% glycerol, 200mM DTT and 0.4% bromophenol blue (all Sigma)) and boiled for 5 minutes at 90ºC. A dual-colour protein ladder (BioRad) was used for molecular weight guidance. Proteins were separated according to size via sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 200v for 60 minutes at room temperature. Co-immunoprecipitation samples were run at a constant voltage of 150v for 120 minutes at room temperature.

Proteins were transferred to a PVDF membrane (BioRad) in a transfer buffer made from 25 mM Tris, 19.2 mM glycine and 20% methanol (10% methanol was occasionally used in the transfer buffer for transfer and detection of very small proteins) at a constant amperage of either 250mA and 100V for 90 minutes at 4ºC or 175mA and 85V overnight at 4ºC.

2.8.3 Immunoblotting

Unspecific binding to the membranes was prevented using 5% skimmed milk powder (Sigma) with 2% bovine serum albumin (BSA) in TBS-T (Tris buffered saline with 0.1% Tween) for 60 minutes at room temperature. Membranes were then incubated overnight with primary antibodies (see Table 3, Chapter 2.2.3 for detailed list).

Application of primary antibodies was followed by incubation with the

appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit, BioRad). Enhanced chemiluminescence (Pierce) was used for detection and GAPDH (Merck Millipore) was used as a loading control. Densities of bands were quantified using FluorS-Multi-Imager (Biorad) and all values, adjusted to GAPDH values before being determined as a percentage of basal or control where possible.

2.9 Data analysis

For statistical analysis student's two-tailed t-tests and ANOVA with post-hoc tukey tests were used where group size was bigger than two. Experiments were performed in at least triplicate using at least three different batches of cells from different individuals where possible. The p-value was used to determine significance of the results; ns (not significant) $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. In figures mean ± SEM is given.

3. Results

3.1 PrP expression and structure

3.1.1 Different anti-PrP^{C} antibodies show different staining patterns in schwannoma cells

The PrP antibody used at the start of the project (PrP 6D11 (mouse) Insight Biotechnology, Wembley, UK) was initially thought to be specific to full-length PrP, however, due to its application specificity and species reactivity another antibody had to be used for immunoprecipitation and immunocytochemistry (PrP [EP1802Y] (rabbit) Abcam, Cambridge, UK). Even though both antibodies showed the exact same Western blotting pattern (size of band, molecular weight of band, etc.), the Abcam antibody that was specific to the C-terminal region of PrP, showed much more nuclear staining than the previous 'full length' PrP 6D11 antibody from Insight Biotechnology, which showed localisation throughout the cytosol and in the cell membrane of schwannoma cells when using the same fixation and staining protocol.

To investigate fully, I purchased two new antibodies that were specifically designed to target either the C-terminal (Nbp1-92285) or N-terminal (Nbp2-38508) portion of the protein. These antibodies confirmed my hypothesis that N-terminal-specific PrP^{C} antibodies localised mainly to the cytosol and membrane with only some nuclear staining (Fig. 9A) whereas C-terminal PrP localised only to the nucleus of most schwannoma cells (Fig. 9B). These findings mean that the antibody specificity must be taken into account when drawing conclusions from the results discussed below, particularly co-localisation and immunoprecipitation results, as different antibodies may have given a slightly different result.

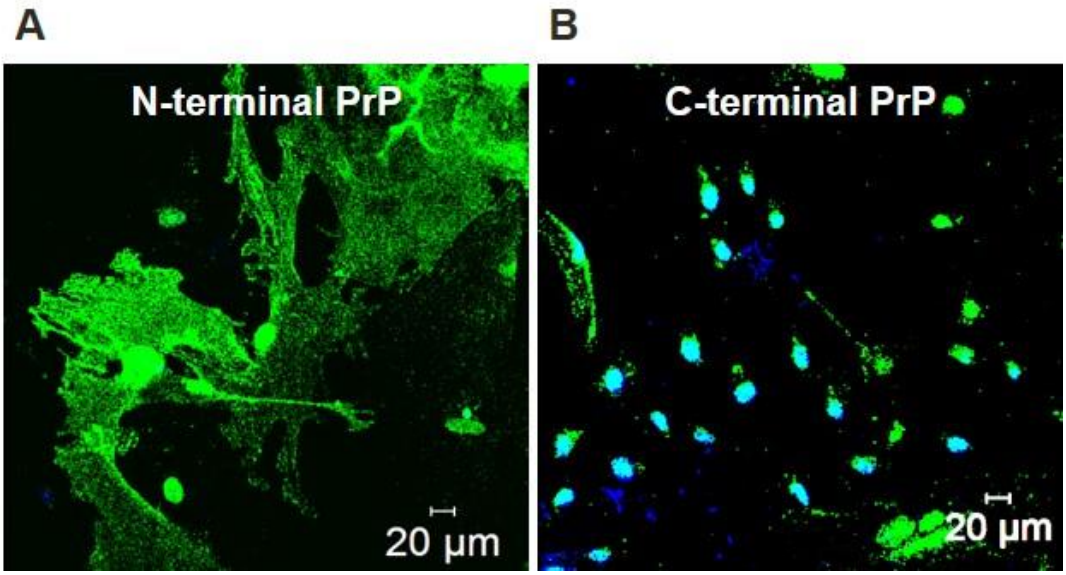

Figure 9. N-terminal PrP is localised throughout the cytoplasm and cell membrane whereas C-terminal PrP is primarily localised to the nucleus.

Schwannoma cells were fixed in 4% PFA, permeabalised with 0.2% Triton X-100 then blocked with 10% NGS to prevent unspecific binding before being incubated either with the PrP antibody specifically recognising the N-terminus (A) or the C-terminus (B) of PrP (green staining). After incubation overnight, cells were washed before havng secondary antibodies and DAPI (blue staining) applied. Immunocytochemical staining demonstrates that different staining patterns occur between the two antibodies with the C-terminal portion only being picked up within the nucleus of schwannoma cells whereas the antibody recognising the N-terminal portion of the protein picked up PrP staining throughout the cells (n=3) PrP^{C} is in green and DAPI is shown in blue.

Upon discovery of the differences in PrP antibody specificity in immunocytochemical staining, I produced a table of the different antibodies used, what they were used for and the staining pattern I see. Certain antibodies had to be used for different techniques due to their suitability for that technique, thus, the terminal specificity was only taken more into consideration post-hoc. For further information please see Table 4.

Table 4: Table of PrP antibodies used and their staining pattern.

Experiment	Antibody	Recognition site	Observations using immunoflurescence
IP (as bait)	PrP (Sigma) (Mouse, IP-specific antibody)	C-terminal	C-term-specific antibodies show mainly nuclear staining.
IP (blot) Western Blotting	PrP (Abcam) (Rabbit)	C-terminal	C-term-specific antibodies show mainly nuclear staining. Western blot results are no different to N-terminal PrP.
ICC/ Immuno-fluorescence	PrP (Santa Cruz (Mouse))	Unspecified	Cytosolic and membrane staining.
IHC	PrP 12F10 (Mouse)	C-terminal (aa142-160)	Cytosol and some nuclear staining in tissues.

PrP antibodies used for each experiment reflect the table above, any differences in antibodies used are noted. Due to pressures with time and resources, antibodies for both C-terminal and N-terminal were not used for each experiment, although this should be investigated fully in the future.

3.1.2 PrP is overexpressed in schwannoma compared to Schwann cells

Firstly I checked whether the *PRNP* gene upregulation previously described in schwannoma (59) translates to an increase in protein levels of PrP. Immunofluorescence using anti-PrP (N-T) antibody shows an increase in expression of PrP in schwannoma compared to Schwann cells (Fig. 10A) and that the PrP in schwannoma is localised throughout the membrane and cytosol but not in the nucleus (Fig. 10A, right panel, white arrows).

Western blotting also shows that PrP is upregulated 3-4-fold in schwannoma compared to Schwann cells (Fig. 10B) and immunohistochemistry also

demonstrates an up-regulation of PrP in schwannoma with a similar expression pattern to that seen *in vitro* (Fig. 10C, right panel) compared to both normal nerve (Fig. 10C, left panel) and a a traumatic neuroma (Fig. 10C, central panel). Immunohistochemical staining was carried out by Dr. David Hilton's team in the Neuropathology department of Derriford hospital.

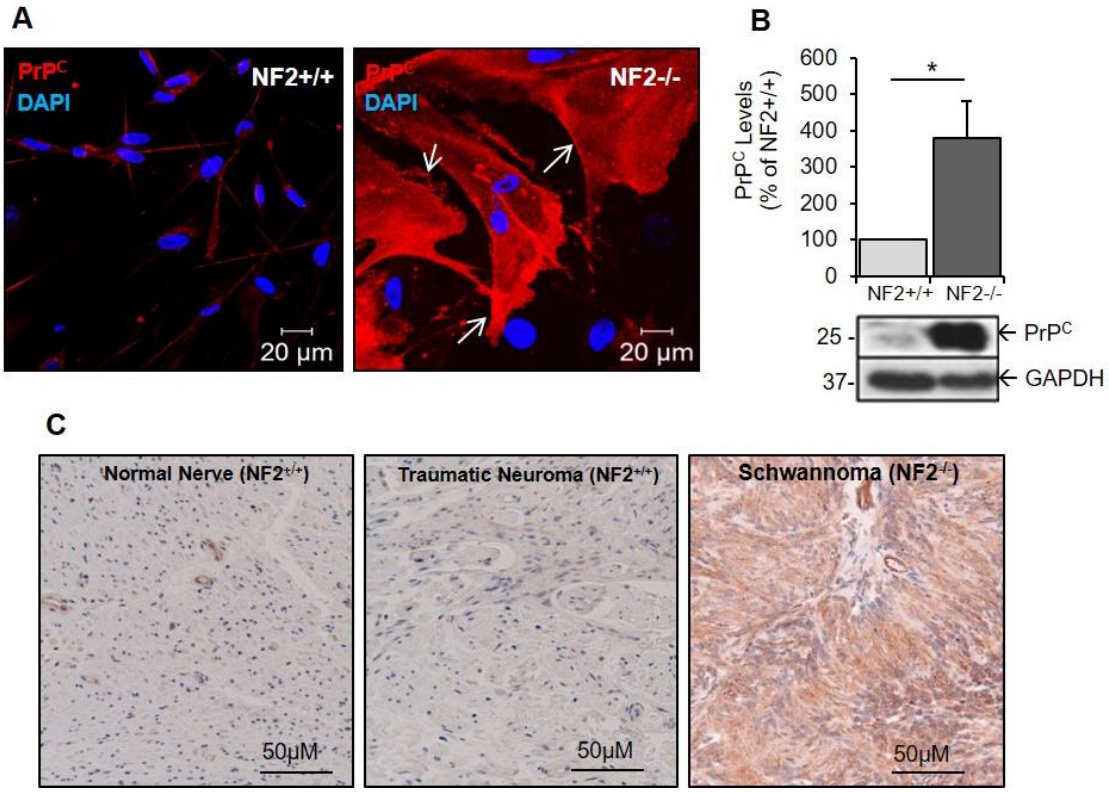

Figure 10. PrP is overexpressed in schwannoma cells and tissue.

Immunofluorescence shows overexpression of PrP (red staining) in schwannoma (A, NF2-/-, right panel) compared to Schwann cells (A, NF2+/+, left panel) (n=5), particularly in membrane ruffles of schwannoma cells (A, right panel, white arrows). Western blotting which showed a significant increase in PrP expression in schwannoma (NF2-/-, B) compared to Schwann cells (NF2+/+, B) (n=5). Immunohistochemistry further demonstrated increased PrP expression (DAB, brown staining) in schwannoma tissue (C, right panel, n=10) compared to both normal nerve (C, left panel, n=5) and traumatic neuroma (C, central panel, n=5). *p<0.05. GAPDH was used as a loading control.

3.1.3 PrP is also overexpressed in meningioma and in Merlin-deficient mesothelioma cell line, TRA cells

My research also aims to make these finding more widely applicable to other NF2-related tumours and other non-NF2, Merlin-deficient tumours. Immunohistochemical staining of meningioma (Fig. 11A) shows that PrP^C is strongly over-expressed in these tumours (Fig. 11A panel ii and iii) compared to normal meninges (Fig. 11A, panel i, bottom) although PrP^C is still more highly expressed in normal brain tissue (Fig. 11A, panel i, top). As meningiomas are more histologically diverse than schwannomas, I looked at both Merlin-positive (as judged by fluorescent *in situ* hybridisation (FISH, carried out by Dr. David Hilton, Derriford Hospital Neuropathology Department) as having an intact chromosome 22q) and Merlin-deficient (from NF2 patients) meningiomas and saw no difference in PrP^C expression levels between the two tumour types (Fig. 11A, ii and iii). Western blotting mirrored immunohistochemistry observations and showed a significant increase in PrP^C expression between Merlin-deficient grade I meningioma samples and Merlin-positive human meningeal cells (HMC) (Fig. 11B and C). Although HMC are not as good as normal human meninges as a control, they were the best available and provided consistent results.

A subset of Human Malignant Mesothelioma (HMM) cells are also Merlin-deficient, two HMM cell lines were used; Merlin-positive HIB cells and Merlin-deficient TRA cells. I show a significant difference in PrP^C expression between the two cell lines, with much higher PrP^C expression in the Merlin-deficient TRA cells than in the Merlin-positive HIB cells (Fig. 11D).

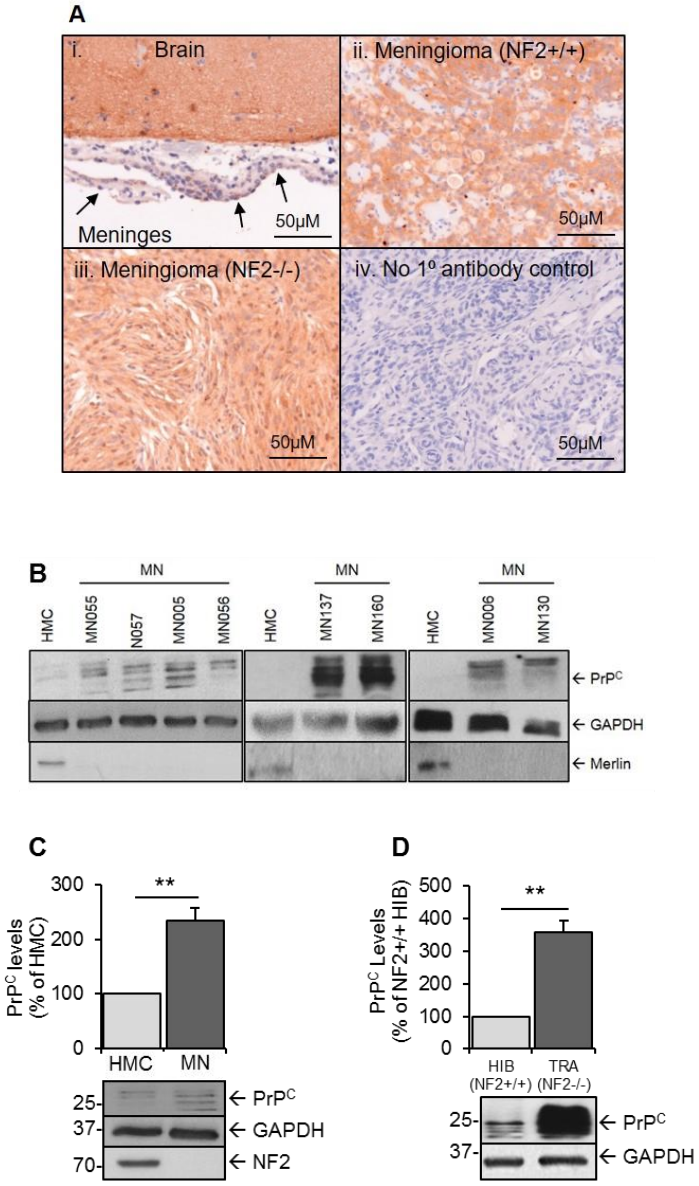

Figure 11. PrP is overexpressed in Merlin-negative meningiomas and human malignant mesothelioma cells.

Immunohistochemistry (n=10 of each sample type) shows that PrP expression is high in normal brain (Ai, top) but shows hardly any expression in normal meninges (Ai, NF2+/+, bottom). PrP expression is increased in both Merlin-positive (Aii, NF2+/+) and Merlin-deficient (Aiii, NF2-/-) meningioma samples compared to normal meninges (Ai). No primary antibody control (Aiv) demonstrates specificity of staining. (B) Western blotting was used to quantify PrP protein expression and showed a significant overexpression of PrP in Merlin-negative meningiomas compared to human meningeal cell line (HMC, B, n=8). Images from each of the NF2-/- Western blot repeats are shown in (C). A significant increase in PrP expression was also seen between Merlin-deficient HMM cells, TRA, compared to Merlin-positive HMM cells, HIB (D, n=3). **p<0.01, GAPDH was used as a loading control.

3.1.4 The PrP expressed in schwannoma is the same di-glycosylated, protease-sensitive form seen in Schwann cells

I aimed to verify whether the PrP present in schwannoma is the protease-sensitive form of the protein and not PrP^{res}, the form associated with the development of prion diseases, which is resistant to degradation by proteinase K. To investigate this, cells were lysed either normally in the presence of a protease inhibitor cocktail or in the presence of proteinase K. Upon treatment with proteinase K there is a strong reduction in PrP^{C} expression (Fig. 12A) suggesting that the PrP present in schwannoma is the cellular, protease-sensitive form of the protein.

In order to check the glycosylation status of the PrP^{C} in both Schwann and schwannoma cells, cells were either treated with the de-glycosylation enzyme PNGaseF or with water as a control. Upon treatment with PNGaseF both Schwann and schwannoma cells display two lower bands on the gel (Fig. 12B bottom arrows), suggesting the presence of both a mono- and un-glycosylated form of the protein whereas the control lysates displayed a higher band around the 30kDa mark, indicating that the PrP present normally in both cell types is the di-glycosylated form (Fig. 12B, top arrow). Schematic diagrams of the different PrP^{C} glycosylation states are shown in Fig. 12C to help visualisation of what occurs upon PNGase F treatment of cell lysates.

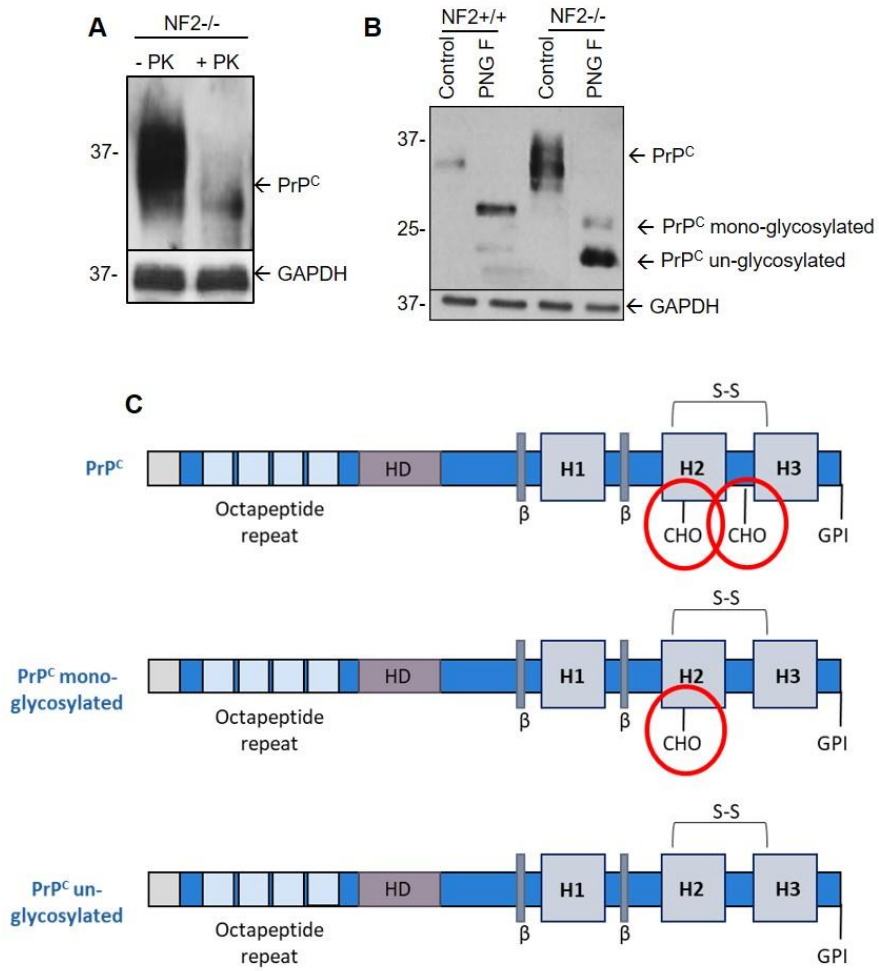

Figure 12. PrP in schwannoma is the cellular, fully glycosylated form of the protease-sensitive protein.

The PrP present in schwannoma was sensitive to degradation by proteinase K (A), unlike protease-resistant forms of the protein (n=3). Treatment with glycosydase enzyme PNGaseF demonstrated the presence of de-glycosylated PrP^C bands in both Schwann and schwannoma cells (B, n=3), suggesting the PrP^C present in schwannoma is in the mature, fully di-glycosylated state. Schematic diagrams of the different PrP^C glycosylation states are outlined in (C). Ns $p>0.05$, *$p<0.05$. GAPDH was used as a loading control.

3.1.5 PrP^C overexpression in schwannoma and TRA is Merlin-dependent but not in meningioma

Next I looked into whether the PrP overexpression in all three of these tumour types is due to loss of the tumour suppressor protein Merlin. There was earlier suggestion that PrP expression in schwannoma is Merlin-dependent, as PrP is

only upregulated in schwannoma and not the Merlin-positive traumatic neuroma (Fig. 10C, central panel). In order to fully investigate whether PrP^C expression is Merlin-dependent, I overexpressed the *NF2* gene using an adenoviral reintroduction. Indeed, it appears that PrP^C expression is dependent, at least partially, on Merlin as reintroduction of the *NF2* gene into schwannoma causes a significant reduction in the expression of PrP^C (Fig. 13A).

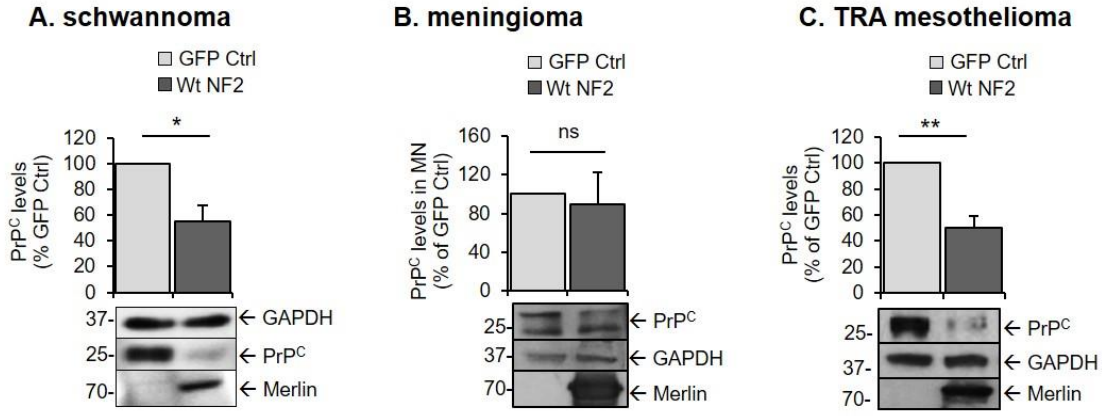

Figure 13. PrP^C overexpression is Merlin-dependent in schwannoma and mesothelioma but not significantly so in meningioma.

Either the *NF2* gene or a GFP control construct were introduced into schwannoma (A, n=5), meningioma (B, n=3) and TRA mesothelioma cells (C, n=3) using an adenoviral system and incubated for 24-48h until a significant number of cells showed GFP expression. Upon reintroduction of Merlin to schwannoma (A) and TRA mesothelioma cells (C), levels of PrP^C significantly reduced. Re-introduction of Merlin into Merlin-deficient meningioma cells had no significant effect on PrP^C levels (B). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, GAPDH was used as a loading control.

Unlike in schwannoma, there appeared to be no difference in PrP expression between Merlin-positive and Merlin-negative meningiomas (Fig. 11A ii and iii), giving an early indication that other factors may be regulating PrP^C overexpression in these tumours. In line with that I find no significant difference

in PrP^C expression upon re-introduction of Merlin into Merlin-deficient meningioma samples (Fig. 13B). Finally, *NF2*-reintroduction into Merlin-deficient TRA cells does also significantly reduce PrP^C (Fig. 13C), again suggesting that the PrP^C overexpression in these cells compared to Merlin-positive HIB cells (Fig. 11D) is due, at least in part, to loss of Merlin.

3.1.6 PrP expression and structure – discussion

PrP mRNA is overexpressed in schwannoma (Fig. 10A-C), as initially determined from the mRNA microarray analysis (59). PrP^C protein overexpression is seen in many other cancer cell lines (83) where it is present mainly in the membrane, where PrP^C is known to reside within lipid rafts (326), and cytosol (Fig. 10A). In several tumours including breast (327) and colorectal (328) cancer, however, PrP^C has been demonstrated in the nucleus. Based on the initial findings of the differential localisation of C and N-terminal PrP (Fig. 9) it could be that these cancers show an elevated expression of C-terminal PrP in the nucleus. Upon further investigation, I found that these previous research papers did use a C-terminal epitope of PrP antibody in their experiments. Similar PrP^C overexpression patterns have been shown previously in neurons and some cancers such as neuroblastoma (reviewed in (97)). This could suggest several different things: Firstly, that the majority of PrP^C in the membrane and cytosol of schwannoma cells is actually lacking the C-terminus but it could also mean that very little of the full-length PrP with the N-terminal makes it to the nucleus, this is odd because several nuclear localisation signals have been shown to be present within the N-terminal region of PrP^C (329). There is some evidence in the literature of C-terminal PrP in the nucleus but it has mainly been shown in prion-infected animal models (reviewed in (329)),

however, the presence of multiple bands when probing with PrP^C antibodies in my data (see Fig. 16A and C), could be indicative of the presence of several species or glycoisoforms of PrP^C present in the same population.

