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Published in:
Oncogene

DOI:
10.1038/onc.2017.200

Publication date:
2017

Document version:
Peer reviewed version

Cellular prion protein (PrP^C) in the development of Merlin-deficient tumours

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Running title: PrP^C in Merlin-deficient tumours

This work was supported by The Laura Crane Youth Cancer Trust and Brain Tumour Research

Abstract

Loss of function mutations in the gene neurofibromatosis Type 2 (*NF2)*, coding for a tumour suppressor Merlin, cause multiple tumours of the nervous system such as schwannomas, meningiomas and ependymomas. These tumours may occur spontaneously or as part of the hereditary condition Neurofibromatosis Type 2 (NF2). Current treatment is confined to (radio) surgery and no drug therapies exist. *NF2* mutations and/or Merlin inactivation are also seen in other cancers including some mesothelioma, breast cancer, colorectal carcinoma, melanoma and glioblastoma. To study the relationship between Merlin-deficiency and tumourigenesis we have developed an *in vitro* model comprising human primary schwannoma cells, the most common Merlin-deficient tumour and the hallmark for NF2. Using this model we show increased expression of cellular prion protein (PrP^C) in schwannoma cells and tissues. Additionally, a strong overexpression of PrP^C is observed in human Merlin-deficient mesothelioma cell line TRA and in human Merlin-deficient meningiomas. PrP^C contributes to increased proliferation, cell-matrix adhesion and survival in schwannoma cells acting via 37/67kDa non-integrin laminin receptor (LR/37/67kDa) and downstream ERK1/2, PI3K/AKT and FAK signalling pathways. PrP^C protein is also strongly released from schwannoma cells via exosomes and as a free peptide suggesting that it may act in an autocrine and/or paracrine manner. We suggest that PrP^C and its interactor, LR/37/67kDa, could be potential therapeutic targets for schwannomas and other Merlin-deficient tumours.

Key words

Cellular Prion Protein (PrP^C), Merlin-deficient tumours, Schwannoma, Neurofibromatosis Type 2 (NF2), 37/67kDa non-integrin laminin receptor

Introduction

Mutations in a gene coding for the tumour suppressor protein Merlin (*NF2*) lead to the development of multiple tumours of the nervous system including schwannomas, meningiomas and ependymomas. These tumours occur spontaneously or as a part of the hereditary disease Neurofibromatosis type 2 (NF2) (1). *NF2* gene mutations/Merlin inactivation are also found in proportion of breast cancer, colorectal carcinoma, melanoma, glioblastoma and mesothelioma (2). Current treatments for NF2, surgery and radiosurgery, are invasive and cannot be used in all patients, therefore new drug-based treatments are needed (3). Schwannoma is the most common Merlin-deficient tumour and the hallmark for NF2 (4). Using a primary human schwannoma *in vitro* cell model we have previously uncovered signalling pathways involved in schwannoma development including; ERK1/2, FAK, Src, NFκB, JNK, PI3K/AKT, p53/MDM2 and the ubiquitin ligase $CRL4^{DCAF1}$ (5-8).

A cDNA array hybridisation and RT-PCR highlighted significantly increased activity of the prion protein (PrP)-encoding gene, *PRNP*, in schwannoma compared to Schwann cells (9). Cellular prion protein PrP^C is a *N*-glycosylated protein, tethered to the cell membrane via a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (10). PrP^C is expressed in CNS neurons and glial cells (10) as well as in PNS axons and associated Schwann cells (11) displaying important biological functions such as myelination maintenance, neural precursor proliferation and adult neurogenesis (12-14). PrP^C is overexpressed in different cancers including colorectal cancer, breast cancer, pancreatic cancer and melanoma regulating proliferation, cell adhesion and survival (15) via key signalling pathways [cyclin D1, ERK1/2, caspase-3 and PI3K/AKT (16, 17)], previously shown to be involved in schwannoma development (18, 19). PrP^C can be released from cells, taking part in autocrine and/or paracrine

signalling (20, 21). PrP^C has been shown to bind to the cell surface 37/67kDa laminin receptor protein (LR/37/67kDa) which could be potentially targeted by anti-LR 37/67kDa antibodies (22) and LR/37/67kDa/laminin interaction inhibitor NSC47924 (23).