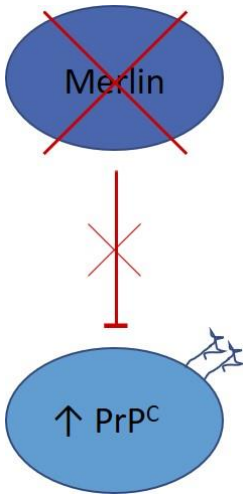

Figure 14. Loss of Merlin leads to overexpression of the di-glycosylated, cellular form of prion protein.

I show that overexpression of PrP in schwannoma is primarily caused by loss of the tumour suppressor protein Merlin. The form of prion protein present in these cells is the cellular, fully glycosylated form and not the protease-resistant form associated with prion diseases.

PrP^C appears to also be upregulated in Merlin-deficient meningiomas compared to normal meningeal tissue and cells (Fig. 11A-C) and in the Merlin-deficient mesothelioma cell line TRA compared to the Merlin-positive HMM cell line HIB (Fig 11D). This is the first time levels of PrP have been investigated in these brain tumour subtypes and, whilst there is evidence in the literature that the prion protein homologue Dpl is upregulated in meningioma (reviewed in (330)) there is nothing in the literature regarding PrP^C expression. These data suggest not only that PrP^C may be overexpressed in a variety of NF2-related tumours but also that PrP overexpression may be caused by loss of the tumour

suppressor protein Merlin and so PrP overexpression may extend to other Merlin-deficient tumours. There are already a number of cancer types reported to show increased levels of PrP, a small fraction of which are deficient in Merlin including breast, prostate, colorectal cancers and glioblastoma multiforme (GBM) (83,331) providing a potential link between these two proteins.

PrP expression in schwannoma appears to be Merlin-dependent as IHC staining shows no upregulation of its expression in the non-Merlin-deficient Schwann cell proliferation known as a traumatic neuroma compared to normal nerve tissues whereas Merlin-deficient schwannoma cells show clear increased expression of PrP compared to controls (Fig. 10C). Importantly however, not all tumours overexpressing PrP^C are Merlin-deficient (e.g gastric cancers). This suggests that there must be other regulatory proteins and pathways involved in PrP^C expression in tumour cells besides Merlin. These other important regulatory pathways could also contribute a small amount to PrP^C overexpression in schwannoma, as Merlin re-introduction failed to decrease PrP^C levels (Fig. 13) by the same amount as they are increased in schwannoma compared to Schwann cells (Fig 10).

Adenoviral reintroduction shows PrP^C to be Merlin-dependent only in schwannoma (Fig. 13A) and TRA cells (Fig. 13C) but not in meningioma (Fig. 13B). The likely reason behind the lack of Merlin-dependent expression in meningioma may be, in part, down to the complexity of these tumours, there are many more subtypes, other mutations and grades (reviewed in (332)) as well as other variables such as additional mutations that are involved in these tumours that may contribute to regulation of PrP^C.

I also investigated whether the PrP present within schwannoma samples was the 'normal' cellular form of prion protein (PrP^C). I found the PrP in schwannoma to be the di-glycosylated, cellular form PrP^C as presence of PrP protein was completely abolished upon treatment with proteinase K (Fig. 12A). This would not have occurred in the PrP present was the scrapie or resistant form (PrP^{Sc} or PrP^{res}). Differences in glycosylation state of PrP may have accounted towards its accumulation within cells due to under or over-processing (333). However, I show that in both Schwann and schwannoma cells in culture PrP is present in the di-glycosylated, fully post-translationally modified form (Fig. 12B and C).

3.2 Control of PrP^C expression

3.2.1 PrP^C overexpression in schwannoma is due to increased gene transcription and not due to altered protein degradation

Next I investigated the mechanisms by which PrP^C overexpression may occur; whether it is solely due to the increased gene expression as previously described (59), or whether altered degradation is causing intracellular accumulation of the protein. In order to investigate gene expression, I used two inhibitors – Actinomycin D (Act. D), an inhibitor of transcription, and cycloheximide (CHX), an inhibitor of protein synthesis. Upon treatment of schwannoma cells with both Actinomycin D (Fig. 15A) and cycloheximide (Fig. 15B) there is a significant reduction in PrP^C expression suggesting that high PrP^C levels in schwannoma are largely associated with increased *PRNP* gene expression and translation. The half life of GAPDH (8h (334)) also differs from that of PrP^C (2.6h (335)), suggesting that PrP^C can be degraded more rapidly than GAPDH upon treatments with actinomycin D and cycloheximide, allowing

for a stable GAPDH band even though PrP^C levels are seen to decrease significantly. Protein estimation took into account for any smaller differences the actinomycin D and cycloheximide may have had on GAPDH protein expression to ensure the same amount of total protein was loaded onto the gel at the start of the experiment.

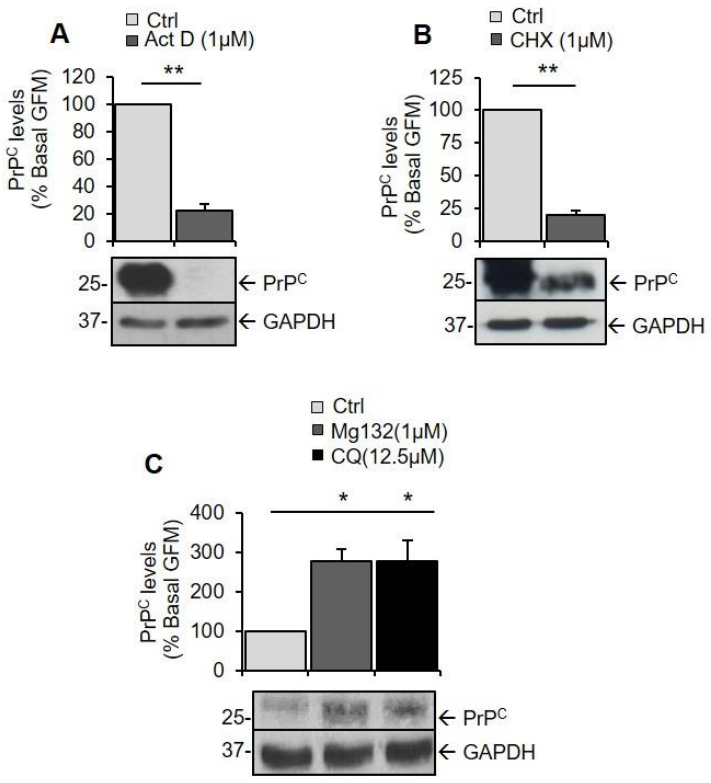

Figure 15. PrP^C in schwannomas is increased due to enhanced gene transcription and is degraded by both proteasomal and lysosomal degradation.

Inhibition of schwannoma gene transcription using the inhibitor Actinomycin D (Act D, A), significantly reduced expression of PrP^C (n=3), as did inhibition of protein synthesis using the translation inhibitor cycloheximide (B, n=5). Inhibition of proteasomal (Mg132) and lysosomal (Chloroquine, CQ) degradation was able to significantly increase levels of PrP^C (C, n=5). *p<0.05, **p<0.01, GAPDH was used as a loading control.

There are several pathways by which cellular proteins are degraded but the most common are lysosomal degradation and proteasomal degradation. Prion

proteins have been previously described to be degraded via both of these pathways (151,336,337). In schwannoma cells treatment with proteasomal degradation inhibitor MG132 (Fig. 15C) and lysosomal degradation inhibitor chloroquine (CQ, Fig. 15C) cause significant increases in PrP^C levels. These results suggest that overexpression of PrP^C in schwannoma is not due to alterations in the degradation pathways that would likely lead to protein accumulation and instead is purely due to gene upregulation and increased transcription of *PRNP*.

3.2.2 PrP^C expression is not regulated by DCAF1

As PrP^C expression in schwannoma is mainly regulated by gene transcription I wanted to investigate the role of two transcription factors – $CRL4^{DCAF1}$ (a broad-range transcriptional regulator potentially acting epigenetically) and NFκB, which have well-defined roles in schwannoma development and pathology (72,213) – to see whether they regulate PrP^C expression and thus may be involved in PrP^C overexpression in schwannoma. Firstly, I used a shRNA construct to knock down expression of *DCAF1* in schwannoma. Upon *DCAF1* knockdown there was no difference in PrP^C expression (Fig. 16A). I also wanted to see whether PrP^C in schwannoma could, instead, reside upstream of DCAF1, potentially regulating its transcription. However, upon knocking down of PrP^C expression there was no significant difference in expression of DCAF1 (Fig. 16B).

3.2.3 PrP^C overexpression in schwannoma may be regulated by NFκB

NFκB has been widely described as a transcription factor, regulating expression of many genes including those involved in cell survival (including BAX, Bcl-2 and XIAP (338-340)) adhesion (such as CD44 and N-CAM (341,342)) and

inflammation (e.g. COX2 (343)). I have previously shown that NFκB expression is upregulated in schwannoma compared to healthy cells (213).

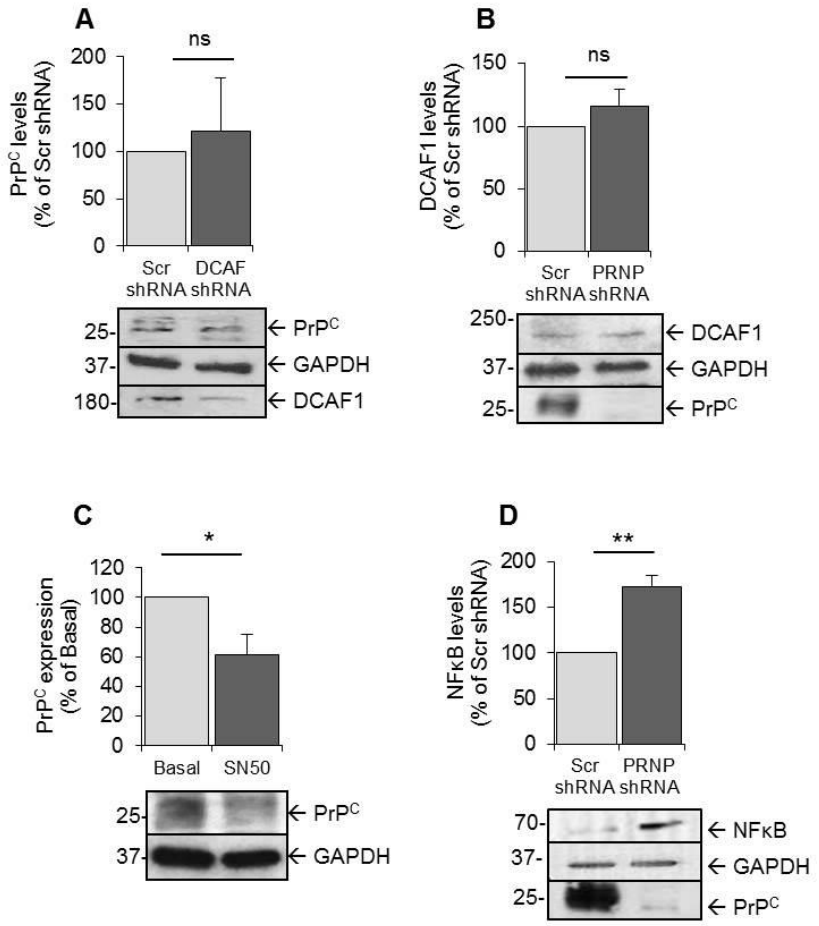

Figure 16. Transcriptional regulation of PrP^C occurs via NFκB but not DCAF1.

Upon knockdown of DCAF1 expression using *DCAF1* shRNA PrP^C levels were not affected (A, n=3). DCAF1 expression was also not altered upon PrP^C knockdown using *PRNP* shRNA (B, n=3). Inhibition of NFκB with SN50 significantly reduced PrP^C expression (C, n=5) but knockdown of PrP^C was also able to significantly increase NFκB p65 expression (D, n=4). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, GAPDH was used as a loading control.

Using an NFκB small molecule inhibitor; SN50 (previously successfully used in schwannoma cells (213)), to inhibit the transcription factor activity of p50/p65 NFκB (the canonical NFκB pathway) by preventing p50 translocation to the

nucleus. (213)Lentiviral knockdown using shRNA for NFκB was not possible as the lentiviral particles I had did not sufficiently reduce NFκB expression and therefore could not be used. Upon treatment of schwannoma cells with SN50 (8µg/ml) for 24 hours, levels of PrP^C significantly decreased (Fig. 16C) suggesting that NFκB plays a role in regulating PrP^C expression in schwannoma. Interestingly, I also checked NFκB expression in cell lysates treated with *PRNP* shRNA even though there is no evidence of PrP^C as a transcriptional regulator, I found that reducing PrP^C in schwannoma also increases NFκB expression (Fig. 16D).

3.2.4 Control of PrP^C expression – Discussion

From the gene array analysis between schwannomas and Schwann cells (59) it appeared that upregulation of PrP^C occurred at the genetic level, with alterations in the transcription of *PRNP*. However, I wanted to check whether the overexpression of PrP^C at the protein level was solely due to alterations in transcription or whether alterations in the normal protein degradation pathway were affecting PrP^C levels. I show using transcription inhibitor actinomycin D and translation inhibitor cycloheximide (Fig. 15) that by reducing global levels of transcription in schwannoma cells in culture, expression of PrP^C can be reduced. This suggests that increased PrP^C expression in schwannoma is due to the increased transcription and translation of *PRNP*. It is not the first time that higher PrP mRNA levels have been shown to be present in tumours; in breast cancer (327) and colon cancer PrP mRNA was shown to be upregulated via microarray analysis (344). To fully quantify the *PRNP* gene expression difference between Schwann and schwannoma cells, quantitative real-time polymerase chain reaction (qRT-PCR) would need to be used.

There are several known nuclear proteins that may act as transcription factors which are upregulated in schwannoma and involved in schwannoma pathological signalling that may be regulating *PRNP* transcriptional upregulation. One of the most well-known is the nuclear E3 Ubiquitin Ligase $CRL4^{DCAF1}$ (DCAF1) which in healthy Schwann cells is negatively regulated by Merlin (50). However, upon shRNA knockdown of *DCAF1* expression, there was no alteration in PrP^C expression (Fig. 16A) suggesting either that DCAF1 is not in control of *PRNP* transcription or that compensatory mechanisms are able to take over to maintain the high levels PrP^C expression.

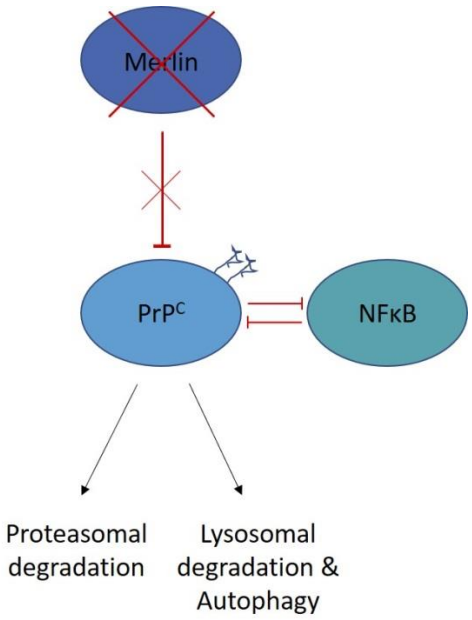

Figure 17. Overexpression of PrP^C in schwannoma is due to increased gene activity, not altered protein degradation and appears to involve NFκB.

Overexpression of PrP^C is likely due to increased gene activity of *PRNP* as seen previously (59) rather than decreased degradation. PrP^C degradation in schwannoma can be blocked by both inhibitors of proteasomal and lysosomal degradation suggesting that PrP^C in these cells is degraded via both pathways. Finally, transcriptional control of PrP^C could be via NFκB but does not appear to involve $CRL4^{DCAF1}$.

Another transcription factor potentially in charge of regulating *PRNP* transcription is NFκB which is upregulated in schwannoma (213) and known to regulate other key proteins associated with schwannoma pathology including c-Jun (345) and AKT (346). Two NFκB inhibitors (BAY11-7082 and Curcumin) were found to significantly reduce VS proliferation in culture (211) suggesting that, targeting NFκB could make for a beneficial therapeutic target in schwannoma cells, although small molecule inhibitors can be unspecific and have multiple off-target effects. Circumin, for example also inhibits phorbol ester-induced protein kinase C (PKC), production of inflammatory cytokines, EGFR, cyclooxygenase 2 (COX2), lipoxygenase and IκB kinase as well as NFκB (347). I used the small molecule inhibitor SN50 to inhibit NFκB translocation to the nucleus and found the action of this drug was able to significantly reduce PrP^C expression levels (Fig. 16C). ER stress, a common mechanism by which PrP^C is upregulated in cells (327), also causes upregulation of NFκB (348). This suggests that PrP^C may be under direct control of NFκB in schwannoma cells. What's more, PrP^C appears to be able to reciprocate this activation of NFκB transcription as knockdown of *PRNP* using shRNA significantly increases expression of NFκB (Fig. 16C), suggesting some kind of reciprocal interaction between these two proteins. No data has, thus far, demonstrated a role for PrP^C as a transcription factor but PrP^C has been shown to reside in the nucleus of some cancer cell types (97), uncovering a potentially interesting new role for PrP^C as a transcriptional regulator in cancer cells.

I then discovered that the increased expression of PrP in schwannoma was purely down to the aforementioned upregulation in *PRNP* gene activity rather

than alterations or complete defects in the PrP degradation pathways, as inhibitors of proteasomal (MG132) and lysosomal (CQ) degradation further increased PrP^C expression (Fig. 15C). PrP^C has been demonstrated widely to be degraded by autophagy and lysosomal degradation (349) and no deficits in either of these pathways have thus far, been demonstrated in schwannoma. In fact, increased autophagic activity has been described in schwannoma before (350) and my own unpublished data showed increased levels of LAMP-1 and LC3, both autophagy-related proteins, in schwannoma compared to Schwann cells (Supplementary Fig. 1).

3.3 PrP^C release from schwannoma cells

3.3.1 PrP^C is released from schwannoma cells via exosomes

It is well known that prions can be released from cells, propagating prion diseases by interacting with surrounding cells. I originally hypothesised that PrP^C was also released from schwannoma cells and acts in both an autocrine and paracrine manner to stimulate surrounding cells. Firstly, I used an ELISA to check the release of PrP^C from Schwann and schwannoma cells into the cell culture medium. I show for the first time, that PrP^C is released from both Schwann and schwannoma cells but significantly more from the tumour cells (Fig. 18A). I wanted to check whether the PrP^C released from schwannoma cells is released as free peptides via cleavage from the cell membrane or exocytosis, or, whether the PrP^C was being released via exosomes, as this could affect how the PrP^C may act on neighbouring cells. I used an exosome isolation kit (Invitrogen) to extract the exosomes from cell culture medium and ran this on a Western blot alongside both total cell medium and the leftover cell

medium after the exosome isolation (termed ‘supernatant’). Concentrated growth factor medium (GFM) from the bottle was used as a negative control and was made with exosome-free FBS throughout the exosome isolation experiments.

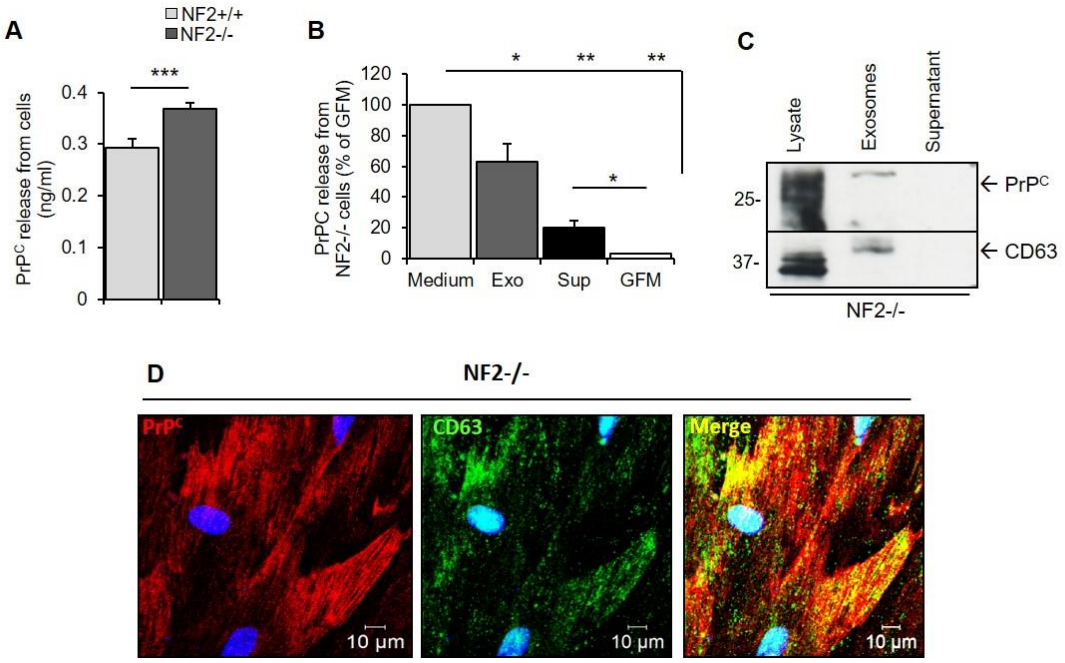

Figure 18. PrP^C is released from schwannoma cells via exosomes.

PrP^C is more highly released from schwannoma cells compared to Schwann cells (ELISA, A, n=3). Upon exosome isolation, it is clear that a large portion of the PrP^C released from schwannoma cells occurs via exosomes (B, dark grey bar, n=3, ELISA). Western blotting demonstrated that PrP^C was present in the exosome fraction although at a much lower level than the PrP^C expressed intracellularly (C, n=3, top panel), CD63 was used as a late endosome/exosome marker (C, bottom panel). Immunofluorescent staining shows co-localisation of PrP^C and CD63 within schwannoma cells suggesting that PrP^C travels through cells in late endosomes before being released via exosomes (D, n=3). *p<0.05, **p<0.01, ***p<0.001.

The results indicate that the majority of the PrP^C released from schwannoma cells into the culture medium (Fig 18B, light grey bar ‘medium’) is via exosomes (Fig.18B, dark grey bar ‘Exo’) and only a small amount is released as free

peptide in the ‘supernatant’ fraction (Fig. 18B, black bar, ‘Sup’). This is further demonstrated by Western blotting (Fig. 18C) in which PrP^C is only visible in the exosome fraction and is present in too small a quantity in the supernatant to be picked up as Western blotting is much less sensitive a technique compared to ELISA. CD63 is used as an exosome marker to validate the isolation (Fig. 18C, bottom panel). Using immunocytochemistry, I show co-localisation between PrP^C and CD63 (late endosome/exosome marker) within the tumour cells (Fig. 18D) suggesting that PrP^C is transported through schwannoma cells within endosomes to the plasma membrane and is released to the extracellular matrix via exosomes.

3.3.2 Non-exosome PrP^C release is not regulated by GPI anchor cleavage

Next I wanted to ascertain the mechanism behind how ‘free’ PrP^C in the culture medium is released from cells. PrP^C has been previously demonstrated to be released from cells *in vitro* via cleavage of the GPI anchor using phosphatidylinositol phospholipase C (PIPLC). However, in schwannoma there is no difference in PrP^C expression or localisation upon treatment with PIPLC, as shown by immunofluorescence (Fig. 19A) and no significant difference in the amount of PrP^C released from schwannoma cells into the culture medium, as shown using ELISA (Fig. 19B). Thus, in schwannoma, free PrP^C is released by mechanisms other than cleavage of the GPI anchor.

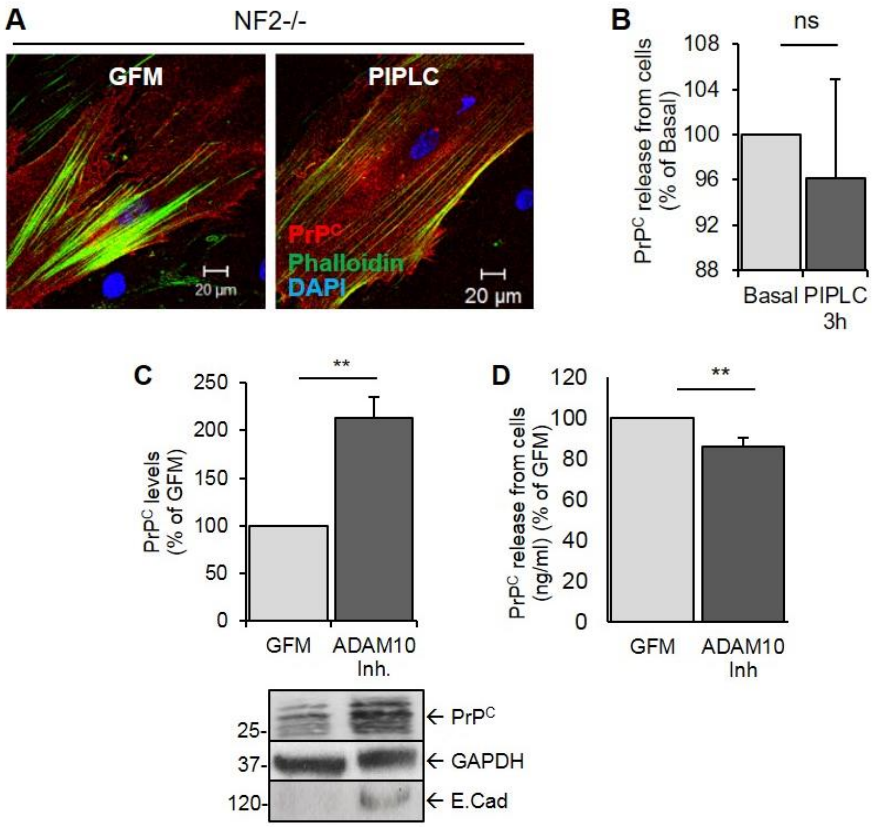

Figure 19. PrP^C is released from schwannoma cells by cleavage by ADAM10 but not by GPI anchor cleavage by PIPLC.

GPI anchor cleavage of PrP^C using PIPLC did not alter intracellular PrP^C expression judged by immunofluorescence (A, n=3), or the PrP^C released from cells into the culture medium (B, ELISA, n=3). Inhibition of α-cleavage by ADAM10 significantly increased intracellular PrP^C level (C, n=5), E-cadherin was used as a positive control as it is known to be cleaved by ADAM10 at the cell surface (351). Inhibition of ADAM10 also significantly decreased the amount of PrP^C released from schwannoma cells as judged by ELISA (D, n=6). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, GAPDH was used as a loading control.