We demonstrate, for the first time, that (a) PrP^C protein is strongly overexpressed in schwannoma compared to control Schwann cells/tissues; (b) that this overexpression is negatively regulated by Merlin and depends on NFκB; (c) PrP^C levels are also highly increased in Merlin-deficient human mesothelioma cell line TRA and Merlin-deficient human meningioma grade I tissues and primary cells; (d) PrP^C increases schwannoma cell proliferation, survival and cell-matrix adhesion acting via LR/37/67kDa and downstream ERK1/2, PI3K/AKT, and FAK signalling pathways; (e) PrP^C is released from schwannoma cells, both via exosomes and also as cleaved peptides. We therefore suggest that decreasing PrP^C levels either by using humanized anti-PrP antibody PRN100 [clinical trials are planned by MRC Prion Unit for sporadic Creutzfeldt-Jakob disease (24)]; or proteasome inhibitor (targeting NFκB) Bortezomib [FDA approved for multiple myeloma (25)]; or targeting PrP^C receptor and/or the, LR/37/67kDa laminin receptor, using inhibitors such as NSC47924 (23) or antibodies (22) could be a good therapeutic strategy for schwannoma and other Merlin-deficient tumours.

Results

Cellular di-glycosylated form or prion protein PrP^C is strongly overexpressed in schwannoma

Immunocytochemistry shows increased PrP staining in Merlin negative human primary schwannoma cells (NF2-/-), throughout the cytoplasm and cell membranes (Figure 1a, right panel) compared to the weak staining seen in control human Schwann cells (NF2+/+) (Figure 1a, left panel). Immunohistochemistry on formalin-fixed, paraffin-embedded tissues also shows a very high level of PrP expression in human schwannoma tumours (Figure 1b, third panel) compared to healthy nerve (Figure 1b, first panel) and traumatic neuroma (Reactive non-neoplastic proliferation of, Schwann cells and perineurial cells caused by injury or trauma) sections (Figure 1b, second panel). These data are confirmed by Western blotting, demonstrating a strong PrP overexpression in schwannoma compared to Schwann cells and tissues (Figure 1c and Supplementary Figure 1a right panel).

To define which form of the PrP is overexpressed in schwannoma (i.e. either the normal cellular PrP^C, or pathogenic infectious proteinase K-resistant PrP^{Res}), cells were either lysed normally with the addition of protease inhibitors or with proteinase K (16). Western blot analysis of schwannoma lysates treated with proteinase K show complete ablation of PrP (Figure 1d, right lane) compared to control (Figure 1d, left lane) suggesting that the cells express cellular PrP^C and not PrP^{Res}.

Mature, di-glycosylated PrP^C has been linked to resistance to apoptosis in several cancers whereas lack of glycosylation is linked to PrP^C to PrP^{Res} conversion (26). Using a PNGaseF de-glycosylation assay we show that the PrP^C expressed in both Schwann and schwannoma cells is di-glycosylated (Figure 1e left panel). Both samples show two clear de-glycosylated bands which account for the presence of

mono- and completely un-glycosylated PrP^C after treatment with PNGaseF. The replicates are highly variable but no difference in the ratio of mono-: un-glycosylated bands between Schwann and schwannoma samples was observed (Figure 1e right panel).

PrP^C overexpression is due to increased transcription and translation and not due to decreased proteosomal degradation

Treatment of schwannoma cells with transcription inhibitor actinomycin D (ActD) (10nM, 24h) (Figure 1f) and translation inhibitor cycloheximide (CHX) (1μM, 24h) reduced PrP^C levels by ~80% (Figure 1g). Additionally, proteosomal degradation inhibitor MG132 (1μM, 24h) increased PrP^C level by ~50% (Figure 1h). These and our previous data on *PRNP* gene activation in schwannoma (9), suggest that increased PrP^C level is due to increased transcription and translation and not due to intracellular accumulation caused by decreased proteosomal degradation (27). Similarly to schwannoma, PrP^C is also upregulated in human Merlin-deficient mesothelioma cell line TRA (28) (Figure 1i) and in Merlin-deficient human grade I meningioma tissues (Figure 1j) and primary cells (Figure 1k and Supplementary Figure 1b) compared to controls.