3.3.3 PrP^C is released from schwannoma by α-cleavage by ADAM10

Upon discovering that PrP^C did not appear to be cleaved and released from the membrane by PIPLC, I investigated potential other mechanisms of PrP^C cleavage. The most well-documented is α-cleavage just after the octapeptide repeat of PrP^C by the membrane metalloproteases ADAM9, ADAM10 and ADAM17, a process also known as ‘ectodomain shedding’ (102,352). I decided

to investigate the role of ADAM10 in PrP^C cleavage as this was the enzyme most well-documented in the literature (353).

Schwannoma cells were incubated for both with GI254023X, a specific ADAM10 inhibitor at a final concentration of 20µM for 24 hours. Cell lysate was collected before being subject to Western blotting in order to analyse PrP^C levels. Data shows that there is a 1.5-2-fold increase in the amount of PrP^C within cells upon treatment with the ADAM10 inhibitor (Fig. 19C) suggesting that ADAM10 plays a small role in the normal regulation of PrP^C expression and release of the N-terminal fragment in schwannoma cells (reviewed in (354)). Although a small amount of full length PrP^C can be secreted from cells (PrP^{Sec}), the majority of uncleaved PrP^C remains in the cell. Cell culture medium was also run using the *PRNP* ELISA which demonstrated a significant decrease in PrP^C release from schwannoma cells upon treatment with ADAM10 inhibitor (Fig. 19D).

3.3.4 PrP^C release from schwannoma cells – Discussion

I show for the first time that PrP^C is released from schwannoma cells and at a higher rate than is released from Schwann cells (Fig. 18A). Although exosomes have been shown to be released from tumour cells in various previous studies (355) and both cellular and scrapie forms of PrP have been shown to be released via exosomes in ROV cells (derived from RK-13 rabbit kidney cells overexpressing PrP) and MOV cells (derived from mouse neuroglial cells overexpressing PrP) (356,357), the two together have never been shown in tumour cells. I show using ELISA, Western blotting and immunofluorescence

that PrP^C is released from schwannoma cells in association with exosomes (Fig. 18B-D).

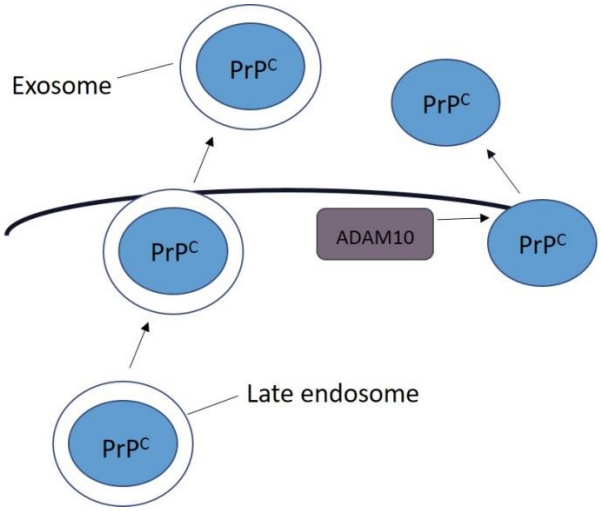

Figure 20. PrP^C is released from schwannoma cells via exosomes and by cleavage by ADAM10.

PrP^C is known to be transported through the cytosol of cells via late endosomes, my data shows that these PrP^C-containing late endosomes are released from cells as exosomes which may then be able to activate cells in a paracrine or autocrine manner. I also show that PrP^C can be cleaved and released from the schwannoma cell membrane by the matrix metalloprotease ADAM10, although other ADAMs such as ADAM9 and ADAM17 may also play a role.

From the exosome isolation ELISA experiments, it was clear that a small portion of the PrP^C detected in the ELISA was not exosome associated (Fig. 18B), thus I investigated other mechanisms of PrP^C release from these cells. Initially investigating whether PrP^C could be released via cleavage of the GPI anchor (358), a process referred to as 'shedding'. However, it was clear that cleavage of the GPI anchor using PIPLC is insufficient to cause release of PrP^C from schwannoma cells (Fig. 19A and B). This raises the question as to whether PrP^C is even membrane-anchored in these cells in the first place or whether it is just trafficked through vesicles and recycled at the cell surface.

I then wanted to investigate whether PrP^C was released instead via its α-cleavage by one of the several metalloproteases that have been reported to be able to cleave PrP^C (102). One of the most widely reported of these metalloproteases is ADAM10, which cleaves PrP^C between Gly^{228} and Arg^{229} (121). I found that inhibition of ADAM10 did indeed cause an accumulation of PrP^C to build up within schwannoma cells and reduce the amount of PrP^C released from these cells suggesting that PrP^C is cleaved and released from schwannoma cells via α-cleavage and not via 'shedding' (Fig. 20).

This has many implications for the treatment of tumours. Recently another tumour suppressor protein shown to be downregulated in schwannoma, p53 (359) has been shown to have 'prion-like' tendencies. Data has suggested that mutant p53 is able to aggregate and turn healthy p53 into oncogenic, mutant p53 in cancer cells (360). If PrP^C has similar oncogenic prion tendencies within these tumour cells it could make investigating PrP^C release from schwannoma cells, and its action on neighboring healthy cells, an avenue that would be interesting to investigate further. Furthermore, it could also make PrP^C a very interesting potential biomarker to consider if PrP^C-containing exosomes could be detected in patient blood (PrP^C and PrP^{Sc} have previously been described in human blood plasma samples (361)) and could help predict re-growth or tumour size and burden for patients in a relatively un-invasive and inexpensive manner when compared to scans and surgeries.

3.4 PrP^C in schwannoma proliferation, adhesion and survival

In order to further evaluate the role of PrP^C in schwannoma development I wanted to look at the effects of knocking down levels of PrP^C. I used two methods to reduce expression of PrP^C in schwannoma cells, lentiviral particles

containing *PRNP* shRNA both homemade TRC lentivirus (Fig. 21Aii) and a pre-made pool containing 5 different shRNA constructs (Fig. 21Ai).

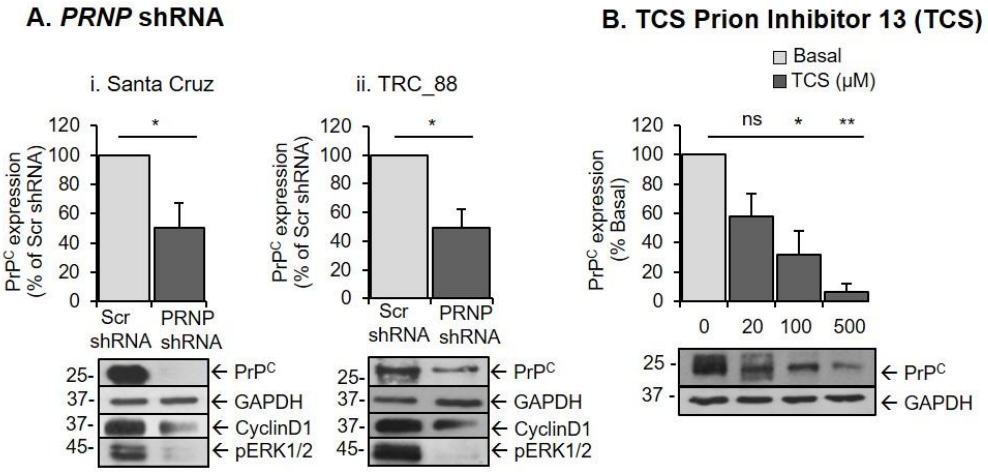

Figure 21. Knockdown of PrP^C using PRNP shRNA and TCS prion inhibitor 13.

Both pre-made *PRNP* shRNA (Ai) and home-made *PRNP* shRNA viral particles made using a TRC gene set (Thermofisher) (Aii) significantly reduce expression of PrP^C as well as cyclin D1 and pERK1/2, as determined by Western blotting (n=5 for each lentivirus). Results from both the pre-made and homemade viruses were combined due to time restraints on making more of the homemade virus. At both 100μM and 500μM concentrations of TCS prion inhibitor 13 PrP^C levels were significantly reduced in a dose-dependent manner (n=6) therefore TCS prion inhibitor 13 was also used in further experiments to reduce PrP^C expression in schwannoma cells. Ns $p>0.05$, *$p<0.05$, **$p<0.01$, GAPDH was used as a loading control.

I also used TCS prion inhibitor 13, originally designed to inhibit PrP^{Sc} accumulation (362), which I show to also affect total PrP^C levels (Fig. 21B), all three methods (shRNA and TCS prion inhibitor) significantly reduced expression of PrP^C in schwannoma cells (Fig. 21Ai, ii and B). As results from the two types of *PRNP* shRNA were identical, I combined the results and continued using mainly the pre-made viral particles due to time constraints and due to limited primary tumour material.

3.4.1 PrP^C contributes to increased schwannoma proliferation and survival

Firstly, I wanted to investigate whether reducing PrP^C levels in schwannoma affects the pathological hallmarks of these tumours – increased proliferation, resistance to apoptosis and increased cell matrix adhesion. Reducing PrP^C levels significantly reduces the number of proliferating schwannoma cells in GFM medium. This was judged by the decreased presence of Ki67 in the nucleus of schwannoma cells upon treatment both with *PRNP* shRNA (Fig. 22C) and TCS prion inhibitor 13 (Fig. 22D), this is further demonstrated in the immunofluorescence images (Fig. 22A and B, red arrows).

Apoptosis of schwannoma cells was judged by expression of cleaved caspase-3, the executioner caspase with a major role in apoptosis (ref?). Numbers of cleaved caspase-3-positive cells increases as PrP^C is reduced (Fig. 22E and F), seen both using immunofluorescence (Fig. 22A and B, green arrows) and Western blotting (Fig. 22G and H). Data suggests a role of PrP^C in schwannoma proliferation and survival as an increase in schwannoma apoptosis and a reduction in proliferation can be observed as PrP^C levels diminish.

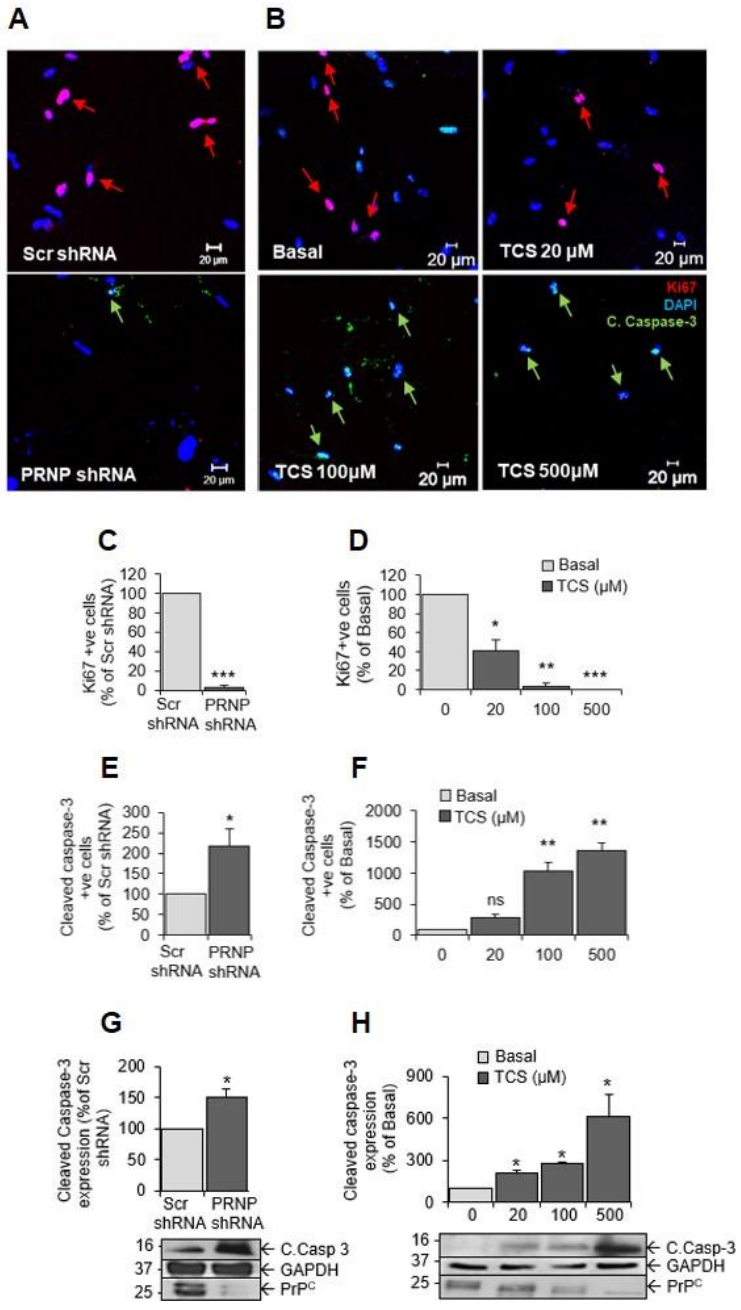

Figure 22. PrP^{C} is involved in schwannoma proliferation and resistance to apoptosis.

Knockdown of *PRNP* leads to a reduction in Ki67-positive cells (A, red arrows and quantified in C, n=3) as does inhibition of PrP^{C} using TCS (B, red arrows, quantified in D, n=3). *PRNP* knockdown also increases the number of cleaved caspase-3-positive cells (A, green arrows, quantified in E, n=3) as does inhibition of PrP^{C} using TCS (B, green arrows, quantified in F, n=3) also observed using Western blotting using both *PRNP* shRNA (G, n=4) and TCS (H, n=5). Ns p>0.05, *p<0.05, **p<0.01, ***p<0.001. GAPDH was used as a loading control.

3.4.2 PrP^C in schwannoma protects against free radical-induced apoptosis but not Bax-mediated apoptosis

There are several mechanisms by which PrP^C is thought to exert neuroprotective effects, thus contributing to cell survival; namely preventing Bax or free radical-mediated apoptosis.

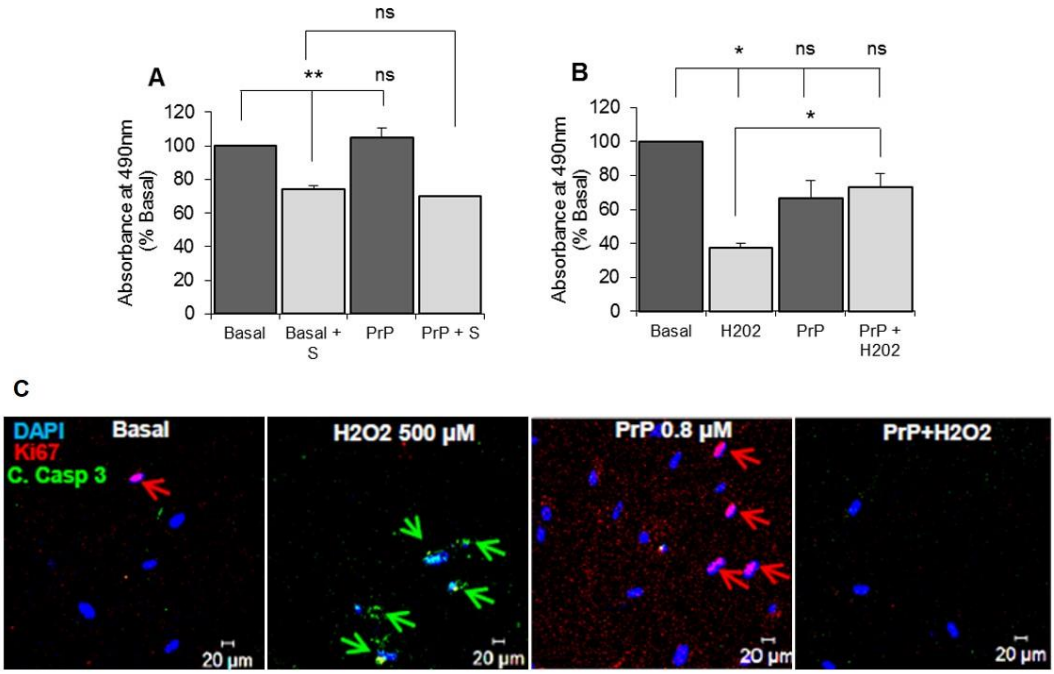

Figure 23. Protection of Bax and free radical-mediated schwannoma apoptosis by PrP.

Treatment of schwannoma cells with H_2O_2 to stimulate free radical-induced apoptosis significantly reduced cell viability, quantified using MTS assay (B) but pre-treatment with PrP peptide led to a significantly reduced cell viability (B, light grey bars, n=3). Staurosporine (denoted as 'S') was used to induced Bax-mediated apoptosis in schwannoma cells (A), but pre-treatment of cells with PrP was unable to protect them from staurosporine-induced cell death, quantified using MTS assay (B. light grey bars). Treatment of schwannoma cells with H_2O_2 induced activation of caspase 3 (C, green arrows) but pre-treatment of these cells with PrP peptide prevents this increase in cells expressing cleaved caspase-3 (C, bottom right panel). Ns $p>0.05$, *$p<0.05$, **$p<0.01$. Where the SEM is very low the error bar cannot be depicted.

I show using MTS assays that pre-treatment with a central portion of the PrP peptide chain (aa105-120, AbD Serotec) protects schwannoma cells from oxidative stress induced by hydrogen peroxide treatment (H_2O_2, Fig. 23A) but not against staurosporine-induced BAX-mediated apoptosis (Fig. 23B).These results mirror what has previously been reported in the literature regarding PrP^C protecting against free-radical mediated cell death via activation of caspase-3 and PKCδ (363), and give a glimpse of the potential mechanisms of PrP^C-mediated schwannoma survival that is seen in this primary cell culture model, therefore providing further potential therapeutic targets to investigate.

3.4.3 PrP^C plays a role is schwannoma pathological cell matrix adhesion

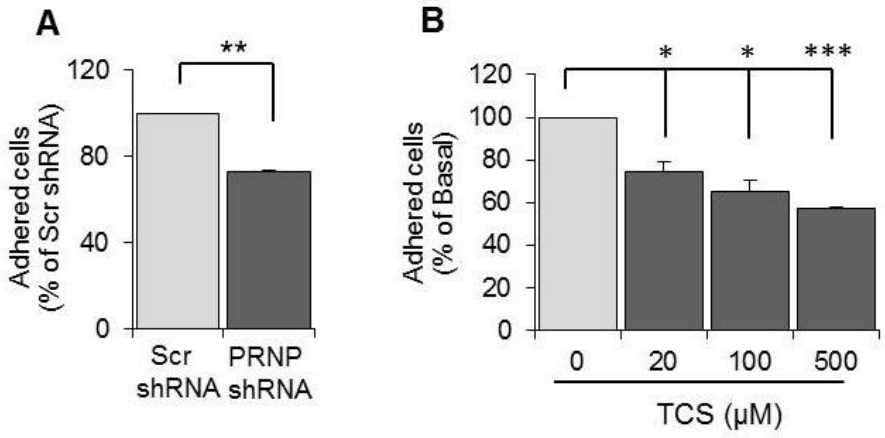

Figure 24. PrP^C plays a role in schwannoma pathological cell matrix adhesion.

Schwannoma cells were infected and selected with either scramble (scr) shRNA or *PRNP* shRNA before being plated in equal numbers for three hours at 37ºC. Knockdown of *PRNP* significantly reduced the number of schwannoma cells that adhered to the pre-coated culture plate within this time frame (A, n=3). For the TCS prion inhibitor experiment cells were split and treated with the inhibitor just before being plated then incubated for three hours. Again, in a dose-dependent manner, reduction of PrP^C significantly reduced schwannoma cell matrix adhesion (B, n=3). *p<0.05, **p<0.01, ***p<0.001.

Aberrant cell matrix adhesion is one of the pathological hallmarks of schwannoma cells *in vitro* and is thought to be due to overexpression of adhesion proteins such as FAK, integrins and their associated ligands such as insulin-like growth factor binding protein -1 (IGFBP-1) (183). Reducing levels of PrP^C significantly reduced the adhesive properties of schwannoma cells in culture after three hours post-plating, seen both using *PRNP* shRNA (Fig. 24A) and TCS prion inhibitor 13 (Fig. 24B).

3.4.4 PrP^C affects schwannoma proliferation, survival and cell matrix adhesion by signalling via cyclin D1, ERK1/2, PI3K/AKT, FAK and p53

To further investigate the role of PrP^C in schwannoma proliferation, survival and cell matrix adhesion I then investigated relevant signalling pathways downstream of PrP^C. The pathways I investigated have all been shown to be key for schwannoma development. Cyclin D1 controls proliferation via regulation of cell cycle progression through G1/S phase, cyclin D1 is upregulated in schwannoma (364) but levels are significantly reduced upon treatment of cells both with *PRNP* shRNA (Fig. 25A, left panel) and TCS prion inhibitor 13 (Fig.25A, right panel). Another pathway over-activated in schwannoma that regulates proliferation is the Raf/MEK/ERK pathway (365). ERK1/2 catalyses phosphorylation of nuclear transcription factors including FOXO3 and STAT1 which then activate genes involved in cell division (366). Levels of both total and phosphorylated, active ERK1/2 are decreased using *PRNP* shRNA (Fig. 25B, left panel, darker bars), levels of both total and pERK1/2 are decreased upon treatment at high concentrations with TCS prion inhibitor 13 (Fig.25B, right panel).

Figure 25. PrP^C acts via key pathways involved in schwannoma proliferation, survival, adhesion and tumourigenesis.

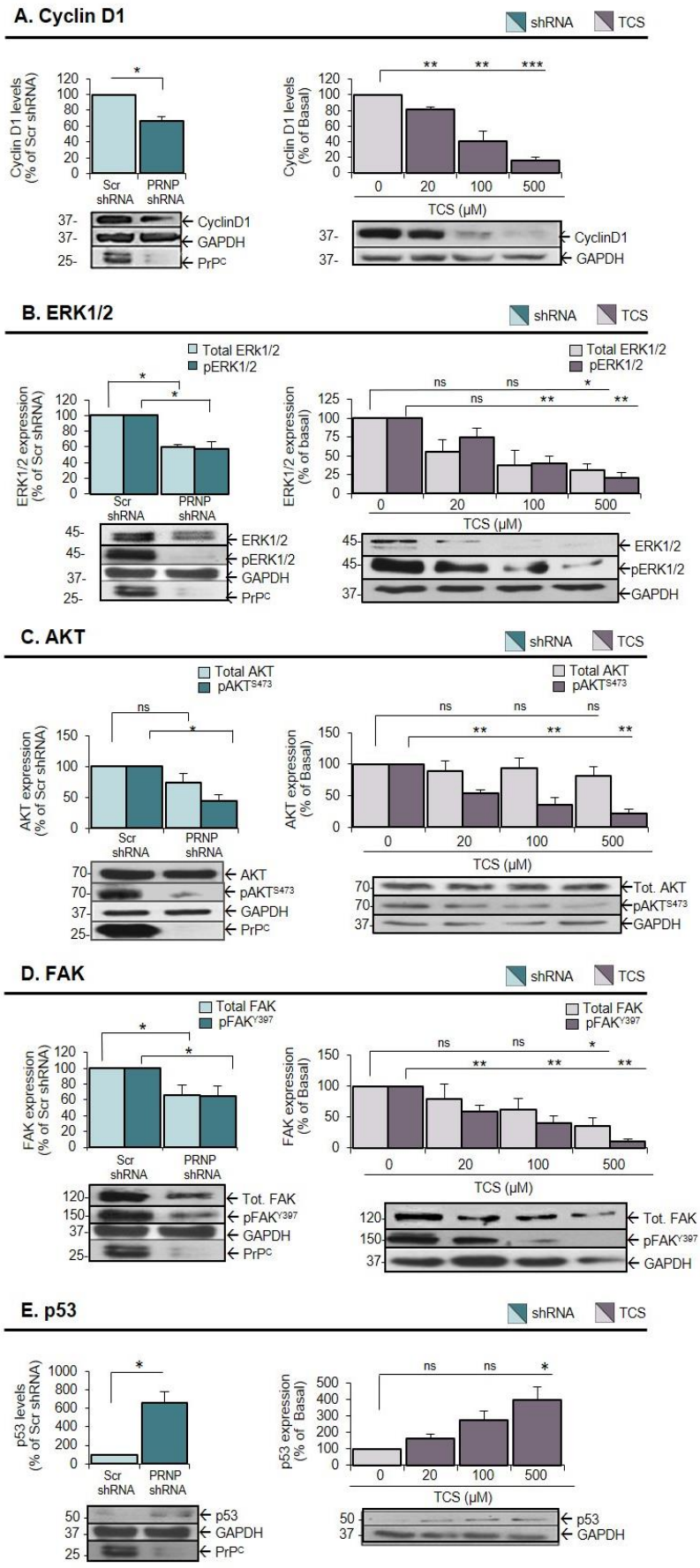

Figure 25. PrP^C acts via key pathways involved in schwannoma proliferation, survival, adhesion and tumourigenesis.

Both PrP^C shRNA and TCS prion inhibitor were able to reduce expression of cyclin D1 (A, n=3). Levels of both total and pERK1/2 were reduced both with *PRNP* shRNA and TCS inhibitor (B, n=5). Levels of pAKT were reduced both with *PRNP* shRNA and TCS (C, both panels, lighter bars) but levels of total AKT were unaffected (C, both panels, darker bars, n=5). Levels of both total and phosphorylated FAK are reduced upon reduction of PrP^C (D, n=6). P53 expression increases as a result of PrP^C reduction in schwannoma cells (E, n=6). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. GAPDH was used as a loading control.

The PI3K/AKT pathway and its downstream effector protein mTOR, have previously been shown to be upregulated in schwannoma (367) and contribute to increased schwannoma cell proliferation and survival (368). Reduction of PrP^C via both *PRNP* shRNA (Fig.25C, left panel) and TCS prion inhibitor 13 (Fig. 25C, right panel) reduces levels of activated AKT phosphorylated at serine 473 (darker bars), but expression of total AKT is unchanged (lighter bars).

FAK is a protein kinase localised mainly to membrane focal adhesions. As its name suggests it has a role in cell matrix adhesion (368) but, via its translocation to the nucleus can also play an important role in cell proliferation (369). It also binds to and inhibits the tumour suppressor activity of p53 (369) which is mutated in at least 50% of all human cancers and has been shown to be reduced in schwannoma tumours (359). I show that phosphorylation of FAK at tyrosine 397 may be dependent on PrP^C expression as reducing PrP^C both with *PRNP* shRNA and TCS prion inhibitor 13 reduced activation of FAK at this phosphorylation site (Fig. 25C right and left panel, darker bars). Levels of total FAK protein are reduced using shRNA and at high levels of prion inhibitor treatment (Fig. 25C right and left panel, lighter bars).

Finally, levels of p53 which are very low in schwannoma are significantly increased using *PRNP* shRNA (Fig. 25D, left panel) and at high levels of TCS prion inhibitor 13 (Fig.25D, right panel).

3.4.5 PrP^C in schwannoma proliferation, survival and adhesion – Discussion

PrP^C has been shown to protect human neurons from Bax-mediated apoptosis (125) and from oxidative stress in neuroblastoma SH-SY5Y cells (113). Resistance to oxidative stress has been previously demonstrated in schwannoma and has been linked to high expression levels of JNK due to their role in suppressing the accumulation of superoxides in the mitochondria (370,371). My data show that treatment of schwannoma cells with PrP peptide is able to protect them from oxidative-stress-induced apoptosis (Fig. 23A) but not from Bax-mediated apoptosis (Fig. 23B) suggesting that the mechanisms via which PrP^C exerts its neuroprotective effects may differ between different cell types.

As well as influencing schwannoma pathological proliferation and survival, it appears that PrP^C also plays a role in schwannoma cell matrix adhesion as knockdown of *PRNP* expression leads to reduced numbers of adhered cells in culture (Fig. 24). There are several well-documented mechanisms via which PrP^C affects cell adhesion including N-CAM (372), integrin β1 (175), laminin (373), and E-cadherin (reviewed in (374)), proteins known to be expressed in Schwann cells, therefore it makes sense to see adhesive properties of schwannoma cells diminish as PrP^C levels decreased.

The pathways via which PrP^C signals to affect proliferation, survival and adhesion are plentiful, so I investigated only a handful of pathways which have been previously shown to be important for schwannoma development

(67,68,183,213,359,368,375-377). I show that PrP^C likely affects schwannoma cell proliferation by acting via several of these pathways; the PI3K/AKT pathway and its downstream effector cyclin D1 are all downregulated upon reduction of PrP^C (Fig. 25 A and C), two pathways previously linked and shown to contribute to cancer cell proliferation and tumourigenesis (378), also ERK1/2 appears to be regulated by PrP^C to contribute to increased schwannoma pathological proliferation.

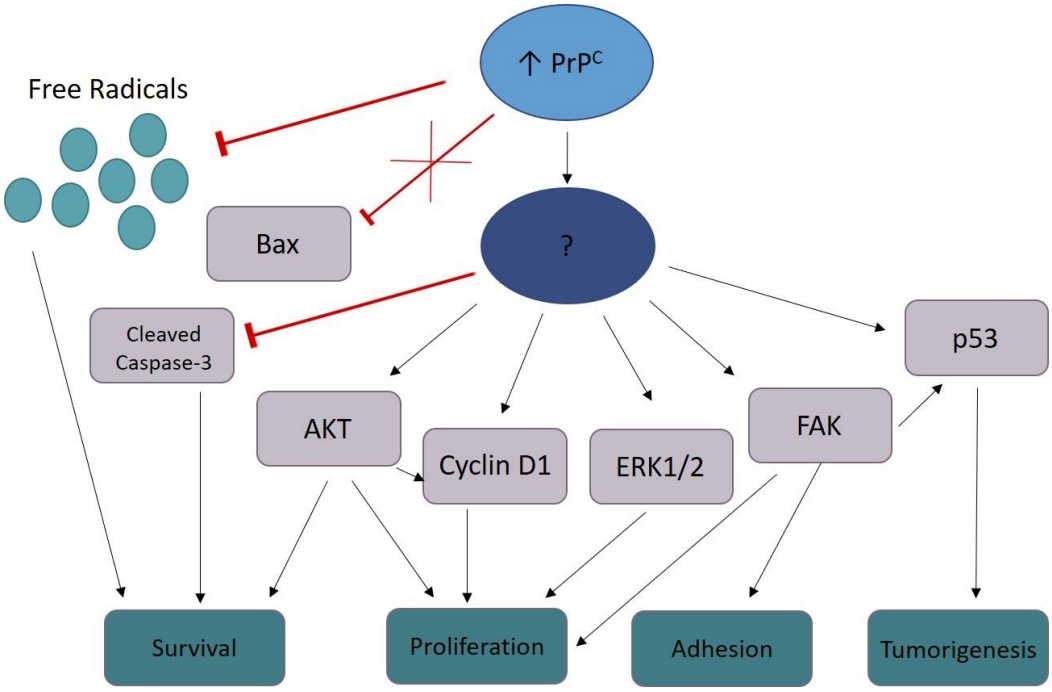

Figure 26. PrP^C acts via several key signalling pathways to promote schwannoma survival, proliferation, cell matrix adhesion and tumorigenesis.

My results show that PrP^C acts to enhance schwannoma survival by blocking the damaging effects of free radicals as well as causing a reduction in caspase-3 and an increase in AKT activation. PrP^C also acts via cyclin D1 and ERK1/2 to increase schwannoma proliferation and FAK, and p53 to increase cell matrix adhesion and tumorigenesis.

Proliferation of schwannoma cells was also likely affected by the reduced ERK1/2 signalling seen upon PrP^C knockdown and inhibition (Fig. 25B) and

also reduction of autophosphorylated FAK (Fig. 25D) known to be involved in cell proliferation and tumour development as well as adhesion (379). Levels of total ERK and FAK, but not AKT, were also significantly decreasued upon *PRNP* knockdown. This shows that there is reduced production of these proteins upon PrP^C depletion which may account for the reduction in phosphorylation seen, suggesting that PrP^C promotes the transcription of ERK1/2 and FAK. The fact that the same result is seen using TCS demonstrates that the reduction in total protein is not an off-target effect of the shRNA (Fig. 25B-D, light grey bars). Reduction of PrP^C likely reduced cell survival by decreasing signalling via the same PI3K/AKT pathway as well as increasing levels of pro-apoptotic cleaved caspase-3 (Fig. 22). Finally reduction of PrP^C affects increases levels of the tumour suppressor protein p53 (Fig. 25E) that is inherently decreased in schwannoma tumours due to FAK signalling leading to its proteasomal degradation (15,183). These results suggest that a treatment that focuses on reducing PrP^C in schwannoma would likely have significant impact in reducing schwannoma pathological proliferation, adhesion and survival (for overview see Fig. 26).

3.5 The effect of PrP treatment and overexpression in Schwann cells

3.5.1 Treatment with PrP increases proliferation, expression of c-Jun and alters Schwann cell morphology

To check the importance of PrP^C in schwannoma development I wanted to look at the effects of treating Schwann cells with the central portion of the PrP peptide (aa 105-120). My initial investigation was to see whether it would induce signalling pathways similar to those shown to be activated in schwannoma.

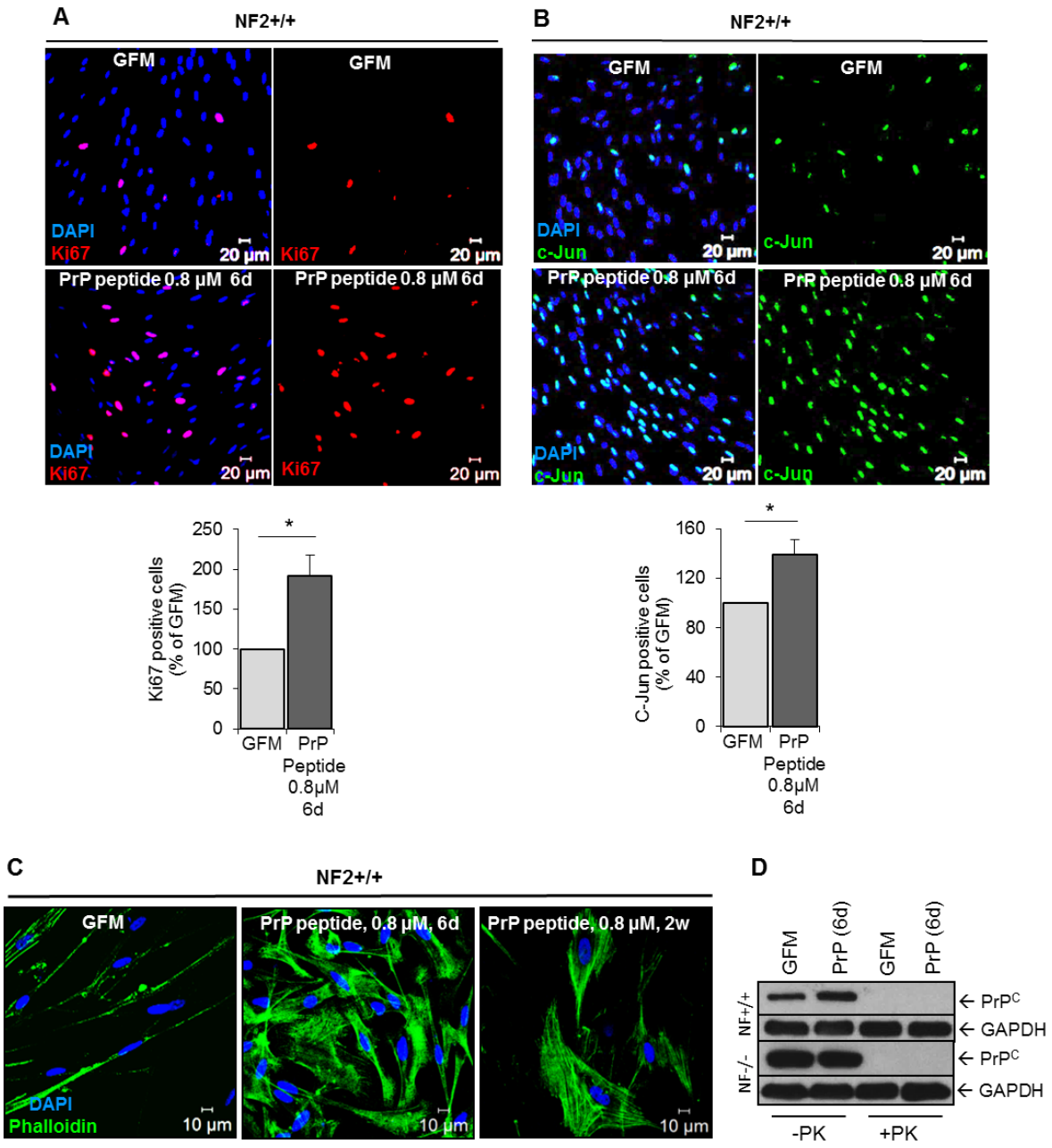

Figure 27. Long-term treatment of Schwann cells with PrP peptide increases proliferation and causes a schwannoma-like phenotype.

Treatment of Schwann cells with a synthetic human prion protein peptide (Abd Serotec) significantly increased the number of Ki67-positive cells (Ai and ii, n=3) and the number of cells staining positive for the master regulator of de-differentiation, c-Jun (Bi and ii, n=3). Long-term treatment of Schwann cells with the PrP peptide also altered cell morphology to that of a more schwannoma-like shape, visualised using immunostaining of cells with phalloidin (C). Long-term treatment of both Schwann and schwannoma cells with PrP peptide was insufficient to transform the PrP^{C} within these cells to PrP^{res} and render it resistant to degradation by proteinase K (PK) (D). *p<0.05. GAPDH was used as a loading control.

Due to extremely limited numbers of control human Schwann cells I looked only at the effects on proliferation (via Ki67 staining) and c-Jun, a proto-oncogene and marker of de-differentiation, shown to be overexpressed in schwannoma (380).

Results show that treating Schwann cells with PrP 105-120 peptide for six days significantly increases the number of Ki67-positive cells (Fig. 27A, red staining, bottom panel) when compared to untreated Schwann cells (Fig. 27A, top panel), cell counts have been quantified in Fig. 24Aii. Treatment of Schwann cells with PrP peptide also increases the number of cells staining positive for c-Jun (Fig. 27Bi) andthis difference is statistically significant (Fig.27Bii).

PrP 105-120 treated Schwann cells alters cell morphology after both six (Fig. 27C, central panel) and 14 days (Fig. 27C, right panel) and cells begin to lose their polarity, become more spread and show signs of altered actin cytoskeleton (phalloidin, green staining) compared to cells cultured only in GFM (Fig. 27C, left panel). The cell shape after treatment with PrP 105-120 is more similar to that of schwannoma cells with more membrane ruffles and increased cell spreading (Fig. 27C), suggesting that overexpression of PrP may be an early event in the pathogenesis of schwannoma tumours.

Finally, several papers have suggested long-term treatment of cells with PrP aa105-120 peptide can cause the PrP^{C} expressed to turn to PrP^{res} (381,382). Therefore, it was essential that we checked whether the PrP^{C} expressed by Schwann cells after PrP 105-120 treatment was still able to be degraded by proteinase K. I show that treatment with PrP 105-120 for six days both in Schwann and schwannoma cells does not affect the ability of PrP^{C} to be

degraded by proteinase K, nor does it affect the endogenous PrP^C levels within the cells (Fig. 27D).

3.5.2 Overexpression of PrP^C increases expression of cyclin D1 and c-Jun

To further back up the results found using the PrP 105-120 peptide, I made a *PRNP* overexpressing virus by cloning the coding section of *PRNP* into a pLenti6.2 vector that uses a CMV promoter to overexpress the gene (full structure of the plasmid can be seen in Fig. 8). I then continued to make lentiviral particles using 293FT cells that were able to effectively and stably deliver the *PRNP* overexpressing clone into primary human Schwann cells. I followed a lentiviral infection protocol to infect Schwann cells with the *PRNP* overexpressing virus before checking protein expression within cell lysates via Western blotting.

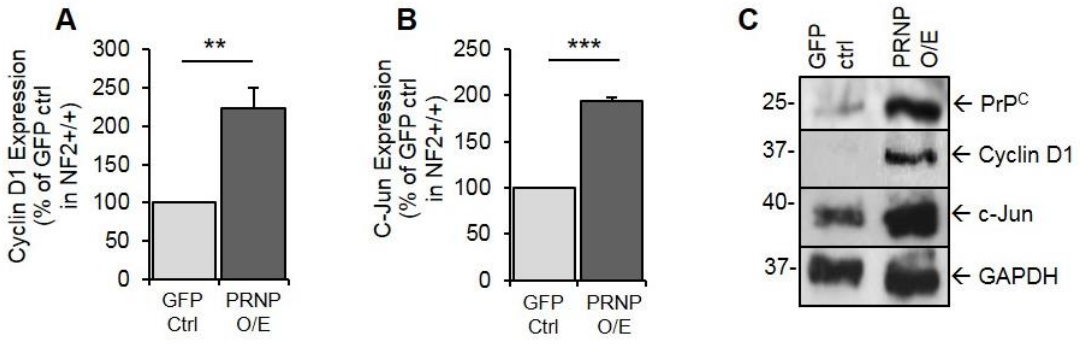

Figure 28. Overexpressing *PRNP* in Schwann cells leads to increased cyclin D1 and c-Jun expression.

Overexpression of *PRNP* in Schwann cells using a homemade *PRNP*-containing lentivirus significantly increased expression of both cyclin D1 (A and C, n=3) and c-Jun (B and C, n=3) compared to cells infected with scramble-control containing lentiviral particles, mirroring the effects seen with treatment using PrP peptide 105-120 (Fig. 27). **p<0.01, ***p<0.001. GAPDH was used as a loading control.

I show that overexpression of PrP^C in Schwann cells increases expression of cyclin D1 (Fig. 28A and C) a key marker of proliferation suggesting that the increase in proliferation of Schwann cells upon treatment with PrP 105-120 (Fig. 27A). This complements my earlier findings where *PRNP* knockdown in schwannoma caused a reduction of cell proliferation (Fig. 22A and G). Levels of c-Jun were increased (Fig. 28B and C) as demonstrated using the PrP peptide (Fig. 27B).

3.5.3 The effect of PrP treatment and overexpression in Schwann cells – discussion

In order to fully understand the effects of PrP^C overexpression in schwannoma I investigated the effects of overexpressing PrP^C in healthy Schwann cells (Fig. 28). I show upregulation of two key proteins known to be involved in schwannoma proliferation. Cyclin D1, a protein that marks the transition between G1 and S phases of the cell cycle, known to be overexpressed in schwannoma tumours alongside many other cancers (383) and Ki67 which is expressed in all active phases of cell division. Upregulation of both of these proteins upon overexpression of PrP^C confirms our earlier findings of PrP^C's role in schwannoma proliferation (Fig.22).

Moreover, upregulation of c-Jun, regulator of Schwann cell dedifferentiation and myelination, is seen in schwannoma (218) and thought to contribute to schwannoma development and pathology (384). The fact that overexpression and treatment of cells with PrP^C leads to overexpression of c-Jun (Fig. 27 and 28), suggests that PrP^C-induced upregulation of c-Jun upon Merlin loss may be

a key initiating stage in the development of schwannoma tumours. An overview of these findings can be seen in Fig. 29.

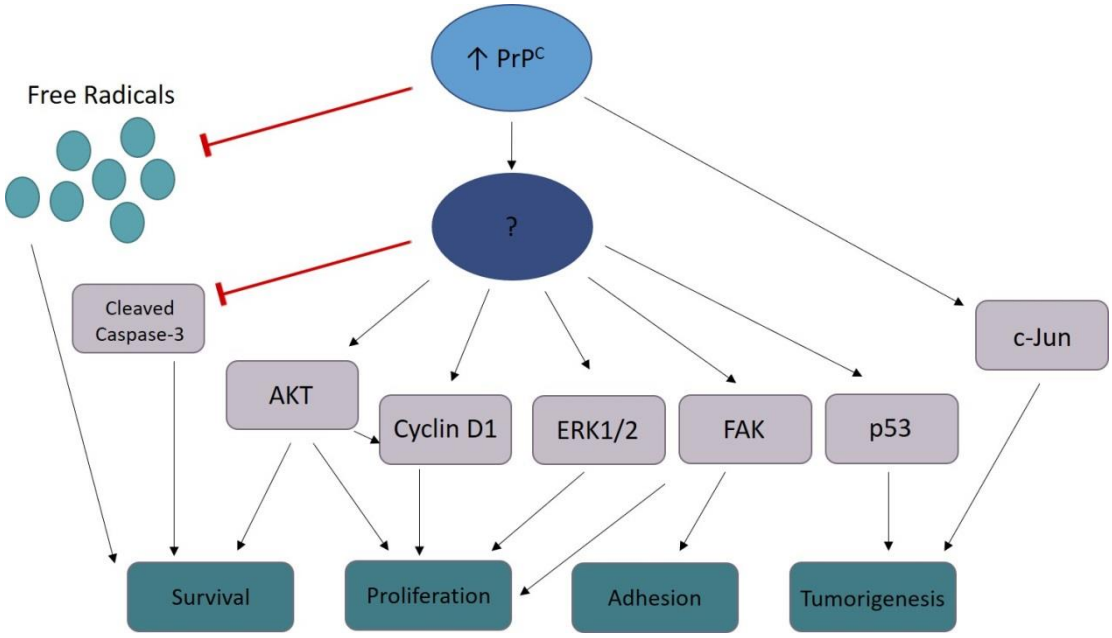

Figure 29. PrP^C also acts via c-Jun to increase proliferation and tumourigenesis in schwannoma cells.

As well as the previously discussed pathways (PI3K/AKT, FAK, cyclin D1 ERK1/2, cleaved caspase-3 and p53), it appears that c-Jun may also be regulated downstream of PrP^C in schwannoma and contribute to tumourigenesis and dedifferentiation in these cells.

3.6 LR/37/67kDa as a receptor for PrP^C in schwannoma

3.6.1 LR/37/67kDa is not differentially expressed between Schwann and schwannoma cells

Next, I looked at potential receptors via which PrP^C could interact with on the schwannoma cell membrane in order to cause the increases in proliferation, adhesion and cell survival that we have thus far demonstrated. Laminin receptor (LR/37/67kDa or LR) is a promising target as it has been shown previously in several different studies to act as a cell surface receptor for PrP^C (128,248). I first checked whether there was and differential expression of the LR/37/67kDa

between Schwann and schwannoma cells. I show that LR is expressed in both Schwann and schwannoma cells (Fig. 30A) but that expression of LR /37/67kDa does not significantly differ between the two cell types.

3.6.2 LR/37/67kDa co-localises and physically interacts with PrP^C in schwannoma

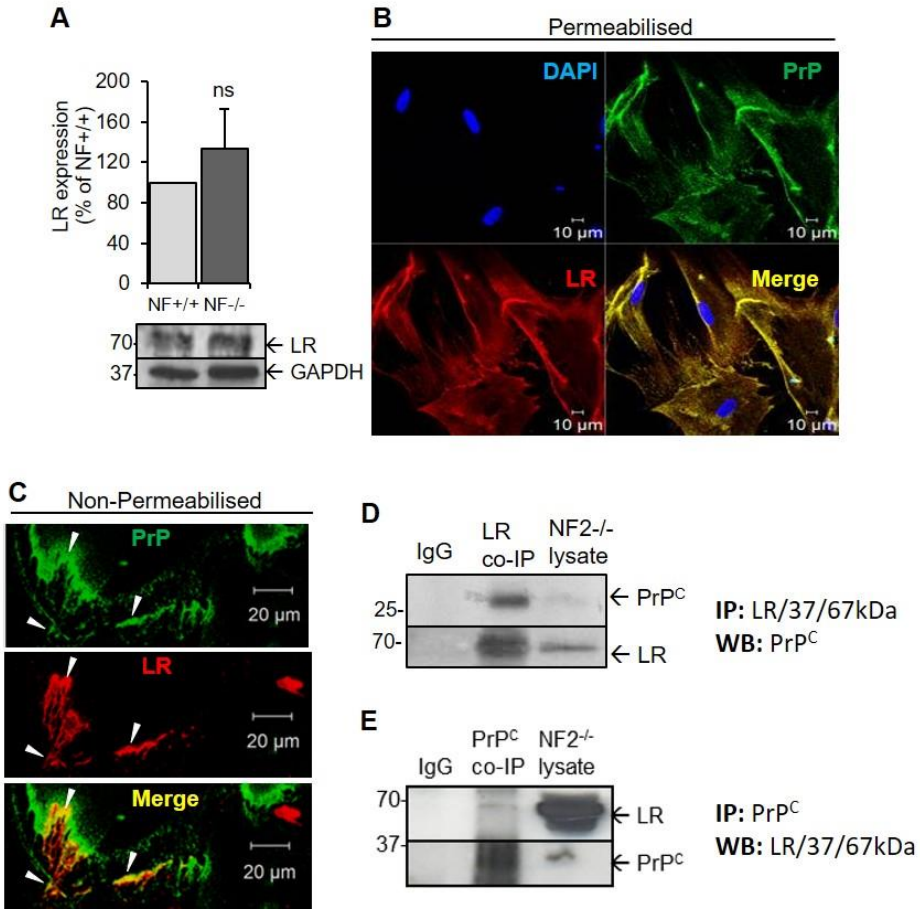

Figure 30. PrP^C physically interacts with LR/37/67kDa in schwannoma cells.

LR/37/67kDa (LR) is not differentially expressed between Schwann and schwannoma cells as seen using Western blotting (A, n=3). PrP^C and LR/37/67kDa co-localise both throughout the cytosol (B, n=3) and in membrane ruffles at the cell surface (C, white arrowheads, n=3). PrP^C and LR/37/67kDa directly interact in schwannoma seen by co-immunoprecipitation both when LR/37/67kDa is pulled down (D, n=5) and when PrP^C is pulled down (E, n=4). Ns $p>0.05$. GAPDH was used as a loading control.

Using immunofluorescence visualised with z-stack confocal microscopy and co-immunoprecipitation, I then looked at whether LR/37/67kDa and PrP^C co-localise and physically interact. It appears that PrP^C and LR/37/67kDa do localise together intracellularly throughout the cytoplasm (Fig. 30B) and in membrane ruffles on the cell membrane of schwannoma cells (Fig. 30C, white arrows).

Upon immunoprecipitation of laminin receptor PrP^C was also pulled down (Fig. 30D) and, likewise, upon immunoprecipitation of PrP^C I also found LR/37/67kDa in complex (Fig. 30E), suggesting a physical interaction between PrP^C and LR/37/67kDa in schwannoma that may be as a receptor-ligand pairing.

3.6.3 Knockdown of LR/37/67kDa significantly affects the ability of PrP^C to activate several key signalling pathways

Having shown that PrP^C and LR/37/67kDa interact together in schwannoma cells, I wanted to ascertain whether LR/37/67kDa does indeed act as a receptor for PrP^C in these cells. To do this I knocked down *RPSA*, the mRNA coding for LR/37/67kDa before stimulating the cells with synthetic human PrP aa105-120 (the same PrP peptide as used in previous experiments). I show that treatment with PrP peptide increases cyclin D1 levels and activation of ERK1/2, AKT and FAK (Fig. 31A-E. light grey bars). Upon knockdown of LR/37/67kDa levels of cyclin D1 and activation of ERK1/2, AKT and FAK are not affected but stimulation with PrP peptide no longer leads to activation of these pathways or increased expression of cyclin D1 (Fig. 31A-E, dark grey bars). This suggests that PrP^C acts via laminin receptor to stimulate ERK1/2, PI3K/AKT and FAK signalling and increase expression of cyclin D1, and could lead to pathological proliferation, adhesion and survival seen in schwannoma cells.