Merlin-reintroduction is sufficient to reduce PrP^C levels in Merlin-deficient-schwannoma and -mesothelioma but not -meningioma cells

To investigate if PrP^C overexpression is due to Merlin deficiency we reintroduced Merlin (*NF2)* wild-type protein into schwannoma, TRA and meningioma grade I cells, using recombinant adenovirus AdNF2 (29). Interestingly, Merlin-reintroduction reduced PrP^C levels in both schwannoma (Figure 2a) and TRA (Figure 2b) cells but

not in meningioma cells (Figure 2c), suggesting that in Merlin-negative meningioma, additional mutations may be involved in the regulation of PrP^C [(30-33) and (34)].

PrP^C expression in schwannoma cells is up-regulated by NFκB

To find potential therapeutic targets downstream of Merlin which are involved in increased levels of PrP^C we inhibited two major transcription regulators in schwannoma, ubiquitin E3 ligase $CRL4D^{CAF1}$ using shRNA (35, 36) and NFκB using SN50 inhibitor (8μg/ml, 72 hours) (5). We demonstrate that PrP^C overexpression in schwannoma cells was not affected by $CRL4D^{CAF1}$ shRNA (Figure 2d) but, instead, was significantly decreased upon treatment with NFκB inhibitor SN50 (Figure 2e and Supplementary Figure 2). Thus, in schwannoma PrP^C overexpression is due to Merlin-deficiency and downstream activation of transcription factor NFκB.

PrP^C is released mainly via exosomes from schwannoma cells

A small but significant increase in PrP^C was detected by ELISA in the medium from schwannoma cells compared to Schwann cells (Figure 3a). Since PrP^C can be released via exosomes (20), we first checked the expression of exosome/late endosome marker CD63 in schwannoma and Schwann cells. Figure 3b demonstrates strong overexpression of CD63 in schwannoma compared to Schwann cells, suggesting higher levels of exosomes/late endosomes in these cells (Figure 3b). Additional ELISA experiments, on GFM media containing exosome-free FCS, show that the majority of PrP^C released from schwannoma cells is released via exosomes and only a small portion as free peptides. The levels of exosome-bound PrP^C (Figure 3c, dark grey bar) are 6-fold higher compared to the levels of free PrP^C (Figure 3c, black bar) present in the supernatant fraction left over after exosomes

were isolated. This was compared to 'whole' cell culture medium which was simultaneously collected and run (Figure 3c, medium light grey bar) and fresh GFM as a negative control (Figure 3c, white bar). A similar pattern was demonstrated by Western blotting where clear bands for PrP^C are seen in the exosome fraction (Figure 3d, second row). PrP^C is a glycoprotein and therefore its molecular weight spans between 30 to 37 kDa(10). No band for PrP^C is detectable in the supernatant fraction of medium remaining after exosome isolation collected from schwannoma cells (Figure 3d, third row) which could be due to lower sensitivity of western blot technique compared to ELISA. Furthermore, immunocytochemistry showed co-localisation between PrP^C and CD63 in schwannoma cells (Figure 3e). The levels of free PrP^C are not altered upon treatment of cells with PIPLC (0.2U/ml, 3h) which cleaves PrP^C at the GPI anchor (37) (Figure 3f) and there is no significant difference in the amount of PrP^C released into the cell culture medium after PIPLC treatment (Figure 3g). Thus free PrP^C (Figure 3c) is released by other mechanisms than PIPLC-mediated cleavage. One alternative mechanism is ADAM10 metalloproteinase-mediated cleavage (38). We demonstrate that upon schwannoma cell treatment with ADAM10 inhibitor GI254023X (20μM) for 3 hours the levels of PrP^C increased in cell lysates (Figure 3h) and decreased in culture media (Figure 3i), suggesting that in schwannoma cells PrP^C cleavage and release is mediated at least partially by ADAM10 metalloproteinase.

Reducing PrP^C levels decreases proliferation and survival of schwannoma cells

To investigate functional relevance of PrP^C overexpression we used two methods to reduce PrP^C expression in schwannoma cells; (1) two sets of *PRNP* shRNA lentiviral