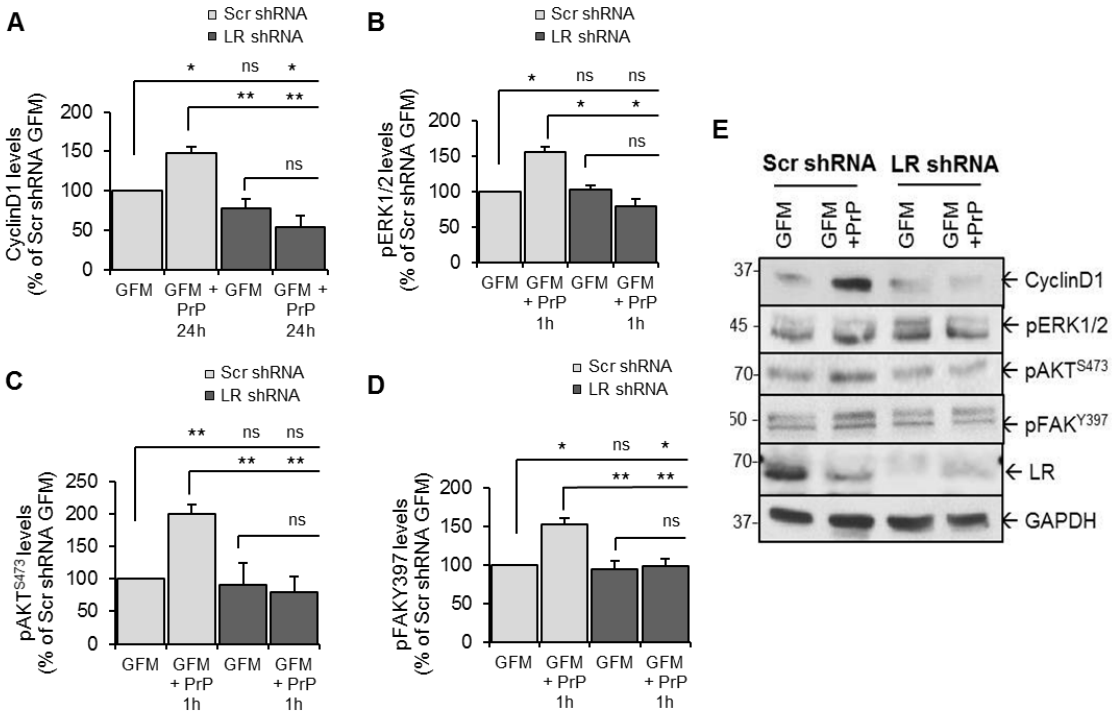

Figure 31. PrP^{C} signals via LR/37/67kDa to activate key signalling pathways in schwannoma.

Upon treatment with PrP peptide (72h) levels of cyclin D1, pERK1/2, pAKT^{Ser473} and pFAK^{Tyr397} are significantly increased (A-D, light grey bars). Knockdown of LR/37/67kDa using shRNA prevented this increase in expression upon treatment with PrP peptide (A-D, dark grey bars) suggesting that LR/37/67kDa is key for PrP^{C}'s activation of downstream signalling pathways. Western blot images are combined in (E, n=5). Ns p>0.05, *p<0.05, **p<0.01. GAPDH was used as a loading control.

3.6.4 LR/37/67kDa as a receptor for PrP^{C} – discussion

Although there appears to be no significant difference in LR/37/67kda expression between Schwann and schwannoma cells (Fig. 30A) there appears to be an important role for this receptor regarding signalling of PrP^{C}. Literature has shown in other cancer cell types that LR/37/67kDa acts as a cell surface receptor for PrP^{C} including in neuroblastoma N2a cell line (128) thus fuelling

the investigation to see whether it plays the same role in schwannoma. PrP^C and LR/37/67kDa both co-localise and physically interact in schwannoma cells (Fig. 30B-E) as shown by both immunofluorescence and co-immunoprecipitation suggesting, as previously shown, that PrP^C may signal via LR/37/67kDa in schwannoma cells.

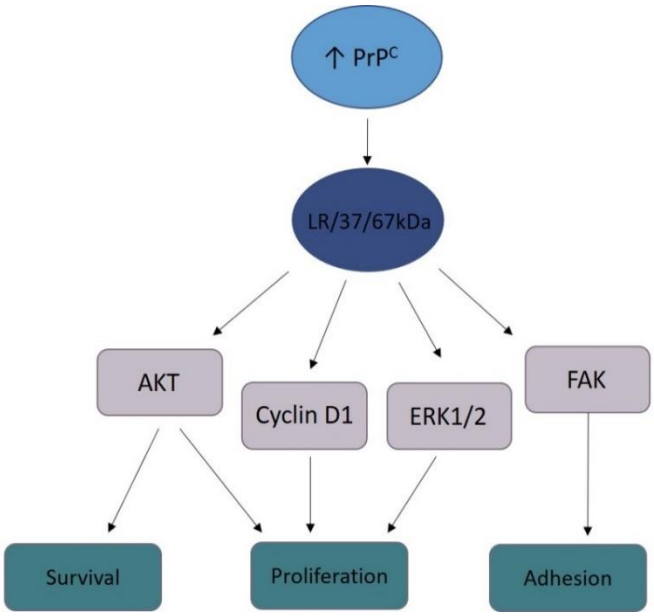

Figure 32. PrP^C signals via LR/37/67kDa in schwannoma cells to activate AKT, cyclin D1, ERK1/2 and FAK pathways.

Data show that PrP^C and LR co-localise and physically interact in schwannoma cells and that knockdown of LR/37/67kDa affects the ability of PrP^C to activate AKT, cyclin D1, ERK1/2 and FAK pathways. Taken together this suggests that LR/37/67kDa acts as a receptor for PrP^C in schwannoma cells.

Moreover, reducing expression of LR/37/67kDa reduced the ability of PrP to activate several key signalling pathways involved in schwannoma development (Fig. 31), namely Cyclin D1, ERK1/2, AKT and FAK, which strongly suggests that LR/37/67/kDa acts as the receptor for PrP^C in schwannoma cells and may be the mechanism via which extracellular or membrane-bound PrP^C signals to induce schwannoma proliferation, survival and cell adhesion (see Fig. 32).

There are several drugs targetting LR/37/67kDa that have been discovered; for example NSC47924 that disrupt the interaction between PrP^C and LR/37/67kDa (385) or monoclonal antibodies raised against PrP^C aimed at inhibiting its interactions (386). Further work would have to be done to elucidate whether any of these drugs would be effective in reducing schwannoma proliferation, adhesion and survival and to check whether the PrP^C released in exosomes is able to stimulate signalling via LR/37/67kDa.

3.7 P-glycoprotein in schwannoma

A recent study linked p-gp expression and PrP^C in cancer cells, showing a co-localisation and interaction between these two proteins (264). Moreover, as expression of *PRNP* has been correlated to poor prognosis and decreased drug sensitivity in some cancer types (387), it has actually been linked to improved survival in other cancer types (388). As of yet, no drug resistance studies have been carried out in schwannoma and this interesting link between p-gp and PrP^C warrants further investigation.

3.7.1 Different p-gp and CD44 antibodies identify different patterns of p-gp expression in schwannoma cells

Similarly to the PrP^C antibodies (Fig. 9), I had to use different antibodies to p-gp and CD44 depending on the experimental technique used. After several experiments I discovered that, whereas all CD44 antibodies showed very similar staining patterns, different p-gp antibodies showed very different immunocytochemical staining (Fig. 33). One antibody raised to aa1-50 of p-gp displayed nuclear staining of p-gp whereas a p-gp antibody raised to a part of

the protein located on the third extracellular loop did not show much nuclear staining and instead showed p-gp to be localised more the the cytosol.

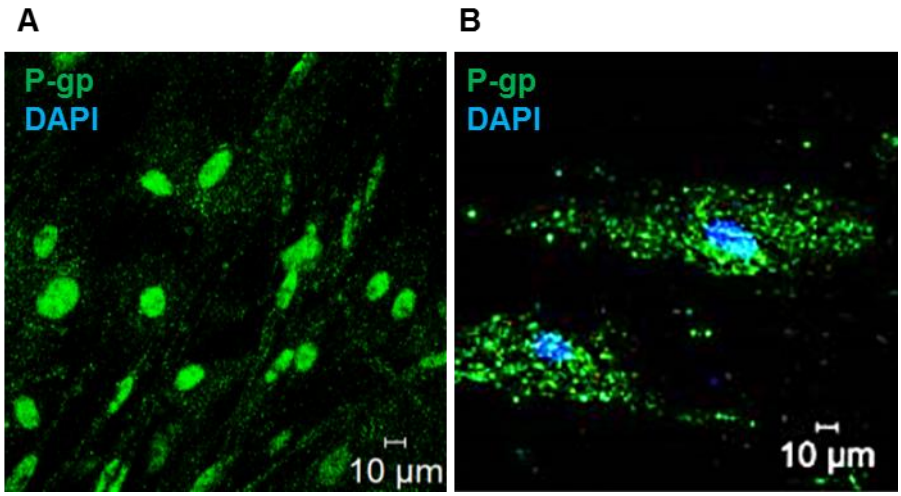

Figure 33. Different p-gp antibodies show different staining patterns.

Two different p-gp antibodies used in various experiments show very different staining patterns in schwannoma cells. The rabbit antibody from Abcam (A, aa1-50 of p-gp) shows mainly nuclear staining with only slight cytoplasmic p-gp expression whereas the mouse antibody from Sigma (third extracellular loop) shows mainly cytoplasmic staining with only a small amount of p-gp localisation in the nucleus (B).

Table 5 outlines the various different p-gp and CD44 antibodies used, the applications in which they were used and their staining pattern, based on immunofluorescent tests. Once again, any antibodies used for experiments other than those outlined below will be described next to the appropriate results.

Table 5: Table of p-gp and CD44 antibodies used and their staining pattern

Experiment	Antibody	Recognition site	Observations
IP (as bait)	P-gp (Abcam)	aa1-50 of human p-glycoprotein	Nuclear staining determined by ICC
	HCAM (CD44, Santa Cruz)	Exon v6 in variable region of human CD44	Cytosolic and membrane staining in ICC
IP (blot)	P-gp (Abcam)	aa1-50 of human p-glycoprotein	Nuclear staining determined by ICC
	CD44 (Novus Biologicals)	Central region (unspecified)	Membrane, cytosol and small amount of nuclear staining in ICC
ICC/ Immuno-fluorescence	P-gp (Abcam (R))	aa1-50 of human p-glycoprotein	Nuclear with only a little cytosolic staining
	P-gp (Sigma (M))	3rd extracellular loop	Mainly cytosolic, some membrane and nuclear staining
	HCAM (CD44, Santa Cruz (M))	Exon v6 in variable region of human CD44	Cytosolic and membrane staining
	CD44 (Novus Biologicals (R))	Central region (unspecified)	Membrane, cytosol and some nuclear staining
IHC	P-gp (Sigma (M))	3rd extracellular loop	Mainly cytosolic, some membrane and nuclear staining.

3.7.2 P-gp is over-expressed and localises to the nucleus of schwannoma cells

Using a blocking peptide alongside a p-gp-specific antibody (anti-p-glycoprotein, Sigma) I show that there are several isoforms of p-gp present in schwannoma (20kDa, 35kDa, 57kDa, 72kDa and 85Kda) but the main, fully glycosylated 170kDa isoform does not appear to be present (Fig. 34A). I show, using Western blotting (Fig. 34B) that most isoforms of p-gp are more highly expressed in schwannoma compared to Schwann cells, part from the 72kDa isoform.

This is mirrored in immunocytochemistry where not only is it clear that p-gp is more highly expressed in schwannoma (Fig. 34C, right panels) compared to Schwann cells (Fig. 34C, left panels) but also that, in schwannoma, p-gp is localised mainly to the nucleus. This has previously been described as being indicative of drug-resistance as it is seen in drug-resistant cells to protect the nucleus from doxorubicin and other chemotherapeutic agents (389). This is also seen using immunohistochemistry where in normal nerve sections the nuclei are mostly unstained (blue in colour, Fig. 34D, top panel, blue arrows) whereas, in schwannoma tumours, many of the nuclei are also stained brown with DAB, depicting the presence of p-gp (Fig. 34D, bottom panel, brown arrows).

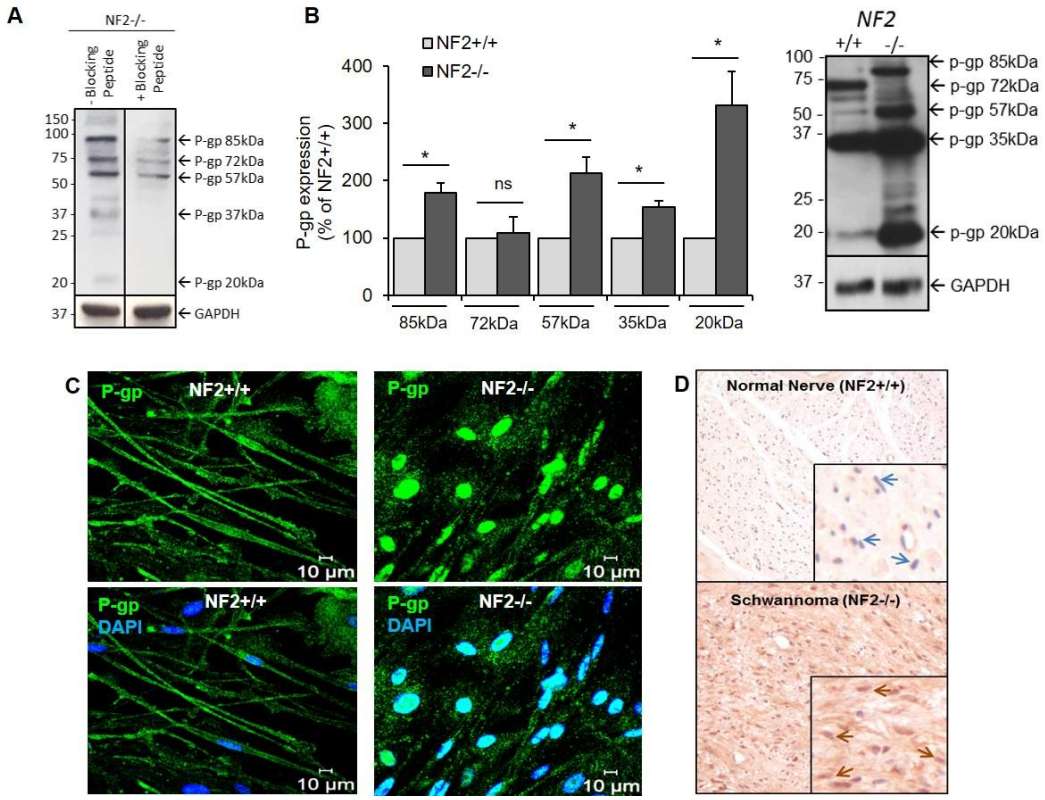

Figure 34. P-gp is overexpressed and localises to the nucleus of schwannoma cells.

P-gp blocking peptide was used to determine which of the multiple p-gp Western blot bands are specific as the main 170kDa isoform is not present. Blocking peptide experiments demonstrate only 20kDa, 35kDa, 57kDa, 72kDa and 85kDa to be specific. All p-gp isoforms apart from 72kDa are overexpressed in schwannoma (NF2-/-) compared to Schwann cells (NF2+/+) (B, n=3). Overexpression of p-gp in schwannoma can also be visualised using immunofluorescence (C, n=3), where it is apparent that the p-gp in schwannoma localises mainly to the nucleus and weakly to the cytosol (C, NF2-/-, right panels). Immunohistochemical staining of normal nerve and schwannoma sections also shows increased p-gp expression in schwannoma (D, bottom panel) compared to normal nerve (D, top panel). IHC also demonstrates more p-gp in the nucleus as well as cytosol of schwannoma (D, brown arrows) compared to normal nerve (D, top panel, blue arrows). Ns $p>0.05$, *$p<0.05$. GAPDH was used as a loading control for Western blots.

3.7.3 P-gp overexpression in schwannoma is Merlin-dependent

I show that the overexpression of p-gp seen in schwannoma is due to loss of Merlin as reintroduction of the *NF2* protein decreases expression of many of the isoforms of p-gp seen in schwannoma (Fig. 35A) apart from the 85kDa band. These results suggest a potential endogenous or intrinsic drug resistance of schwannoma tumours that may help explain why they are unable to be targeted using traditional chemotherapeutic agents but also flags up as a warning that any future drug could cause further drug resistance in these cells.

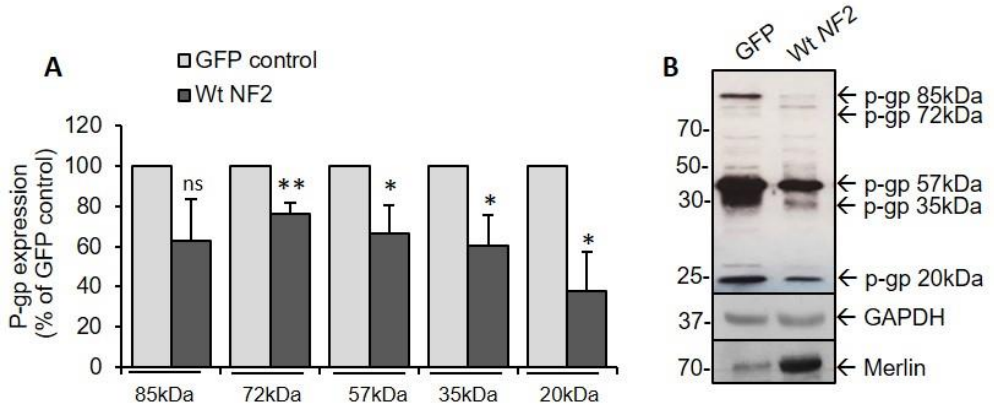

Figure 35. P-gp expression in schwannoma is Merlin-dependent.

Adenoviral reintroduction of the *NF2* protein into schwannoma cells leads to a significant increase in the expression of all p-gp isoforms (72kDa, 57kDa, 35kDa and 20kDa) but not the 85kDa isoform (A, n=3). Western blot bands can also be visualised (B). Ns $p>0.05$, *$p<0.05$. GAPDH was used as a loading control.

3.7.4 LR/37/67kDa and p-glycoprotein co-localise in schwannoma but not Schwann cells

There is evidence to suggest a role for LR/37/67kDa in multi-drug resistance (390), particularly as the laminin receptor precursor LRP, also known as MGr1-Ag, an MDR-associated protein, is involved in adhesion-mediated drug

resistance through activation of several key pathways I show to be activated by PrP^C/LR interaction in schwannoma including FAK/PI3K and MAPK (269). I show, in schwannoma, that LR/37/67kDa seems to co-localise with p-gp in membrane ruffles (Fig. 36B). I also demonstrate the absence of this co-localisation between p-gp and LR/37/67kDa in human primary Schwann cells in culture (Fig. 36A), suggesting that this interaction with LR could be acquired by p-gp upon loss of Merlin from the cells.

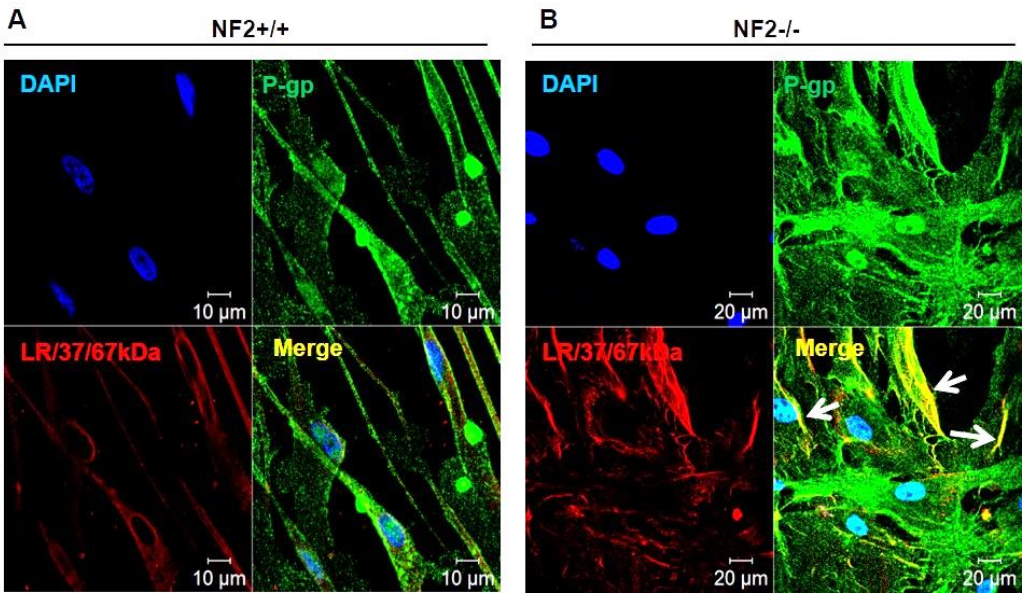

Figure 36. P-gp co-localise with LR/37/67kDa in schwannoma but not Schwann cells.

Immunofluorescent staining shows that p-gp (Sigma) and LR/37/67kDa do not co-localise in Schwann cells (A, NF2+/+, n=3) but do co-localise in membrane ruffles of schwannoma cells (B, NF2-/-, white arrows, n=3), suggesting a possible interaction between the two proteins. DAPI was used to visualise nuclei.

To confirm it I would need to reintroduce Merlin into schwannoma cells and again check for p-gp and LR/37/67kDa interaction as well as using co-

immunoprecipitation to determine whether a physical interaction between LR/37/67kDa and p-gp takes place in schwannoma cells.

3.7.5 P-gp in schwannoma – Discussion

PrP^C overexpression has been linked to multi-drug resistance (MDR) in several different cancer types including gastric cancer (184) and has been referred to as an MDR-associated protein via its interaction with the major MDR protein p-gp (264). Interestingly, there are also links between LR/37/67kDa and drug resistance; as laminin receptor precursor (LRP) is also known as MGr1-Ag, an MDR-associated protein that has been shown to interact with PrP^C (268). This evidence suggests a role for both LR/37/67kDa and PrP^C in MDR. MGr1-Ag promotes cell adhesion-mediated drug resistance via interaction with laminin (LA) which, in turn, results in Bcl-2 upregulation and thus protection from chemotherapy-induced apoptosis (391).

I first of all wanted to explore the possibility of intrinsic MDR in schwannoma cells before going on to investigate whether any drug-resistance could be caused by an interaction of PrP^C with LR/37/67kDa or not. Initially I investigated endogenous levels of the key MDR protein p-glycoprotein between Schwann and schwannoma cells and show, using a variety of methods, that p-gp expression is upregulated in schwannoma but at various isoforms and not the complete 170kDa peptide (Fig. 34). I speculate that the upregulation of p-gp in schwannoma could be the reason that drug interventions fail in these patients, for example in a recent study investigating the treatment of NF2 with Bevacuzimab, a VEGFR inhibitor (392). By targeting p-gp expression in schwannoma it would be interesting to see whether these tumours become more susceptible to drug treatments.

I show that overexpression of p-gp in schwannoma is linked to loss of Merlin as re-introduction and overexpression of the *NF2* gene leads to a significant reduction in the majority of the isoforms of p-gp (Fig. 35). No link between the expression of these two proteins have been shown before, this could prove interesting to investigate within more malignant NF2-related tumours including grade III meningiomas (393), for which chemotherapy is sometimes used as a last resort treatment option when resection or radiosurgery are inefficient, although itself is not very effective (394). Finally, in this section, I show a potential interaction, between LR/37/67kDa and p-gp in schwannoma (Fig. 36) as previously predicted by the fact that LRP/MGr1-Ag is a MDR-associated protein. This relationship requires further investigation as immunofluorescence cannot confirm direct interaction but it could suggest that PrP^C may act via LRP/LR/37/67kDa to affect MDR in schwannoma cells, a very tentative prediction at this stage but something that could greatly impact schwannoma therapeutics and provide an even bigger role for PrP^C/ LR/37/67kDa interaction inhibitors in schwannoma.

3.8 Control of p-glycoprotein expression by PrP^C

Using immunofluorescence visualised by z-stack confocal microscopy, I show that there is a slight peri-nuclear co-localisation between PrP^C (Santa Cruz,N-T antibody) and p-gp (Abcam aa1-50 of human p-glycoprotein) in schwannoma cells (Fig. 37 A) However, no apparent interaction between PrP^C and p-gp in schwannoma was found using immunoprecipitation and pull down of PrP^C (Fig. 37B).

Although this seem to rule out any direct interaction between the two proteins, at a perinuclear location, there could be an indirect mechanism by which PrP

and p-gp interact that is yet to be elucidated. Additionally, I will need to repeat co-IP experiments using antibodies targeting the third extracellular loop of p-gp and the N-terminal of PrP which I show detect both proteins at the membrane and the cytosol (Fig. 9 and Fig. 33). A co-IP using antibodies targeting the aa1-50 of p-gp and C-terminal of PrP both detecting nuclear proteins would also need to be carried out in order to fully understand whether PrPC/p-gp interact in schwannoma cells.

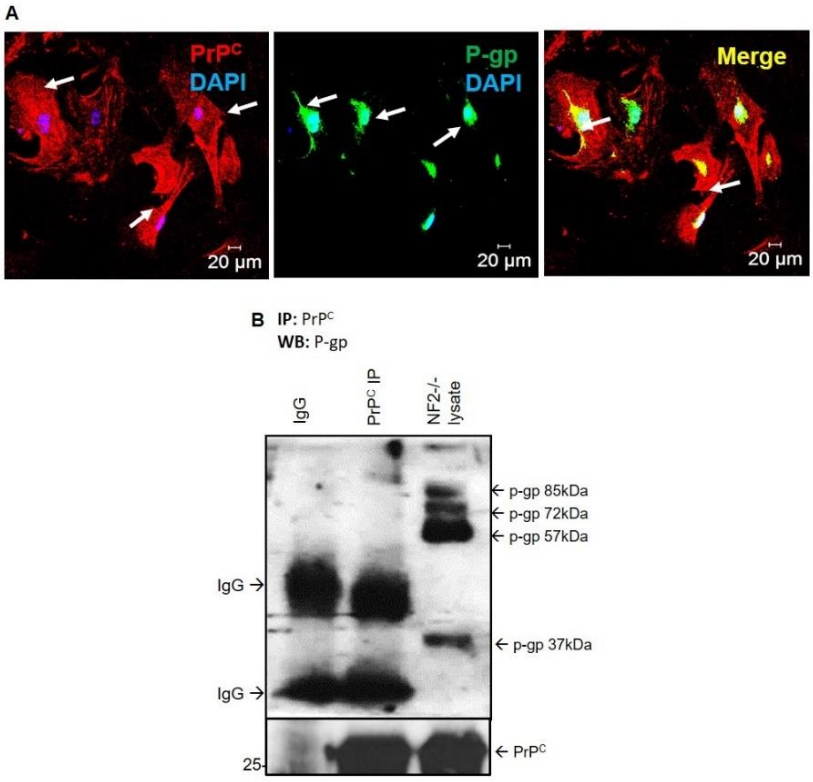

Figure 37. PrP^C and p-gp co-localise in the peri-nuclear region of schwannoma cells but appear not to directly interact.

Immunofluorescent staining of schwannoma cells with both p-gp (Abcam) and PrP^C (Santa Cruz) suggests a small amount of co-localisation in the peri-nuclear region of schwannoma cells between these two proteins (A, right hand panel, white arrows, n=3). However, upon immunoprecipitation of PrP^C, p-gp was unable to be detected (B, n=3).