particles; (2) an anti-prion agent TCS and both techniques strongly reduced PrP^C expression (Supplementary Figures 3ai, 3aii and 3b). Since, *PRNP* shRNA lentiviral particles from Santa Cruz and *PRNP* shRNA lentivirus from GE Dharmacon displayed similar efficiency for PrP knock down and the decrease of downstream signalling pathways (Supplementary Figures 3ai and 3aii) we combined the data for both shRNA's. We demonstrate that the number of proliferating, Ki67-positive cells, and total cell number (DAPI) were significantly reduced upon treatment with *PRNP* shRNA (Figures 4a, 4i and Supplementary Figure 4a) and TCS (Figures 4b, 4j and Supplementary Figure 4b), as were levels of proliferation marker, cyclin D1 (Figures 4c and 4d). Cells show an increase in apoptosis, detected by cleaved caspase-3 immuno-labelling, upon depletion of PrP^C using both *PRNP* shRNA (Figures 4e and 4i) and TCS (Figures 4f and 4j), and confirmed by Western blot analysis (Figures 4g and 4h). The percentage of alive cells was significantly decreased both using TCS and *PRNP* shRNA (Supplementary Figures 4c and 4d). We also demonstrate that PrP synthetic peptide (amino acid residues 105 - 120 of the human prion protein, N-terminal) (0.8μM, 72 hours) has a cyto-protective role in schwannoma; rescuing cells from hydrogen peroxide H_2O_2 (500μM, 12 hours) mediated cell death (Figure 4k, l m and Supplementary Figure 4e).

Reducing PrP^C levels decreases cell matrix adhesion in schwannoma

Schwannoma display increased cell-matrix adhesion (39) and PrP^C has been shown to affect cell adhesion via interactions with laminin and the neural cell adhesion molecule (NCAM) in hippocampal neurons (10). Therefore, we investigated whether reducing levels of PrP^C would decrease cell adhesion to the laminin/poly-lysine cell matrix. After knockdown of *PRNP* mRNA, cells were less capable of adhering to the

cell matrix (Figure 4n). The same reduction in adhesion was also seen upon depletion of PrP^C using TCS (Figure 4o), n=3). These data suggest that PrP^C is involved in the pathological cell-matrix adhesion of schwannoma.

Decreasing expression of PrP^C alters activity and expression of several key signalling pathways known to be involved in schwannoma development

We next analysed whether signalling via the key pathways (PI3K/AKT, ERK1/2 and FAK) known to be involved in schwannoma proliferation, survival and cell-matrix adhesion were affected by reducing PrP^C. The MAPK pathway has previously been linked to PrP^C signalling (40).

ERK. Levels of total ERK1/2 are significantly reduced upon treatment with *PRNP* shRNA and high concentrations of TCS (Figures 5a and 5b, dark grey bars). Active pERK1/2 expression (light grey bars), was also significantly reduced with *PRNP* shRNA and at both 100μM and 500μM TCS inhibitor concentrations (Figures 5a and 5b, light grey bars).

AKT. Although a slight tendency for total AKT to decrease upon PrP knockdown and TCS treatment, the values are non-significant (Figures 5c and 5d dark grey bars). Levels of active pAKT, however, significantly decrease with *PRNP* shRNA and TCS (Figures 5c and 5d, light grey bars).

FAK. Total FAK is significantly reduced after *PRNP* knockdown and upon treatment with high levels (500μM) of TCS (Figures 5e and 5f, dark grey bars). Levels of auto-phosphorylated FAK^{Y397} are significantly reduced with *PRNP* shRNA and also at all concentrations of TCS (Figures 5e and 5f, light grey bars).

Thus PrP^C is linked to increased expression and activation of ERK1/2, FAK and the activation of PI3K/AKT pathway in schwannoma cells which are known to be

involved in schwannoma development (18, 19).

Treatment with PrP peptide and overexpressing PrP^C increases Schwann cell proliferation and expression of c-Jun

Next we looked at the effects of increasing PrP^C levels in Schwann cells to see if a pathological phenotype could be induced. First, we used a PrP synthetic peptide to mimic the effect of free PrP in cell culture media (41, 42). Schwann cells were stimulated for 6 days with 0.8μM of PrP peptide before looking at levels of proliferation (Ki67) and expression of the proto-oncogene c-Jun which is involved in Schwann cells proliferation and overexpressed/activated in schwannoma via c-Jun N-terminal kinase (JNK) leading to increased tumour proliferation and survival (43). Both the number of Ki67 positive cells (Figure 6a, red staining) and c-Jun positive cells (Figure 6b, green staining) increased in cells treated with PrP (105 – 120) peptide compared to untreated cells in GFM media. Cell morphology examined by the cytoskeletal F-actin marker phalloidin, showed that Schwann cells looked more like schwannoma tumour cells with an increase in cell spreading and membrane ruffling after treatment with PrP peptide (0.8μM) for 6 days and two weeks compared to untreated cells (Figure 6c). Cell area was measured using ImageJ software in n=52 cells (6 days) and in n=50 cells (2 weeks) (Figure 6c right panel).