3.8.1 PrP^C regulates p-gp expression in schwannoma cells

Initially I used the *PRNP* overexpressing virus from previous experiments to look at the effects of overexpressing *PRNP* on p-gp levels in Schwann cell cultures. I found that overexpressing PrP^C led to an increase in expression of most p-gp isoforms but strangely led to a decrease in levels of p-gp 72kDa (Fig. 38A).

I then performed the reverse experiment, treating schwannoma cells with both *PRNP* shRNA (Fig. 38B) and TCS prion inhibitor 13 (Fig. 38C) before checking p-gp expression. I show that both *PRNP* shRNA (Fig. 38B) and TCS prion inhibitor 13 (Fig. 38C) decrease levels of p-gp in schwannoma cells using Western blotting, again with some variability in the effect on specific isoforms.

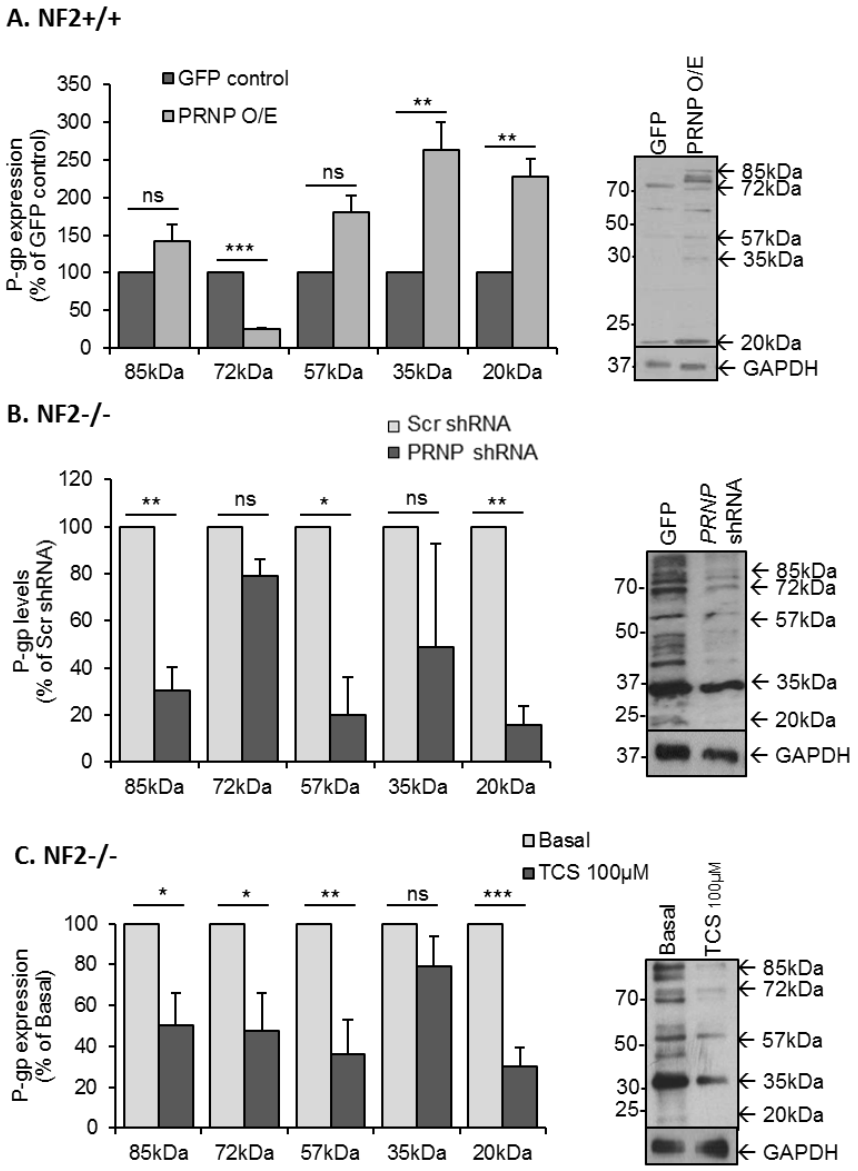

Figure 38. P-gp expression is regulated by PrP^{C} in schwannoma cells.

Overexpressing PrP^{C} in Schwann cells leads to increased expression of the majority of p-gp isoforms, although expression of p-gp 72kDa is significantly *reduced* (A, n=3). Knockdown of PrP^{C} in schwannoma leads to an overall reduction in p-gp expression although, again, not all isoforms appear to be affected in the same way with the 72kDa isoform again not producing a significant result (B, n=5). Reduction of PrP^{C} using TCS prion inhibitor leads to a significant reduction in all p-gp isoforms (C, n=4). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. GAPDH was used as a loading control.

3.8.2 Control of p-glycoprotein expression by PrP^C – discussion

Previous research has suggested that PrP^C and p-gp act together in complex within multidrug resistant cells to promote invasion and resistance to apoptosis of breast cancer cells in culture (264). Preliminary experiments show that there is a slight perinuclear co-localisation between p-gp and PrP^C in schwannoma and no direct interaction using co-IP, thus, further work needs to be done with immunofluorescence to denote whether C-terminal PrP^C co-localises with nuclear p-gp and whether cytosolic PrP^C and p-gp interact using the various antibodies denoted in Table 6. Furthermore, a co-IP specific p-gp antibody to nuclear and cytosolic p-gp needs to be used and checked against both C-terminal and N-terminal PrP^C antibodies. This will allow me to fully understand the complex interaction between these proteins and their many variants.

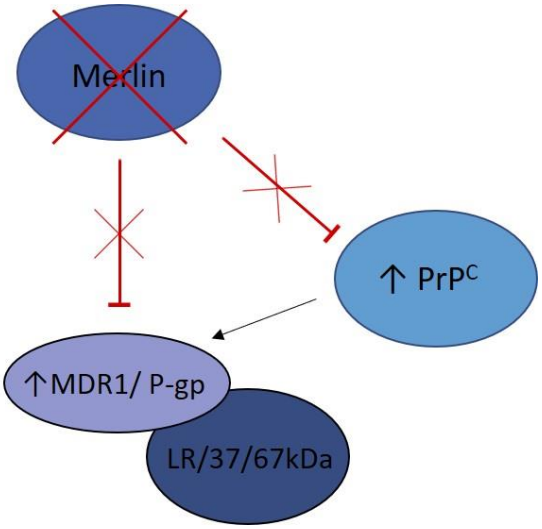

Figure 39. Merlin and PrP^C modulate p-gp overexpression in schwannoma cells.

Results show that p-gp is overexpressed in schwannoma in a Merlin-dependent manner and that p-gp interacts physically with LR/37/67kDa, a known receptor for PrP^C. The high levels of PrP^C in schwannoma also appear to lead to the overexpression of p-gp seen in these cells. Targeting PrP^C may in schwannoma patients produce a secondary effect of contributing to a reduction in p-gp expression which could be beneficial in a clinical setting.

However, it does appear that p-gp expression is dependent on PrP levels as overexpression of PrP^C in Schwann cells led to increased p-gp expression (Fig. 38A) and knockdown of *PRNP* had the opposite effect (Fig. 38B and C). This provides further evidence for PrP^C as a potential transcriptional regulator both upstream and downstream of several key signalling pathways involved in both tumourigenesis (ERK1/2, AKT, FAK, p53) and MDR (p-gp).

72kDa p-gp is the predicted size of transmembrane domains 1 and 2 of p-gp that are de-glycosylated with endoglycosylase H, an enzyme that removed high mannose and oligosaccharide chains from N-linked glycoproteins (395) but there is little in the literature to prove any significance of this portion of the protein in cancer development. The protein picked up at 72kDa may, in fact, actually be breast cancer resistance protein (BCRP) which has been shown to be overexpressed alongside p-gp in drug-resistant triple negative breast cancer cells (396). This is only speculative, further experiments would be required to determine whether BCRP is expressed in schwannoma and, if so, whether it is picked up by p-gp antibody in these cells.

3.9 CD44, the magical linker between PrP^C and p-gp?

3.9.1 CD44 is overexpressed in schwannoma and meningioma cells in a Merlin-dependent manner

CD44 and its variant isoforms have been shown to be overexpressed in many different tumour types including being overexpressed in schwannomas compared to Schwann cells (296). Firstly I checked the glycosylation state of CD44 present in schwannoma cells to assess whether there was any deficiency

in proper glycosylation that could account for some of the pathological features of schwannomas; CD44 glycosylation has an important role in cell adhesion, particularly via its binding partner hyaluronan (HA) (286), interesting data has shown that inhibition of CD44 glycosylation actually enhances HA binding (397). Glycosylation of CD44 is also essential in controlling the switch between 'active' and 'inactive' forms of CD44, making it a key post translational modification for this protein (398).

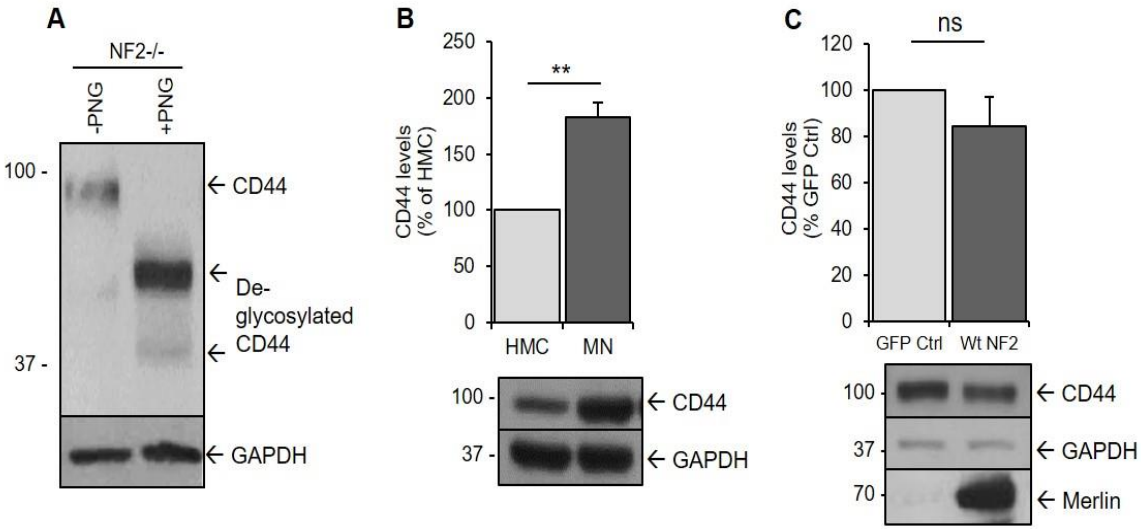

Figure 40. CD44 is overexpressed in meningioma as well as schwannoma but its expression is not Merlin-dependent.

CD44 is in its fully di-glycosylated state in schwannoma cells as shown using PNGaseF de-glycosylation assay (A, n=3). CD44 is also overexpressed in primary, Merlin-negative, grade I meningioma cells compared to HMC control (B). Upon adenoviral reintroduction of Merlin into schwannoma cells, there is no significant difference in CD44 expression compared to GFP control virus (C, n=3). Ns $p>0.05$, $^{**}p<0.01$. GAPDH was used as a loading control.

Results confirm that the CD44 present in schwannoma cells appear to be in a glycosylated state seen by two separate de-glycosylated bands upon treatment with PNGaseF (Fig. 40A). In the literature, there is evidence of a minimum of 5

N-linked glycosylation sites (286) suggesting that although the CD44 may only be partially glycosylated in schwannoma. This lack of full glycosylation may influence and participate in the enhanced schwannoma cell matrix adhesion as shown previously (58).

Next, I wanted to look at whether CD44 was differentially expressed in other NF2-related tumours, primarily between HMC and grade I, Merlin-negative meningiomas. CD44 expression (both standard and several variant isoforms) in meningiomas has previously been described as ‘weak’ in immunohistochemical staining of tumour sections (399). However, in our cell culture model, I show that there is a significant increase in CD44 expression (approximately 1.5-2-fold) in Merlin-deficient Grade I meningiomas compared to the HMC control cells (Fig. 40B).

To investigate the notion as to whether CD44 overexpression in NF2-related tumours is Merlin-dependent I used adenoviral infection to reintroduce and overexpress the *NF2* gene in schwannoma cells. I show that there is no significant difference in CD44 expression between schwannoma cells infected with a GFP-control compared to those infected with the NF2-containing virus (Fig. 40C) thus, allowing the conclusion that the overexpression of CD44 in schwannoma is not Merlin-dependent. This contradicts previous work that show knockdown of Merlin in Schwann cells leading to an upregulation of CD44 [300]. The same experiment would need to be carried out in the future with primary human meningioma grade 1 cells in order to fully understand these results.

3.9.2 CD44 partly co-localises and interacts with serveral p-gp isoforms in schwannoma

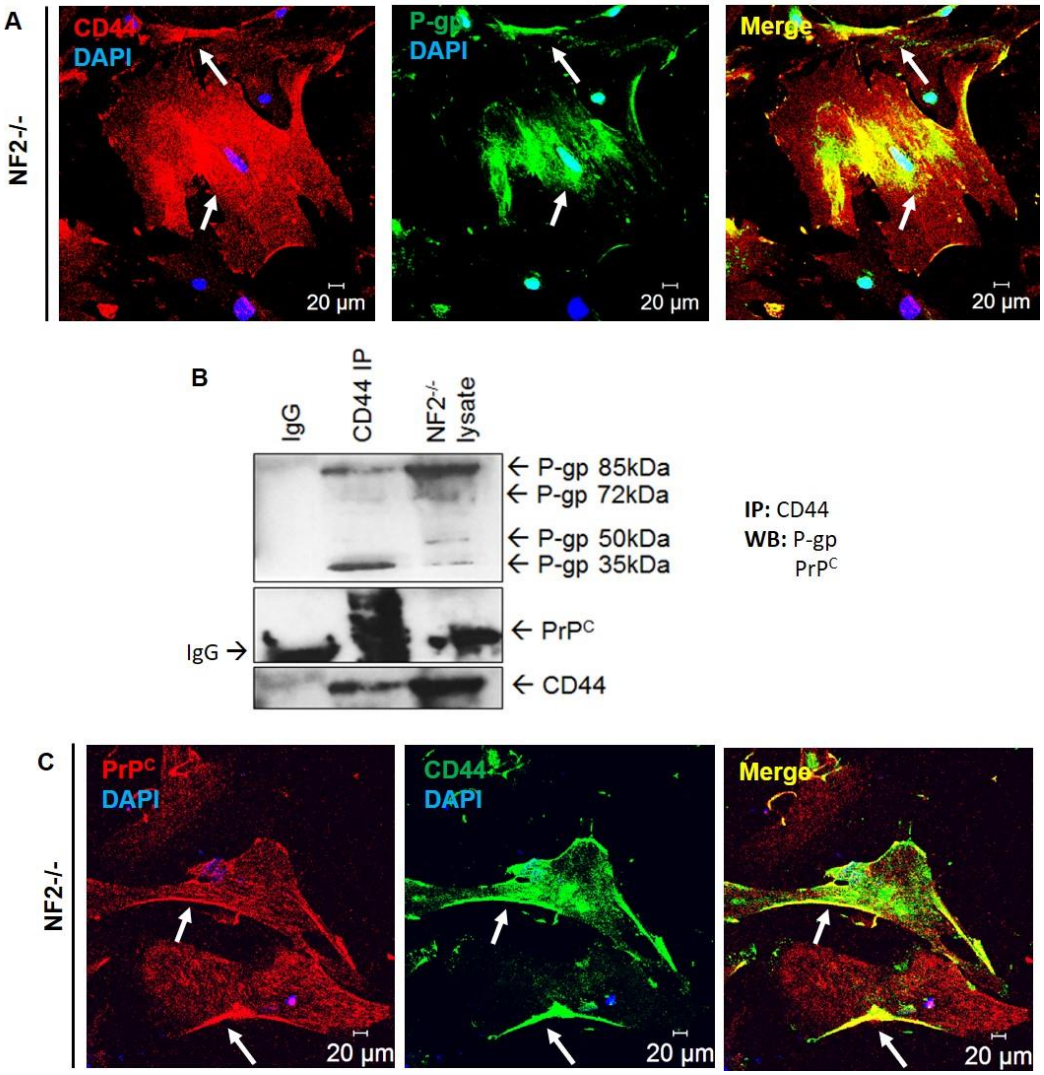

Figure 41. CD44 interacts with PrP^C and with several p-gp isoforms in schwannoma cells.

Immunofluorescent z-stack technique shows co-localisation of CD44 and P-gp (Abcam) in schwannoma both at the membrane and peri-nuclear (A, white arrows, n=3). CD44 immunoprecipitation demonstrated a physical interaction between CD44 and PrP^C (B, n=3) which was also seen using immunofluorescence, z-stack confocal microscopy particularly at membrane ruffles (C, n=2, white arrows).

To check whether CD44 could be an interaction mediator between p-gp and PrP^C I investigated whether CD44 and p-gp interact in schwannoma cells. Using

immunofluorescence, I show that CD44 and p-gp co-localise in the peri-nuclear region and in membrane ruffles of schwannoma cells upon visualisation using z-stack confocal microscopy (Fig. 41A).

Pull down of CD44 showed interaction with only two of the bands normally seen for p-gp in schwannoma – 35kDa and 85kDa (Fig. 41B), however, pull down of p-gp was unsuccessful, thus the results were unable to be confirmed in both directions. This suggests an interaction between CD44 and some p-gp isoforms within schwannoma cells.

3.9.3 PrP^C and CD44 physically interact in schwannoma cells

I next wanted to investigate whether CD44 interacted with PrP^C to see whether PrP^C could interact indirectly with p-gp via a CD44-containing complex. CD44/PrP^C interaction has been demonstrated in several other malignant cancer cells (319) but never in schwannoma. Using immunoprecipitation, it is clear to see bands for PrP^C in the lysate and also in the CD44 IP, suggesting a direct interaction between these two proteins (Fig. 41B, n=3). This gives further evidence to my hypothesis that PrP^C may be in complex with both CD44 and p-gp in schwannoma, something suggested in previous reviews to occur in other breast cancer and cancer stem cells (CSC's) (400). To further evidence this claim, immunofluorescence staining also shows co-localised staining patterns between the two proteins at the cell membrane (Fig. 41C, white arrows). Unfortunately, due to limited material and time constraints, I was unable to follow up this experiment by repeating the IP using a PrP^C pull-down which would be required in future work to confidently demonstrate the interaction between these three proteins, whilst also investigating the role of the N and C-terminals of PrP^C within this interaction.

3.9.4 Reduction of PrP^C decreases expression of CD44

I then decided to investigate the relationship between PrP^C and CD44 further. Using *PRNP* shRNA (Fig. 42A) and TCS prion inhibitor 13 (Fig. 42B), it appears that, like p-gp, expression of CD44 is also dependent of PrP^C as genetic knockdown of *PRNP* using shRNA decreases expression of CD44 (Fig. 42A). Treatment with TCS prion inhibitor 13 (100µM, 72 hours) also causes a significant reduction in CD44 expression, visualised using Western blotting (Fig. 42B). This is in line with previous data (401) suggesting that PrP^C may act as a transcriptional regulator of both p-gp and CD44. However, more experiments would have to be done to confirm the exact mechanism by which PrP^C affects the expression of these two proteins.

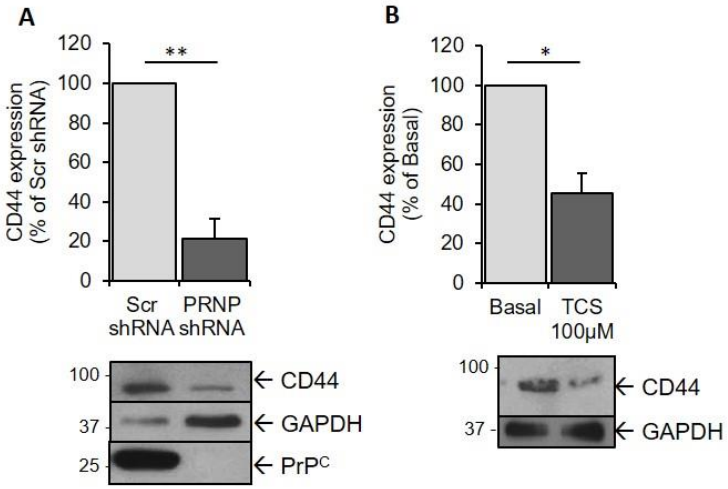

Figure 42. CD44 expression is controlled by PrP^C in schwannoma.

CD44 expression is significantly reduced upon knockdown of *PRNP* in schwannoma cells (A, n=3) and after 72h treatment of schwannoma cells with TCS prion inhibitor (B, n=3). *p<0.05, **p<0.01. GAPDH was used as a loading control.

3.9.5 CD44 acts as a cell surface receptor for PrP^C in schwannoma

Similarly to the experiment investigating whether LR/37/67kDa acts as a cell surface receptor for PrP^C in schwannoma (Fig. 32), I also investigated the possibility that CD44 has a role as a receptor for PrP^C as CD44 is known already to act as a hyaluronan (HA) receptor on the surface of various cell types and also acts to link substrates to the actin cytoskeleton via its ability to interact with ERM proteins (398). To investigate the role of CD44 as a PrP^C receptor in schwannoma cells, I stimulated cells with PrP aa105-120 both before and after knockdown of CD44 expression using *HCAM* shRNA. I conclude, as I showed previously (Fig. 32) that stimulation of schwannoma cells with PrP peptide aa105-120 activates signalling via cyclin D1, ERK1/2, AKT and FAK compared to basal cells cultured in GFM (Fig. 43A-E, light grey bars). Upon knockdown of *CD44,* the basal levels (not treated with PrP peptide aa105-120) of cyclin D1 and $pFAK^{Y397}$ are unchanged (Fig. 43A and D) whereas levels of both pERK1/2 and pAKT are significantly reduced (Fig. 43B and C). Additionally, knocking down expression of CD44 makes PrP peptide aa105-120 unable to activate any of the aforementioned signalling pathways (Fig. 43A-E, dark grey bars), suggesting that CD44 does function as a receptor for PrP^C in schwannoma to activate cyclin D1, ERK1/2, AKT and FAK signalling pathways. The ability of CD44 knockdown to significantly decrease basal levels of pERK1/2 and pAKT also suggests that CD44 alone may play a role in both ERK1/2 and AKT signalling in schwannoma via various other signalling pathways, independently of PrP^C.

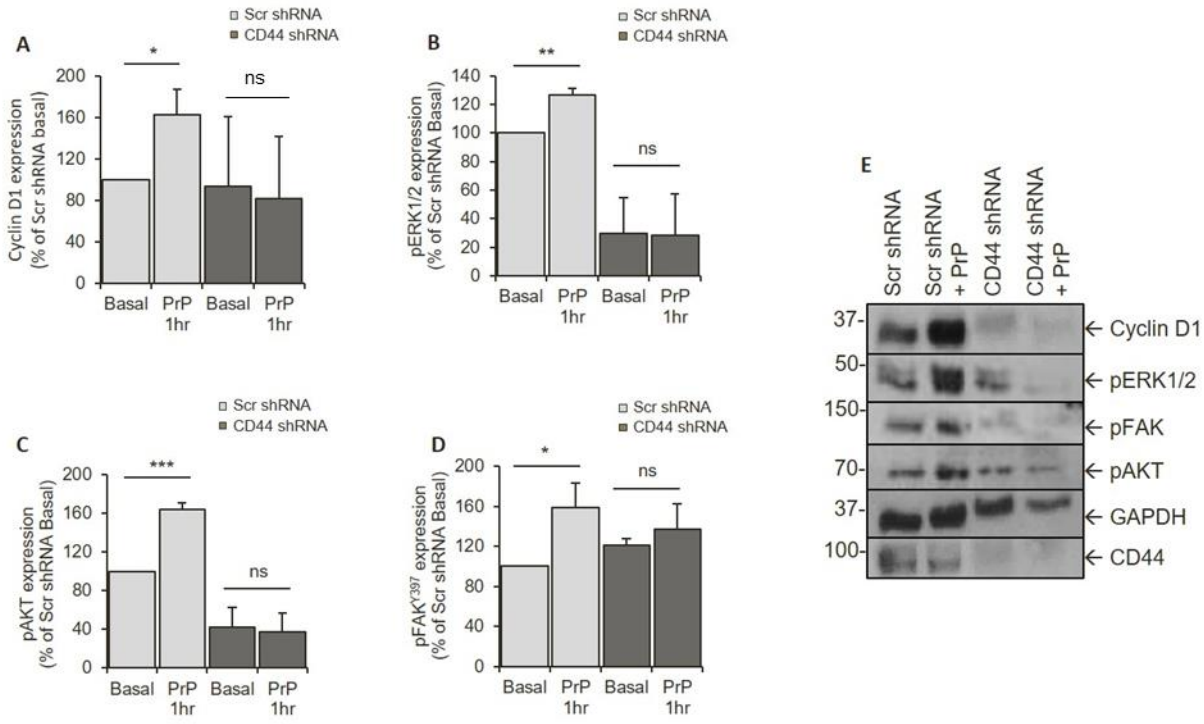

Figure 43. PrP^{C} signals via CD44 to activate key signalling pathways in schwannoma.

As shown previously (Fig. 31), treatment of schwannoma cells with PrP peptide increases levels of cyclin D1, pERK1/2, pAKT and pFAK (A-D, light grey bars). Knockdown of CD44 reduces the ability of PrP to activate each of these signalling pathways (A-D, dark grey bars). Changes in activation of the various signalling pathways can be visualised using Western blotting (E, n=5). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. GAPDH was used as a loading control.

3.9.6 CD44, the magical linker between PrP^{C} and p-gp? Discussion

It has been known for two decades now that CD44 is upregulated in schwannoma (296) but, in that time, very few experiments have been carried out to look at alternative receptor functions of CD44 other than its primary ligand hyaluronan (HA), interaction with which is inhibited by Merlin (402). Work has shown that schwannomas, like Schwann cells, normally express CD44s (standard isoform) and that schwannomas express only a few of the 17 CD44 variants (403).