Next, we checked if PrP (105 – 120) peptide treatment had caused the transformation of endogenous PrP^C protein into protease-resistant PrP^{Res} (44). Cells were lysed either in the presence of protease inhibitors or instead with proteinase K after being treated for six days with PrP (105 – 120) peptide. Results show that cells still expressed normal protease-sensitive PrP^C after treated with PrP (105 – 120) (Figure 6d).

Moreover, we produced *PRNP* overexpressing lentiviruses using a pLenti6.2/V5-DEST plasmid (Supplementary Figure 5), to overexpress full length PrP^C in Schwann cells. DNA from *PRNP* coding region (NCBI Reference Sequence: NG_009087.1) from two separate transformation starter colonies (O/E1B, Figure 6e and O/E2B, Supplementary Figure 6) were used. Western blotting shows that overexpression of PrP^C significantly increases levels of both Cyclin D1 and c-Jun in Schwann cells compared to the GFP-containing control (Figures 6e-g and Supplementary Figure 6). These data confirm our results obtained using *PRNP* shRNA and TCS in schwannoma cells indicating the crucial role of PrP^C in schwannoma development.

PrP^C employs LR/37/67kDa to regulate cellular functions

Membrane-bound as well as externally added PrP^C has been previously shown to interact with the LR/37/67kDa observed in murine N2a neuroblastoma and hypothalamic neuronal mouse GT1 cell lines (23). We demonstrate that LR/37/67kDa is expressed in schwannoma cells (Supplementary Figure 7a).

LR/37/67kDa co-localises with PrP^C in the cytosol, cell membrane and membrane ruffles in permeabilised schwannoma cells (Figure 7a) and in membrane ruffles of non-permeabalised cells (Figure 7b). Additionally, LR/37/67kDa interacts with PrP^C in schwannoma cell lysates demonstrated by co-immunoprecipitation using both proteins as bait (Figures 7c and 7d). Stimulation of starved schwannoma cells with synthetic PrP (105 – 120) peptide for 24 hours increased expression of cyclin D1 (Figures 7e, light grey bars and 7i, row 1) and, after one hour increased activation of ERK, AKT and FAK (Figures 7f-h, light grey bars and 7i, rows 2-4) which was reverted using LR/37/67kDa shRNA (Figures 7e-h, dark grey bars and 7i, rows 1-4). We used a mock PrP peptide to show that the effect of the synthetic PrP (105 – 120)

peptide is specific (Supplementary Figure 7b).

Discussion

In the current study we found, that PrP^C is expressed at protein level in Merlin-deficient human primary schwannoma cells and tissues compared to controls. Surprisingly, this increase is due to upregulated *PRNP* transcription and translation *per se* and not by, as previously reported, decreased proteasomal degradation (27). We have also revealed that PrP^C overexpression translates to other Merlin-deficient tumours, such as human Merlin-negative mesothelioma cell line TRA and human Merlin-negative grade I meningioma cells/tissues. Interestingly, Merlin re-introduction strongly reduced PrP^C levels schwannoma and TRA cells but not in meningioma. This could be due to mutation in additional tumour suppressors, observed in Merlin-deficient meningiomas, such DAL-1 (differentially expressed in adenocarcinoma of the lung)/4.1B protein (30, 31, 45), BCR (breakpoint cluster region) (32), or SMARCB1 (46) which requires further investigations.. We also revealed that PrP^C overexpression in schwannoma cells is regulated by transcription factor NFκB which we previously shown is regulated by Merlin (6). NFκB has been shown to be involved in PrP-mediated invasion and migration in breast cancer cells (47) and can be activated by PrP peptide PrP(106-126) in human monocyte-derived dendritic cells (48), however its role in the regulation of PrP^C expression has never been reported and importantly opens a new therapeutic angle.