There is a large amount of literature on CD44 expression in meningiomas but the conclusions drawn are varied, particularly with so many variants of CD44 to be investigated. Studies have shown that CD44s is expressed in the range of “strong” to “negative” in meningiomas using immunofluorescence (404) whilst other studies have shown variants of CD44 to be expressed in line with transition of tumour cell differentiation to a more epithelial type such as secretory meningiomas (405). I show a high expression of CD44 in grade I, Merlin-deficient meningiomas compared to ‘normal’ human meningeal cell line HMC (Fig. 40B). Although this is in line with what is seen in schwannoma it appears that the cause of CD44 overexpression in these tumours is not due to loss of Merlin as re-introduction of the *NF2* gene did not lead to a significant reduction in CD44 expression in schwannoma (Fig. 40C). Thus, there must be an alternative pathway leading to the genetic upregulation of CD44 in schwannoma that isn’t Merlin-dependent or due to alterations in glycosylation status of CD44 as the CD44 expressed in schwannoma appears to be fully glycosylated (Fig. 40A).

Although CD44 is predicted to be the missing link in the interaction between p-gp and PrP^C having been shown to interact with both p-gp (316) and PrP^C in cancer cells (319), it appears it might not be quite as simple a relationship as previously hypothesised. Although p-gp and CD44 co-localise in schwannoma cells (seen using immunofluorescence Fig. 41A), determining whether a direct interaction between the two protein occurs using immunoprecipitation proved difficult as p-gp pull-downs were all unsuccessful. This may well be due to the fact that low-salt lysis buffer was unable to sufficiently lyse the nuclear component of the schwannoma cells where the majority of p-gp is retained.

Much more work is required to fully elucidate the relationship between p-gp and CD44 in schwannoma cells with further co-IP and immunofluorescence experiments required, testing both of the p-gp antibodies against CD44 and PrP^C.

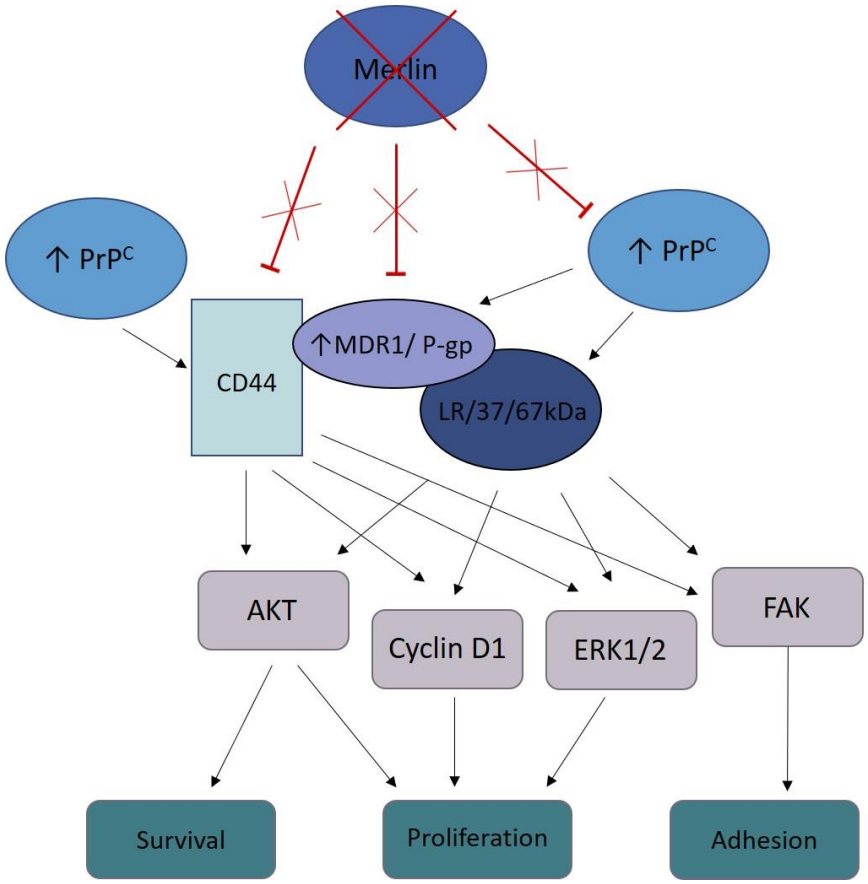

Figure 44. PrP^C can act via CD44 to activate AKT, cyclin D1, ERK1/2 and FAK which, in turn, enhance schwannoma survival, proliferation and adhesion.

I show that CD44 expression is controlled by PrP^C and that CD44 and PrP^C physically interact. CD44 also acts as a receptor for PrP^C via which downstream signalling pathways cyclin D1, AKT, ERK1/2 and FAK can be activated to enhance schwannoma proliferation, adhesion and survival.

PrP^C and CD44 do appear to interact (co-IP, Fig. 41B) in schwannoma cells, suggesting that this still could be a mechanism of reduced therapeutic efficiency in schwannoma cells and inhibition of this interaction could be interesting to

target therapeutically. Moreover, PrP^C appears to be controlling expression of CD44 in schwannoma, further complicating the picture of PrP^C/CD44 interaction and upregulation. These data, taken together, suggest that PrP^C lies upstream of both p-gp and CD44 but may also interact with both proteins in order to mediated tumorigenesis.

Finally, CD44 is known to act as a cell surface receptor in various cancer cell types and has a variety of ligands including other cell adhesion molecules laminin and integrin β1 (reviewed in (406)) and is able to transduce extracellular stimuli to activate intracellular signalling pathways, with the intracellular domain having been shown to interact with various cytoplasmic proteins including ERM proteins (of which Merlin is a member) and Fyn kinase (which PrP^C has been shown to interact with (406)).

Therefore, I wanted to check whether CD44 acts as a receptor for PrP^C in schwannoma cells. Results show that PrP aa105-120 is able to act via CD44 to activate ERK1/2, PI3K/AKT, cyclin D1 and FAK. This suggests that CD44, like LR/37/67kDa, acts as a cell surface receptor for PrP^C and may explain the co-localisation and interaction demonstrated between the two proteins (Fig. 41). As well as abolishing the ability of PrP^C to activate these key signalling pathways, knockdown of CD44 appears to dramatically reduce pERK and pAKT levels, even without PrP stimulation (Fig. 43, Fig 44). This mirrors previous findings that CD44 signals via ERK1/2 and PI3K/AKT to protect chronic lymphocytic leukaemia (CLL) cells from spontaneous and drug-induced apoptosis (407) suggesting that the same may be happening in schwannoma cells and that targeting CD44 expression alone, as well as CD44/PrP^C interaction could produce promising therapeutic effects.

3.10 Drug resistance in schwannoma

For the next portion of this study I wanted to investigate the effects of inhibiting PrP^C using TCS prion inhibitor alongside two drugs that are already clinically approved for other conditions. Firstly, Sorafenib (commercially known as Nexavar, Bayer), a tyrosine kinase inhibitor that targets several tyrosine kinases including PDGFR and VEGFR that is currently in Phase 0 clinical trials for schwannoma but has been approved for use in some types thyroid cancer (408), advanced renal cell carcinoma (409) and in hepatocarcinoma (410). Secondly, PSC833 (commercially known as Valspodar, Amdray), a non-immunorepressive cyclosporine-D analogue that acts as a chemosensitizer and multidrug resistance modulator to reverse the p-gp efflux pump. Although some studies have been successful (411,412) the benefits of its use have yet to be fully determined. PSC833 has not been successful in other clinical trials when given with a combination of several other chemotherapeutic agents including vincristine, doxorubicin and dexamethasone (known as VAD). This was due to increased toxicity and lack of improved treatment outcome in PSC833/Valspodar-treated patients compared to VAD therapy alone (413).

3.10.1 MTS assay demonstrates that inhibition of PrP and p-gp can enhance the effects of Sorafenib

I wanted to investigate the effects of inhibiting PrP^C and p-gp on Sorafenib treatment and cell proliferation and survival in human primary schwannoma cells to see whether a multi-pronged approach would be more beneficial as a therapeutic intervention. This experiment had two treatment time points (72 hours, Fig. 45A or 14 days, Fig. 45B) in order to demonstrate both the short and long-term treatment effects of these drugs. Cells were pre-treated with PSC833

or TCS prion inhibitor 13 (for laboratory use only). PSC833 has been shown to be effective in combination with Methotrexane as a treatment to peadeatric acute leukaemia. I chose to use a concentration of 4µg/ml of PSC833 to inhibit p-gp as a phase I clinical trial in these patients show plasma serum concentrations ranging from 1.1-7.8µg/ml with toxicity appearing in some patients around 4.4µg/ml serum concentration level (414). Maximum tolerated dose of PSC833 in patients was 12.5mg/kg/day for 5 days which resulted in varying plasma concentrations of the drug from 1.4µg/ml to 5.2µg/ml.

Cells were pre-treated with PSC833 or TCS, followed by treatment with Sorafenib (1µM). The medium was exchanged and cells were re-treated every 48 hours to maintain a stable concentration of the drug, as the half-life of Sorafenib is 25-48 hours. MTS assay was used to determine cell viability, whether that be due to increased cell death or decreased cell proliferation.

I show that Sorafenib significantly decreases schwannoma cell viability/ proliferation by ~30% after 72 hours (Fig. 45A), interestingly, no further decrease was seen after 14 days (Fig. 45B) which may either be due to an upregulation of p-gp pumps that efflux the drug from cells or simply because the concentration used was insufficiently high to cause more cell death than this. Treatment with TCS prion inhibitor 13 (20µM) alone significantly decreased schwannoma viability after 72 hours (by ~60%) as predicted by the previous proliferation and survival assays (Fig. 22), even though this concentration of TCS was never enough to significantly decrease PrP^C levels (Fig.21).

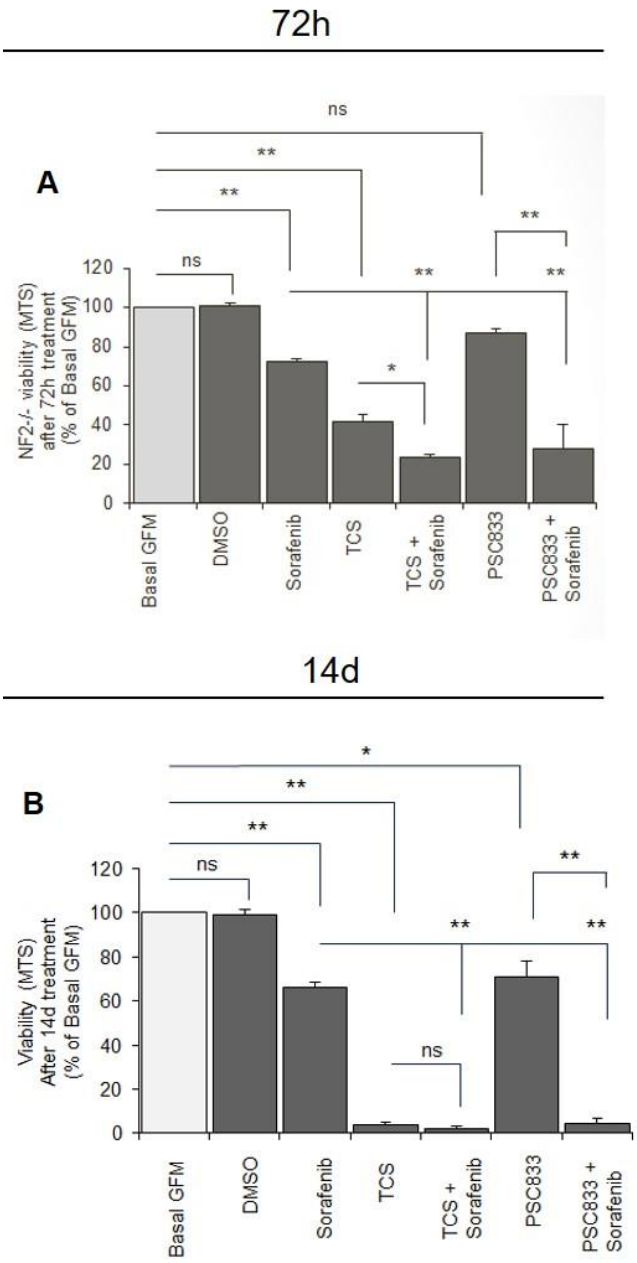

Figure 45. Treatment of schwannoma cells with TCS prion inhibitor or a combination of Sorafenib and PSC833 (Valspodar) is more effective at reducing cell viability than treatment with sorafenib alone.

Schwannoma cells were treated with either DMSO, Sorafenib alone, Sorafenib in combination with TCS or PSC833, TCS alone, or PSC833 alone for either 72 hours or 14 days before undergoing an MTS assay to ascertain cell viability. At both time points (A and B) Sorafenib treatment significantly decreased schwannoma cell viability. Viability was further decreased when Sorafenib treatment was combined with either TCS or PSC833 (A and B). Although combined treatment of Sorafenib and TCS reduced viability more than TCS alone at the 72 hour time point, this difference was abolished by day 14 (A and B). Ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The effects of TCS on schwannoma viability were even more apparent after the 14 day treatment (~90% reduction in viability) (Fig. 45B), suggesting that this drug is even more powerful than Sorafenib at reducing schwannoma viability. A combination treatment of both TCS and Sorafenib was only beneficial at the 72 hour timepoint (Fig. 45A), the addition of Sorafenib had no significant additive effect at the longer timepoint due the effectiveness of TCS alone (Fig. 45B).

Use of the p-gp inhibitor PSC833 alone had no significant effect on viability (MTS test) after 72 hours but did significantly decrease viability (~30%) after 14 days (Fig. 45A and B). However, PSC833 treatment in combination with Sorafenib, decreased viability levels dramatically, more that treatment with Sorafenib alone, both at 72 hours (~80%) (Fig. 45A) and 14 days (~90%) (Fig. 45B). The fact that combination therapy of Sorafenib and the PSC833 was able to overcome the plateau in viability seen with the Sorafenib treatment alone after 14 days, suggests that upregulation of p-gp may have been preventing the continual decrease of viability in these cells.

Nevertheless, treatment with PSC833, even in combination with Sorafenib was not as effective as TCS alone after 14 days, suggesting that a monotherapy of just TCS (if it were ever approved for human use) may be better than a dual therapy intervention that could initiate drug-drug interactions and cause a large number of adverse side effects. More investigations need to be carried out to investigate the mechanisms of action of TCS before it can even be considered for clinical trials, as my work (415) is the only published data available which uses this product. Thus, for the time being it may be beneficial to use Sorafenib and PSC833 in combination for schwannoma patients as both have been used clinically previously and PSC833 has been reformulated to make it more

bioavailable and reduce its toxicity profile (414). The reformulation was due to reports from a phase III trial in acute myeloid leukaemia (AML) patients show that Valspodar (PSC833) was associated with excessive mortality and had no beneficial effect on complete remission rates (416), hence has so far been unsuccessful in clinical trials. I therefore suggest, for future experiments, trying a lower concentration of Valspodar (PSC833, ~1.1µg/ml) to in combination with Sorafenib to increase the effects of Sorafenib whilst minimising any residual toxicity caused by the reformulated PSC833/ Valspodar.

3.10.2 Treatment with both TCS and PSC833 may also improve inhibition of downstream signalling pathways by Sorafenib in schwannoma

To back up the results shown by the MTS assay, I looked into the effects of the combined drug treatments on downstream signalling in schwannoma cells. For these experiments I used a single timepoint of seven days (an intermediate between the 72 hours and 14 days of the previous experiment) to minimise the quantity of primary cells used and due to time restraints on the project. Cells were initially pre-treated with PSC833 (p-gp inhibitor, Valspodar) or with TCS prion inhibitor before treatment with Sorafenib. Samples were then lysed and run on SDS-PAGE to quantify the expression and activation of downstream signalling proteins. I show that levels of both pERK1/2 and pAKT are significantly decreased upon treatment with Sorafenib (1µM) (Fig. 46A, B and C), showing that the drug is effective

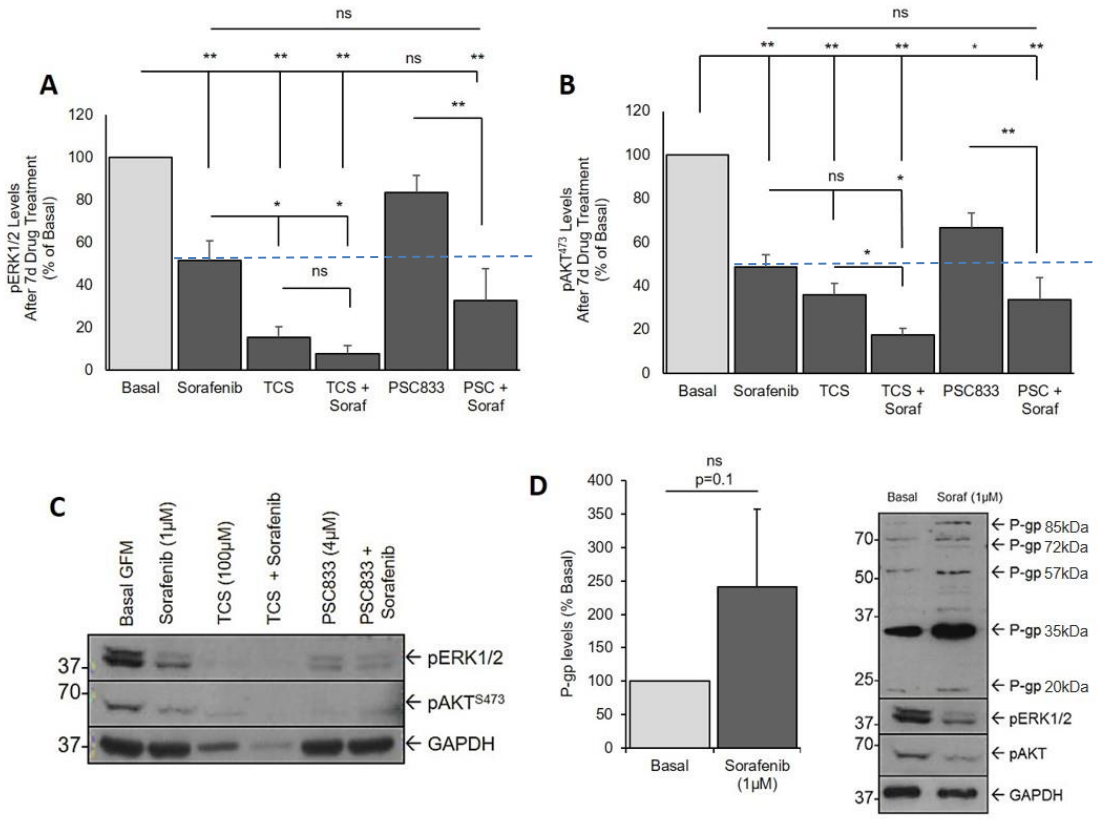

Figure 46. Combined inhibition of p-gp, PrP^{C} and RTKs in schwannoma leads to a reduced signalling through key pathways involved in tumourigenesis.

Sorafenib treatment of schwannoma cells in culture alone significantly reduces expression of pERK1/2 and pAKT (A and B). pERK1/2 levels significantly reduce upon treatment of schwannoma cells with TCS (20μM) alone and in combination with Sorafenib (1μM) (A, n=3), pAKT is also significantly reduced when Sorafenib is combined with TCS compared to Sorafenib treatment alone (B, n=3). PSC833 (4μM) reduces pAKT (B) but not pERK1/2 (A) levels when treated with PSC833 alone. Neither pERK1/2 (A) or pAKT levels are affected when treated with PSC833 in combination with Sorafenib compared to sorafenib treatment alone. This can be visualised in (C). Upon Sorafenib treatment p-gp expression is increased, although not significantly (D, n=4). Ns p>0.05, *p<0.05, **p<0.01, ***p<0.001. GAPDH was used as a loading control.

Levels of pERK and pAKT were reduced with TCS (Fig. 46A, B and C), as previously described in Fig. 25. Combined treatment of TCS and Sorafenib decreased pERK1/2 levels more than Sorafenib alone but not further than TCS alone (Fig. 46A-C) and had additive effect on pAKT inhibition (Fig. 46B and C).

PSC833 significantly decreased pAKT but not pERK1/2 levels. (Fig. 46A and C). Combined treatment of PSC833 and Sorafenib had, however, no significant additive effect on the inhibition of any of the pathways (Fig. 46A, B and C) which contrasts my previous results on cell proliferation/survival observed in Fig. 45. Further experiments must be performed in order to decipher the mechanisms by which PSC833 aids Sorafenib in the inhibition of proliferation/survival in schwannoma cells.

P-gp levels were also investigated to see whether Sorafenib was able to induce an increase in p-gp levels, thus potentially being able to potentiate basal (intrinsic) MDR and/or contribute to acquired MDR in schwannoma cells if the treatment ever cleared clinical trials. Sorafenib treatment did increase levels of p-gp in most schwannoma cells tested but the variability was high and the result insignificant.

To diminish potential side effects, the next step would be to decrease Sorafenib concentration from 1µM to 100nM (plasma concentration, due to risk of hypertension, cardiac arrhythmias and bleeding), TCS from 20µM to 1-5µM (although there is no pulished data at this concentration, I tested several concentrations and found that these were the lowest doses to have a significant effect on both PrP^C expression and schwannoma viability) and PSC833 from 4µM to 1.1µM and find the lowest and most effective dosage and combination of the three drugs in schwannoma cells.

3.10.3 Drug resistance in schwannoma – discussion

The drug I chose to focus my studies on was Sorafenib, a multi-kinase inhibitor that is currently undergoing phase 0 clinical trials in NF2 patients. Previous data suggests that p-gp is strongly expressed on the endothelial cells of blood

vessels at both the blood brain barrier and also at the blood-tumour barrier (417) and prevents cytotoxic agents (including chemotherapy and other anti-cancer drugs) from entering the CNS and tumour. My data suggests that p-gp appears to help prevent Sorafenib transferring from the bloodstream and into the tumour. A recent study shows that although it plays a role, p-gp is not the main culprit for reducing brain and/or tumour accumulation of Sorafenib. Instead, it has been suggested another ABC-protein - ABCG2 (also known as breast cancer resistance protein, BCRP) - causes drug resistance to Sorafenib in mice (418) and in human hepatocellular carcinoma (419).

There is evidently a form of intrinsic drug resistance in schwannoma caused by upregulation of p-gp within these cells (Fig. 34). However, it appears that drug treatments, such as Sorafenib, may further potentiate p-gp levels (Fig. 46D) indicating that some NF2 patients may also develop acquired resistance. Gene expression analysis using quantitative real-time polymerase chain reaction (qRT-PCR) would be able to quantify MDR1 gene expression between patients. The high variability in p-gp expression upon Sorafenib treatment could account for individual differences and be the reason why different tumours respond differently to treatment, making basal p-gp levels a potential marker for drug efficiency. Those patients whose p-gp levels increase dramatically upon treatment may be less likely to respond well to the drug treatment for a longer period of time. However, we cannot exclude that some patients may have very high (‘saturated’) endogenous levels of p-gp which cannot be further potentiated by drug treatment and be resistant to the treatment from the very beginning. Again, this is something that requires further investigation.

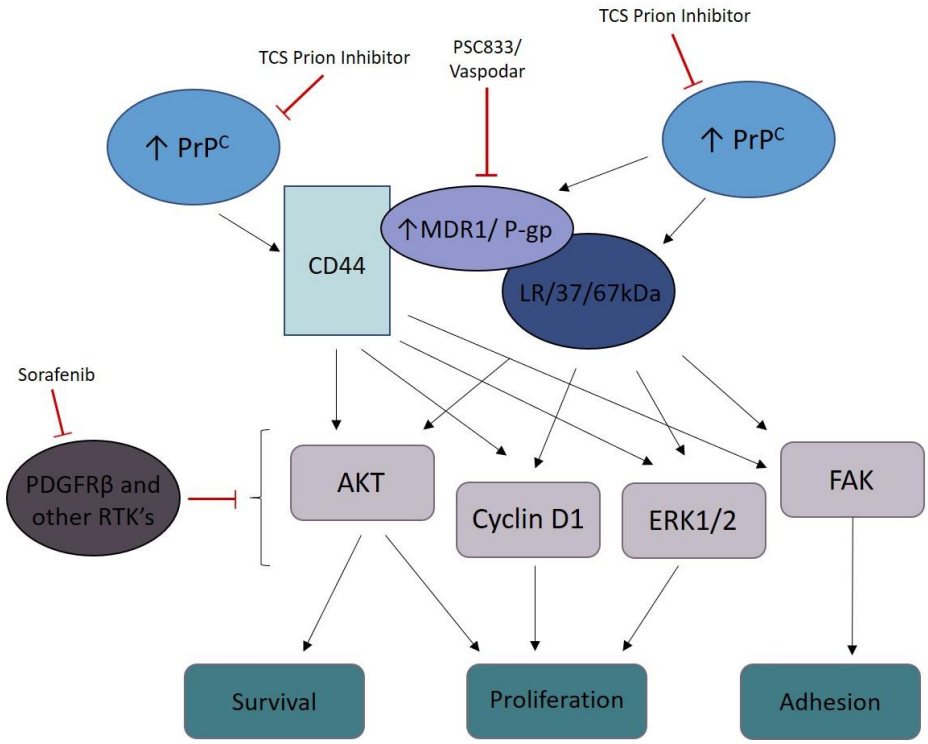

Figure 47. Combined treatment of schwannoma cells with TCS prion inhibitor, PSC833 and Sorafenib could be more beneficial than a single drug treatment approach.

Results show that single drug treatment of the multi-kinase inhibitor Sorafenib alone does not dramatically decrease schwannoma cell viability and may also potentiate p-gp levels in these cells. Therefore, a combination or either Sorafenib and p-gp inhibitor, PSC833 or TCS prion inhibitor could prevent further upregulation of p-gp and allow blockade of downstream signalling pathways, ultimately leading to a decrease in schwannoma proliferation, cell matrix adhesion and survival moreso than treatment alone with sorafenib.

These findings also sway the evidence to suggest that personalised and multi-pronged therapeutic approaches may be the best way forward for treating schwannoma patients, as well as potentially patients with other NF2-related tumours, in the clinic. A potential dual therapy for two drugs already approved for clinical trials has been uncovered in the course of this research project – Sorafenib and PSC833 (Vaslpodar) which together reduce tumour survival/

proliferation and pERK1/2 and pAKT pathways (Fig. 46 A-C, also outlined in Fig. 49). A combination of these two drugs and could be of potential therapeutic use for NF2 patients, if the dosage can be lowered significantly enough to reduce the side effects (including neutropenia (420)) which currently are restricting the widespread use of both of these drugs in the clinic.