The pathological relevance of PrP^C overexpression is emphasised by its increased release from schwannoma cells compared to Schwann cells which suggest an effect via auto- and/or paracrine mechanisms (49).. Interestingly, PrP^C is released both as free (cleaved) peptide,, by ADAM10 metalloproteinase cleavage, and in association with exosomes. Although, there are previous observations showing exosome-mediated release of PrP^C from various cell types such as activated

platelets, rat and mouse cortical neurons and mouse neuroblastoma cells (50-52) our findings demonstrate this mechanism for the first time in human primary tumour cells.

Concerning the mechanism of action, we have shown that PrP^C contributes to schwannoma development by increasing the expression of the cell cycle regulator cyclin D1 resulting in pathologically enhanced proliferation. Although one study performed on cortical neurones showed no effect of PrP on cyclin D1 levels (53), most studies agree with our findings (54). PrP^C-deficient fibroblasts displayed a significant down-regulation of cyclin D1 (54) and, in gastric carcinoma, PrP^C accelerated the G1/S phase transition via induction of cyclin D1 through PI3K/AKT pathway (55), supporting our finding on the role of PrP^C in cyclin D1 expression and AKT activation. We also demonstrate that ERK1/2 activation, which is involved in schwannoma proliferation (56), is potentiated by PrP^C. This is in agreement with other studies reporting the role of MAPK pathway in PrP^C-induced neuritogenesis (53). PrP^C in schwannoma cells contributes to increased pathological survival of these cells, which parallels the majority of work in the field (57). However, a link between PrP^C overexpression and increased cell death via caspase-3 has been reported in murine TSM1 neuronal cells and HEK293T cells (57). Importantly, we found that PrP^C has cytoprotective effect in schwannoma, since pre-treatment with PrP (105 – 120) peptide rescued schwannoma cells from H_2O_2 oxidative stress-mediated cell death (58).

PrP^C plays a role in cell matrix adhesion of schwannoma cells in accordance with previous findings revealing PrP^C interaction with adhesion molecules such as LR/37/67kDa (59) and integrins (60). FAK, a component of focal adhesion complexes is overexpressed in schwannoma (5). Previous findings have shown that

PrP^C silencing induces focal adhesion remodelling (61), now our findings strengthen the link between PrP^C and FAK as we demonstrate that reducing levels of PrP^C decreases cell-matrix adhesion as well the activity and expression of FAK. Essentially, we have shown by knock down experiments that the PrP^C-mediated activation of cyclin D1, ERK1/2, AKT and FAK pathways is due to PrP^C interaction with LR/37/67kDa, suggesting that LR/37/67kDa acts as a cell surface receptor for PrP^C in schwannoma cells.

These results were complemented by either treating Schwann cells with synthetic PrP (105 – 120) peptide or by overexpressing *PRNP*. Both treatments dramatically changed Schwann cells' morphology, increased cell proliferation and the expression of the major regulator of Schwann cell proliferation c-Jun (62-64).

In summary, we demonstrate that PrP^C protein is overexpressed in human schwannoma cells and tissues and plays a part in schwannoma proliferation, cell matrix adhesion and survival. Importantly, PrP^C overexpression was also observed in human mesothelioma cell line TRA and in human grade I meningioma cells and tissues. PrP^C is released in schwannoma cells by exosomes and as free peptides which may contribute to tumourigenesis by autocrine or/and paracrine signalling. PrP^C interacts with and acts via LR/37/67kDa to activate cyclin D1, ERK1/2, PI3K/AKT and FAK in schwannoma cells. Thus, PrP^C and LR/37/67kDa, are good potential therapeutic targets for schwannoma and other Merlin-deficient tumours. PrP^C-mediated signalling can be inhibited in two main ways: firstly, by targeting PrP^C directly using humanized anti-PrP antibody PRN100 (MRC Prion Unit for sporadic Creutzfeldt-Jakob disease) (24) or using the proteasome inhibitor (targeting NFκB) Bortezomib (FDA approved for multiple myeloma treatment) (25); secondly, by blocking LR/37/67kDa using inhibitors such as anti-LR/37/67kDa antibodies (22),

polysulfated glycans (59), small interfering RNAs (65) and LR/37/67kDa-Laminin interaction inhibitor NSC47924 (23).

Material and Methods

Cell cultures

After informed consent, schwannoma and meningioma grade I specimens were collected from surgical procedures and normal nerve from post-mortem donors. Schwannoma and Schwann cells were cultured in growth factor medium (GFM): DMEM; 100U/ml pen/strep; 2.5 µg/ml Insulin (Thermo Fisher 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) at 37ºC in 10% CO2 (66). Meningioma cells were grown in DMEM containing glucose (4.5 g/l), 10% FBS, 100 U/ml penicillin/streptomycin and 1% glutamine. Human meningeal cells (HMC, Sciencell, CA, USA) were grown in the recommended HMC medium (Sciencell) at 37 °C and 5% CO_2. Human mesothelioma cell lines HIB and TRA were cultured in DMEM, containing 10% FBS and 100 U/ml pen/strep at 37 °C and 5% CO2. (39). Human schwannoma and meningioma grade I primary cells (passages 1-4) and tissues used in this study are Merlin-negative (Supplementary Figure 1a and Figure 1k). All Schwann cell cultures are S100 positive (Supplementary Figure 1b). The n number states number of biological repeats.

Chemicals

TCS anti-prion agent 13 was from Tocris Bioscience (67), Proteinase K, MG132, Cycloheximide (CHX), Actinomycin D (Act. D), Phosphatidylinositol Phospholipase C

(PIPLC) and DAPI were from Sigma (MO, USA). Synthetic PrP peptide (AA105-120, KTNMKHMAGAAAAGAVVGGLG) was from AbD Serotec (Bio-Rad, CA, USA) and mock peptide (NGAKALMGGHGATKVMVGAAA) was kindly provided by Dr. M. Salmona of the Instituto Ricerche Farmcologiche Mario Negri, Italy. NFκB inhibitor SN50 was from Calbiochem (La Jolla, CA, USA) and ADAM 10 inhibitor GI254023X from Sigma (Sigma, MO, USA).

Exosome Isolation

Cells were cultured for 7 days in medium containing exosome-free FBS (Gibco). Exosomes were isolated using total exosome isolation reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Enzyme-linked Immunosorbent assay

Cells were cultured for 3 days in GFM; medium was collected and concentrated 10x using AmiconUltra® filters (Merck Millipore, MA, USA). PrP^C was detected using Prion Protein (*PRNP*) ELISA kit (CUSABIO, Hubei, China, ABIN821046) according to the manufacturer's protocol.

Immunocytochemistry

Immunocytochemistry was performed as described in Flaiz et. al 2009 (68) using anti-human-PrP^C (Santa Cruz Biotechnologies, CA, USA), anti-CD63 (Merck Millipore), anti-c-Jun (Cell Signalling Technology, MA, USA) and anti- LR/37/67kDa (Novus Biologicals, CO, USA) antibodies. Alexa Fluor 488-labeled phalloidin (Life Technologies, CA, USA) was used to visualize actin filaments (1:100; Molecular Probes, Eugene, OR). Multitrack imaging was performed using a Zeiss Confocal

LSM510.

Immunohistochemistry

Tissue sections were pre-treated with EDTA buffer at pH 9 for 30 min, before overnight incubation with the anti-PrP^C12F10 (1:1000; Bioquote, York, UK). Detection was performed with the Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK).

Proliferation and survival assays

Proliferation and survival assays were carried out using immunofluorescence, after 72 hours incubation with viral particles or treatment with TCS prion inhibitor, with anti-Ki67 (Dako) and anti-cleaved caspase-3 (Cell Signalling Technologies) antibodies. Multitrack imaging was performed using a Zeiss Confocal LSM510.

MTS test

Schwannoma cells were pre-treated with 0.8μM of PrP peptide for 72 hours followed by addition of H_2O_2 (500μM) for additional 12 hours. MTS test was performed according to manufacturer's protocol (Promega).

De-glycosylation assay

Cell lysates were treated with 5% sodium dodecyl sulphate, 1M Dithiothreitol, 0.5M Sodium phosphate buffer (pH7.5), 10% Triton X-100 and PNGase F (Promega, WI, USA) at 37ºC for 1-3h before being run on SDS-PAGE.

Adhesion assay

Cells were seeded onto pre-coated 96-well plates for three hours using different

conditions: DMEM and 20μM, 100μM and 500μM anti-prion agent TCS.. *PRNP* and scramble shRNA were selected prior to being split and plated. Cells were counted and adhesion assay was performed as described previously in Utermark et al (39).

Merlin re-introduction

Merlin (*NF2*) wild type (recombinant adenovirus AdNF2) and control GFP-containing vector adenoviruses were a kind gift from J. Testa (29). Cells were treated with virus for 24 hours and then incubated with fresh GFM for additional 24 hours and lysed.