4. Conclusions and future work

4.1 Summary of results

- Cellular PrP is overexpressed in schwannoma and contributes to schwannoma pathological proliferation, adhesion and survival.
- PrP^C is cleaved at the cell surface of schwannoma cells by ADAM10, as well as being released via exosomes.
- PrP signals via both LR/37/67kDa and CD44 receptors to activate AKT/PI3K, ERK1/2, FAK, cyclin D1 pathways.
- P-gp is intrinsically overexpressed in schwannoma and may form a interact with CD44 and PrP^C
- Inhibition of PrP^C may be beneficical as a treatment option for schwannoma patients, either alone or in combination with the multi-kinase inhibitor Sorafenib.
- Individuals respond differently to drug treatments suggesting a role for personalised medicine in treating schwannoma and other NF2-related tumours.

4.2 PrP^C signalling in schwannoma

As seen in many other tumours, the di-glycosylated cellular form of PrP is overexpressed in schwannoma due to upregulation of the *PRNP* gene caused, in part, by loss of Merlin from these cells. Whereas a similar Merlin-dependent overexpression of PrP^C is seen in human malignant mesothelioma cells, in meningiomas there may be additional genetic mutations that account for upregulation of PrP^C as opposed to solely the loss of *NF2*. PrP^C is released from cells both via exosomes and by α-cleavage from the cell membrane. Extracellular PrP^C is able to activate key signalling pathways involved in schwannoma development including ERK1/2, cyclin D1, PI3K/AKT, FAK and p53 to contribute to schwannoma pathological proliferation, adhesion and survival via direct interactions with both LR/37/67kDa and CD44 receptors on the cell surface (Fig. 48).

My findings agree with other schwannoma studies from our research group, suggesting that inhibitors of common signalling pathways, such as those I have demonstrated to be downstream of PrP^C, could be used as therapeutic targets in these tumours (62,63,67,68,183,213,359,368,375-377). Previous work has shown that targeting only specific isoforms of key signalling molecules such as PI3Kδ (via a drug known as Idelisib) can be very effective and also much more selective than first-generation pan-PI3K inhibitors (421) although it has only currently been approved for treatment of chronic lymphocytic leukaemia, indolent B-cell non-Hodgkin's lymphoma and relapsed small lymphocytic lymphoma. Initially, direct inhibition of PrP^C appears an ideal therapeutic target as most side effects seen in animal trials (upon *PRNP* knockdown) appeared initially to only demonstrate altered circadian rhythms (81). By inhibiting PrP^C,

expression of neurotoxic Dpl may be increased and *PRNP* gene knockdown may lead to altered myelination, cognitive defecits, increased susceptibility to oxidative stress and increased excitotoxicity (81). This suggests that more of an indirect approach to inhibiting PrP^C or just reducing PrP^C levels back to basal levels rather than complete abrogation of the protein would, instead, be a more beneficial therapeutic option. The MRC Prion Unit have a clinical trial planned which utilises humanised anti-prion antibodies as a potential new treatment for CJD (422) in a plan to inactivate the PrP^C present in patients and prevent it from aggregating and causing neurodegeneration.

Further investigation using *PRNP*$^{0/0}$ mice may also be beneficial as no prior studies have shown whether mice lacking PrP^C have a reduced incidence of tumour development. Crossing a $PRNP^{0/0}$ mouse with an NF2-/- mouse could provide extensive insights to whether targeting PrP^C is a good option for treatment of NF2 and Merlin-deficient tumours in general. Plus, as mentioned previously, PrP^C could also act more as a biomarker for tumour development, load and prognosis due to the fact it is released in exosomes and may be detectable in the blood of patients or those individuals susceptible to developing NF2. This would make a huge difference in the diagnosis of these patients and would allow doctors to measure their prognosis in an easy, cost-effective and non-invasive manner.

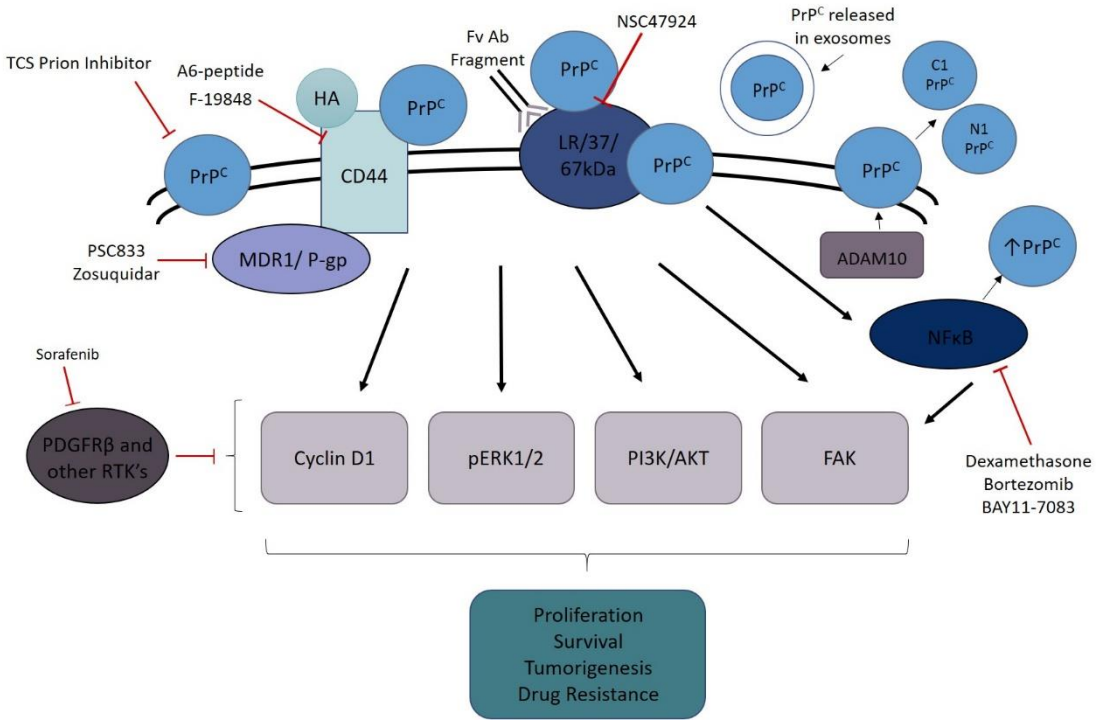

Figure 48. Overview of PrP^C signalling in schwannoma and potential therapeutic targets.

Extracellular PrP^C acts via both CD44 and LR/37/67kDa at the cell membrane of schwannoma cells to activate cyclin D1, pERK1/2, PI3K/AKT and FAK pathways, which contribute to schwannoma proliferation, cell matrix adhesion, survival and tumourigenesis. It is likely both that released PrP^C and intracellular or cell membrane bound PrP^C may also be able to activate the same response to extracellular PrP. Finally, there is evidence to suggest LR/67kDa is able to signal to NFκB (423). NFκB is then able to stimulate PrP^C expression and potentially also CD44 expression (424). Use of antibody fragments and specific inhibitors could be of use to prevent signalling of PrP^C through both LR/37/67kDa and CD44 in schwannoma. Future work may involve looking at inhibitors of some of these elements including A6 peptide or F-19848 to inhibit CD44, an fv antibody fragment to LR/37/67kDa or NSC47924 which blocks the interaction of PrP^C with LR/37/67kDa or even NFκB inhibitors dexamethasone, bortezomib and BAY11-7083 which all may act as potential therapeutic targets in schwannoma patients, particularly those already approved for clinical use.

Further investigation using inhibitors of PrP^c receptors would give an even better understanding of PrP^C interaction with these receptors. Inhibitors of the LR/37/67kDa-PrP^C such as NSC47924 which alters LR cell surface localisation

and interaction with PrP^C (385) or use of Fv antibody fragments to inhibit LR/37/67kDa which have already been found to have therapeutic effects in prion disease mouse models (386). Both of these therapies are yet to go into clinical trial and require further testing before they can be used but they both provide an interesting therapeutic approach that could translate to treatment of schwannoma and other NF2-related tumours.

The interaction between NFκB and PrP^C proved to be interesting, in that NFκB positively regulated PrP^C expression and appears to be an interesting intermediate of PrP^C signalling within schwannoma cells. Although it was beyond the scope of this project to investigate, other research using human hepatoma (Huh-7) and human cervical carcinoma HeLa cells lines shows control of CD44 expression to be downstream of NFκB signalling, however only in the non-canonical NFκB pathway (424) not the p65 NFκB pathway that was investigated here. Other inhibitors of NFκB such as Bortezomib (a proteasome inhibitor that is FDA approved for multiple myeloma (425)) or Dexamethasone (a synthetic anti-inflammatory corticosteroid shown to reduce NFκB activation and in phase II trials for multiple myeloma (426)) could also potentially be re-profiled to help in the reduction of NFκB and PrP^C in schwannoma and other Merlin-deficient tumours.

Inhibitors of CD44-PrP^C interaction would also be interesting to study with potential benefits, not only for reducing PrP signalling but also for resistance to drug therapy. The only CD44 inhibitor currently available is F-19848, which inhibits binding of HA to CD44 and can inhibit CD44-induced HA degradation (427) making it an interesting potential therapeutic target for schwannoma

patients as both HA and CD44 are overexpressed in schwannoma (296,297). Plus, it would be interesting to see if PrP^C/CD44 binding can be altered upon treatment with this novel inhibitor. Furthermore, A6-peptide a urokinase-derived peptide currently in phase II trials for ovarian cancer, is able to modulate CD44 activity, providing a promising new therapeutic option to reduce signalling in schwannoma cells. This drug has not shown any dose-limiting toxic effects in animal studies, providing us with a new candidate to take forward into the schwannoma cell culture model and potentially also into clinical trials in the future (428).

Compensatory activation of proteins upon selective inhibition of signalling pathways is a well-known phenomenon in cancer cells where inhibition of one signalling pathways leads to an upregulation of signalling through other pathways with similar effects to counteract the drug and prevent the cells from dying. An example of this cross talk and compensatory activation is the Ras-ERK and PI3K/AKT pathways which are able to cross-talk and feed into the same downstream pathways. Therefore inhibiting only the Ras-ERK arm of the pathway to target tumour proliferation and survival may lead to increased activity of the PI3K/AKT pathway to counteract this (429). This mechanism is another, non-p-gp associated, mechanism of drug resistance that should always be considered when developing drugs for tumour cells. Compensatory upregulation of receptors and activation of alternative signalling pathways is the reason why targeting a single protein at the beginning of a signaling cascade is not always effective, therefore potential new therapeutic targets should consider a multi-factorial approach to prevent the compensatory upregulation or activation of other pathways that will mask any effects of the given drug. Due to

the similarities seen with regards to the decrease in signalling via ERK1/2, cyclin D1, FAK and AKT upon both LR/37/67kDa and *CD44* shRNA knockdown it could be that decreased expression of each of these receptors leads to a compensatory upregulation of the other receptor, thus masking some of the effects of knocking down each receptor separately. A dual knockdown of both receptors would be interesting to investigate further in the future and to check whether drug treatments show the same decreases in signalling pathways as shRNA.

Unfortunately, it was only towards the end of the project that I discovered that C-terminal PrP^C existed in schwannoma cells and localised to the nucleus (Fig.10). Therefore, I was not able to investigate the importance of this C-terminal portion of PrP, what it was doing in the nucleus and whether the C- and N-terminals of PrP^C interact with different proteins within their separate locations. To fully understand the role of the C- and N-terminal portions of PrP^C in schwannoma, I would ideally like to repeat the key experiments looking at PrP^C levels in schwannoma, particularly investigating whether different PrP^C terminal proteins interact differently with LR/37/67kDa, p-gp and CD44 and also which terminals are involved in signalling and release, these experiments would need to be carried out using C- and N-terminal specific antibodies side-by-side. The C- and N-terminal PrP^C antibodies show the same results when used for Western blotting (data not shown) but show distinct differences in cellular localisation (Fig. 10). I would want to investigate the effect of TCS prion inhibitor 13 and *PRNP* shRNA on cellular localisation of C-terminal PrP^C, look at whether an N-terminal PrP^C antibody would show different tissue staining pattern than seen in Figure 11 and, most importantly, check co-localisation and interaction

between p-gp and C-terminal PrP^C. Once the exact roles of C- and N-terminal PrP^C have been deciphered, the rest of the results discussed in this thesis can be put into context. Although, the fact that these two segments of PrP^C exist in schwannoma cells, further backs up the evidence to suggest the occurance of α-cleavage, the cleavage process that leads to their formation.

4.3 Schwannoma resistance to drug treatments

The data discussed in this report, particularly the data relating to drug resistance and potential therapeutic targets, strongly suggests that a multi-pronged, personalised medicine approach may be the most beneficial way forwards towards a drug treatment for schwannoma and other NF2-related tumours. By doing this, patients are more likely to respond to treatment, have fewer side effects and also have fewer relapses due to drug resistance caused by compensatory receptor upregulation. P-gp inhibitors such as PSC833 or drugs such as zosuquidar, which sensitises p-gp expressing cells to drug treatments, approved for use in AML (430), could be useful aids in the treatment of schwannoma or other Merlin-deficient tumours which present with a high p-gp expression load but, based on my findings, may not benefit patients as a sole treatment option. Combined therapy of Sorafenib with a P-gp inhibitor does however look like a promising potential therapeutic intervention that warrants further investigation (Fig. 45 and 46).

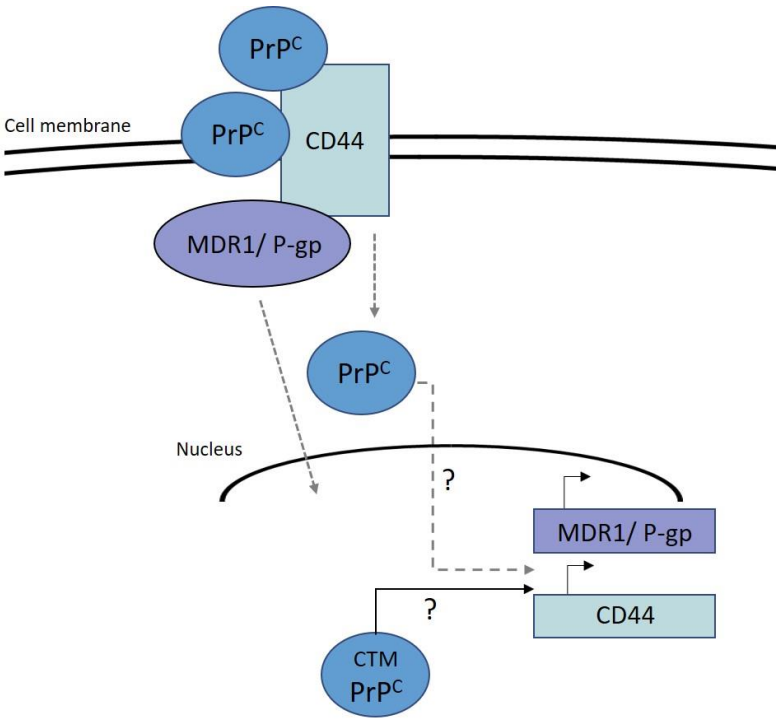

Figure 49. Hypothesis of interplay between PrP^C, p-gp and CD44 in schwannoma.

From data outlined in this thesis, I hypothesise that CD44 acts to link PrP^C and p-gp at the cell membrane as p-gp and PrP^C do not appear to directly interact in schwannoma cells. CD44 may also use this interaction to initiate translocation of both p-gp and the C-terminal portion of PrP^C to the nucleus in schwannoma cells. In the nucleus, it appears that PrP^C exherts some control over expression of p-gp and CD44, potentially at the transcriptional level. More experiments are required to fully understand whether this is the case.

Within this, PrP^C looks to be a particularly promising therapeutic target due to minimal disturbances caused by its inhibition and its role upstream of many of the key pathways involved in schwannoma development and pathology. However, unlike some of the other targets discussed during this report, there is no currently approved PrP^C inhibitor and much more work is needed before any PrP^C inhibitor is released for clinical trials, it looks to be an interesting molecule which deserves further attention within this field of research. Hence why anti-prion antibody, PRN100, is being considered as a potential future treatment for

CJD within the MRC Prion Clinic at University College London. Furthermore, due to the likely interplay between PrP^C, p-gp and CD44 within schwannoma cells (Fig. 49), a combination of inhibitors targeting one of more of this complex of protein could not only help reduce schwannoma signalling by indirectly targeting PrP^C but also increase the tumour response to any drug treatment given due to the reduction of p-gp, this combinatorial approach is a much more realistic, short-term goal for schwannoma patients.

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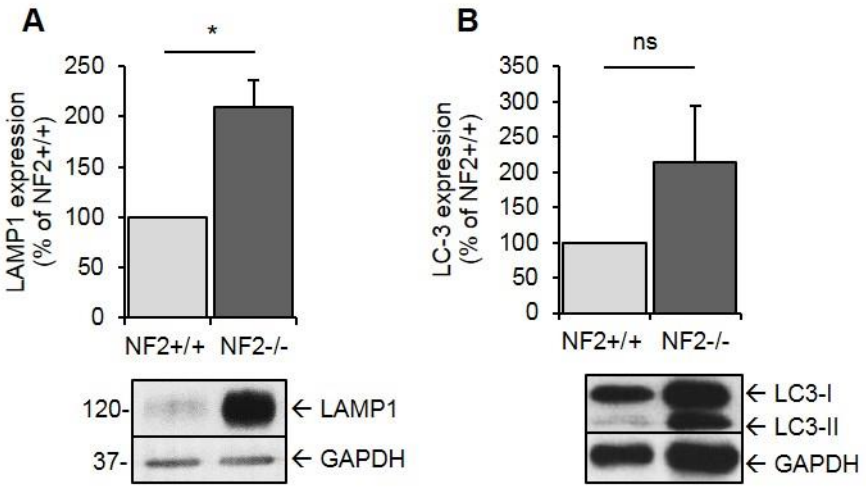
Appendix

6. Supplementary data

6.1. Upregulation of autophagy-related proteins in schwannoma compared to Schwann cells

Lysosome-associated membrane protein-1 (LAMP1) is a glycoprotein associated, as its name suggests, with lysosomes during lysosomal degradation and autophagy (431). LAMP1 expression is significantly upregulated in schwannoma compared to Schwann cells (Sup. Fig. 1A). LAMP1 expression has previously been correlated with the metastatic potential of tumours (432,433). Although schwannomas are mainly benign tumours of Schwann cells, overexpression of LAMP1 in these tumours may be contributing to schwannoma pathobiology and/or development.



Supplementary Figure 1. Upregulation of autophagy-related proteins in schwannoma compared to Schwann cells. LAMP1 is significantly overexpressed in schwannoma (A) compared to Schwann cells (n=3). Although the antibody detected two isoforms of LC3 (I and II) it is LC3-II that is most associated with autophagosomes and thus, autophagy, hence only LC3-II expression was quantified (B). However, it appeared that both isoforms of LC3 showed increased expression in schwannoma compared to Schwann cells, though this increase was not statistically significant (n=3).

Whereas LAMP1 is involved both in normal lysosomal degradation and autophagy, microtubule-associated protein 1 light chain 3 (LC3) is essential for autophagy (434). Increases of LC3 can be seen in schwannoma compared to Schwann cells but these increases are not statistically significant (Sup. Fig. 1B).

The effects and exact roles of increased LAMP1 and LC3 in schwannoma require further investigation, far beyond the scope of this project. It can, however, be predicted from the high levels of LAMP1 that lysosomal degradation and autophagy are not altered in these cells, providing further evidence that the increased accumulation of PrP^C within schwannoma cells is not due to deficits in lysosomal degradation (refer to chapter 3.2.1).

7. Abbreviations

Table 6. Table of abbreviations

Abbreviation	Full terminology
å	Angstrom (unit of measurement equivalent to 0.1nm)
aa	Amino acid
ABC	ATP-binding casette
Act. D	Actinomycin D
ADAM	A disintegrin and metalloproteinase domain
ADM	Adriamycin
ADP	Adenosine di-phosphate
AKT	V-Akt Murine Thymoma Viral Oncogene-Like Protein also known as protein kinase B (PKB)
AML	AcuteMyeloid Leukaemia
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
ATPB1	ATP binding cassette sub-family B member 1, another name for p-

	glycoprotein
BAX	Bcl-2-associated X protein
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma-2
BCRP	Breast cancer resistance protein
BenMen	Benign meningioma-1 cell line
bp	Base pairs
BSA	Bovine serum albumin
CAM-DR	Cell adhesion-mediated drug resistance
CD44	Cluster of differentiation 44
CD44s	Cluster of differentiation 44 standard isoform
CD44v	Cluster of differentiation 44 variant
CD63	Cluster of differentiation 63
CDA II/ HEMPAS	Congenital dyserythropoietic anemia type II
CHX	Cycloheximide
cIAP-1	Cellular inhibitor of apoptosis protein-1
CJD	Creuzfeld Jacob Disease
CLL	Chronic Lymphoblastic Leukaemia
CMV	Cytomegalovirus
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
COX2	Cyclooxygenase 2
CpG	5'—C—phosphate—G—3'
CQ	Chloroquine
$CRL4^{(DCAF1)}$	Cullin ring ligase 4 DDB1-CUL4 associated factor 1
CSC's	Cancer stem cells
ctm	C terminal
ctrl	Control
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dpl	Prion protein dublet/doppel
DPX	Distyrene, plasticiser, xylene mix
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
ERK	Extracellular signalling-related kinase
ERM	Ezrin, radixin, moesin
FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Foetal Bovine Serum
FERM	4.1 ezrin, radixin, moesin
FISH	Fluorescent *in situ* hybridisation
FFI	Fatal Familial Insomnia
FN	Fibronectin
FOXO3	Forkhead Box O3
Fv	Antibody variable domain
G5P	Gene 5 protein
GAG	Glycosaminoglycan
GAPDH	Glyceradehyde 3-phosphate dehydrogenase
GBM	Glioblastoma Multiforme
GFM	Growth factor medium
GFP	Green fluorescent protein

Golgi QC	Golgi quality control
GPI	Glysosyl-phosphotidylinositol
GPI-PSS	GPI anchor peptide signalling sequence
GSS	Gerstmann-Sträussler-Scheinker disease
GTP	Guaosine tri-phosphate
HA	Hyaluronan
HCAM	Homing cell adhesion molecule (also known as CD44)
HGF	Hepatocyte growth factor
HMC	Human meningeal cells
HMM	Human malignant mesothelioma
HRP	Horseradish peroxidase
HSPG	Heparin sulphate proteoglycan
hTERT	Human Telomerase Reverse Transcriptase
IBMX	3-isobutyl-1-methylxanthine
ICC	Immunocytochemistry
IFU	Infectious units
IHC	Immunohistochemistry
IGF1	Insulin-like growth factor-1
IGF1R	Insulin-like growth factor-1 receptor
IgG	Immunoglobulin G
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IκK	IκB kinase
IP	Immunoprecipitation
$iPrP^{C}$	Insoluble cellular prion protein
JNK	c-Jun N-terminal kinase
Kb/kbp	Kilobase/kilobase pairs
kDa	Kilodalton
KLF4	Kruppel-like factor 4
LA	Laminins
LAMP1	Lysosomal membrane-associated protein-1

LC3	Microtubule-associated protein 1A/1B-light chain 3
LR/37/67kDa	37/67kDa non-integrin laminin receptor
LRP	Laminin receptor precursor
LZTR1	Leucine zipper-like trancriptional regulator-1
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MDR	Multi-drug resistance
MDR1	Multi-drug resistance gene 1
MEK	Mitogen-enhancing kinase
Merlin	Moesin Ezrin Radixin-like protein
MGr1-Ag	MGr1 antigen
MMP11	Matrix metalloprotease 11
MN/Men	Meningioma
MOI	Measure of infectivity
MOT	Molecular Targets
mRNA	Messenger RNA
mTOR	Mammalian inhibitor of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NCAM	Neuronal cell adhesion molecule
NF2	Neurofibromatosis Type 2
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nt	Nucleotide
ntm	N terminal
nm	Nanometre
O/E	Overexpressed/ overexpression
$P75^{NTR}$	P75 neutrotrophin receptor
PAK	P21-activated kinase
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDGFRβ	Platelet-derived growth factor receptor β
PFA	Paraformaldehyde
P-gp	p-glycoprotein
PI3K	Phosphatidylinositol 3-kinase
PI-PLC	Phosphatidylinositol phospholipase C
PK	Proteinase K
PKC	Protein kinase C
PMS	Phenazine methosulfate
PMSF	Phenylmethane sulfonyl fluoride
PNG	PNGaseF
PNS	Peripheral nervous system
PRNP	Gene coding for the cellular prion protein
PRND	Gene coding for the cellular prion protein dublet/Doppel
Pro-PrP	Incompletely processed prion protein
PrP^{C}	Cellular Prion Protein
PrP^{Res}	Form of the prion protein resistant to degradation by proteinase K
PrP^{Sc}	Scrapie form of the prion protein that is responsible for transmission of prion disease
PRT	Testes-specific prion protein
PVDF	Polyvinylidene fluoride
Q-PCR	Real-time quantitative PCR
R&D	Research and development
Rac	Ras-related C3 botulinum toxin substrate
Raf	C-Raf Proto-Oncogene, Serine/Threonine Kinase
REC	Research Ethics Committee
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPSA	Gene coding for 37/67kDa non-integrin laminin receptor

RTK	Receptor tyrosine kinase
scr	Scramble gene sequence
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	Secreted
SEM	Standard error of mean
Sho	Shadoo
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SMARCB1	SW1/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily B, member 1
SNP	Single nucleotide polymorphism
Src	Proto-oncogene tyrosine-protein kinase Src
STAT1	Signal transducer and activator of transcription 1
Sup.	Supernatant
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween
TCS	Tocris prion inhibitor 13 / TCS prion inhibitor 13
TM	Transmembrane domain
TNF	Tumour necrosis factor
TRC	The RNA consortium
TRK	Tropomyosin kinase
UPR	Unfolded protein response
VEGFR	Vascular endothelial growth factor receptor
VS	Vestibular schwannoma
WHO	World Health Organisation
wt	Wild type
XIAP	X-linked inhibitor of apoptosis protein

8. Publications

The following publication has been removed due to copyright restrictions:

Provenzano. L, Ryan. Y, Hilton. D.A, Lyons-Rimmer. J, Dave. F, Maze. E.A, Adams. C.L, Rigby-Jones. R, Ammoun. S, Hanemann. C.O. **Cellular Prion Protein (PrP^C) in the development of Merlin-deficient tumours**. 2017. Oncogene, 36(44): 6132-6142. DOI: 10.1038/onc.2017.200