Knock-down experiments using shRNA's

Cells were incubated with shRNA and scramble control viruses for 48 hours, before selection with puromycin for 72 hours. *PRNP* shRNA lentiviral particles (Santa Cruz Biotechnologies); pool of three to five expression constructs each encoding a different, target-specific 19-25nt region of PrP^C were used to knock down *PRNP* expression plus control hairpin shRNA particles. Results were combined with lentivirus made from TRCN0000083488, part of a TRC *PRNP* shRNA, glycerol set (GE Dharmacon, CO, USA). 37/67kDa Laminin Receptor shRNA (h) lentiviral particles (Santa Cruz Biotechnologies) were used to knock down LR/37/67kDa (h) (pool of three to five expression constructs that encode a 19-25 nt (plus hairpin)).

***PRNP* overexpressing clone**

DNA from *PRNP* coding region (NCBI Reference Sequence: NG_009087.1) from two separate transformation starter colonies (1B and 2B) were cloned into a pLenti6 overexpressing vector (Invitrogen). Lentiviral particles were produced by transfection into HEK293FT cells using Fugene 6 (Promega).

Co-Immunoprecipitation

Primary human schwannoma cells (1mg) lysed in low-salt buffer was incubated overnight with 1µg normal IgG (Santa Cruz Biotechnologies) or 1µg anti-PrP antibody (Sigma), 37/67kDa anti-laminin receptor (Abcam) in the presence of protein G agarose beads (GE healthcare).

Western Blotting

After Western blotting (39) membranes were incubated with primary antibodies: anti-PrP, anti-cleaved caspase-3, anti-cyclin D1, anti-total MAPK/ERK1/2, anti-total FAK, anti-pFAK Tyr^{397}, anti-total AKT, anti-pAKT Ser^{473}, anti- active MAPK; anti-$pThr^{183}$-$pTyr^{185}$-ERK1/2; Promega), anti-37/67kDa laminin receptor (Novus Biologicals), anti-CD63 (Merck Millipore), second anti-PrP^{C} (AbD Serotec; N-terminus) and anti-Merlin antibody (Cell Signalling) (D1B8 clone cat. number 6995). Horseradish peroxidase-conjugated secondary antibodies (BioRad) and chemiluminescence (Thermo Fisher Scientific) were used for detection. GAPDH (Merck Millipore) was used as a loading control. Densities were quantified using FluorS-Multi-Imager with Quantity One software (Biorad).

Statistics

Unpaired student's two-tailed t-test was used in all pairwise comparisons and analysis of variance (one-way Anova) followed by post-hoc tukey test for multiple comparisons. F-test was used to compare variances and standard deviations between the groups compared to meet the assumption of the statistical test. Experiments were performed in at least triplicates using at least three different batches of cells from different individuals. In some experiments samples from more

than three different individuals were used. ns (not significant) $p>0.05$, * $p<0.05$, **$p<0.01$, ***$p<0.001$. In figures mean ± SEM is given.

Conflict of interest

The authors declare no conflict of interests.

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Acknowledgments

The authors thank Mario Salmona for mock PrP peptide and Joseph Testa for Merlin adenovirus. This work was supported by The Laura Crane Youth Cancer Trust.

Figure legends

Figure 1.

Expression of PrP^C in human Schwann cells (NF2+/+) and schwannoma cells (NF2-/-). (**a-c**) PrP is overexpressed in schwannoma compared to Schwann cells (a, n=5; b, n=20; c, n=5). PrP localises to the membrane ruffles and throughout the cytosol of schwannoma cells (a, n=5). (**d**) PrP^C in schwannoma is degraded upon treatment with proteinase K (PK), n=5. (**e left and right**) Glycosylation patterns of PrP^C between Schwann cells (NF2+/+) and schwannoma (NF2-/-) cells show no difference in the ratio of mono- and un-glycosylated PrP^C between these two cell types (n=5). (**f and g**) Treatment of schwannoma cells with actinomycin D (ActD) (f, n=5) and with cycloheximide (CHX) (g, n=6) reduces PrP^C expression in schwannoma cells. (**h**) Inhibition of proteasomal degradation by MG132 increases PrP^C levels in schwannoma cells, n=6. **(i)** PrP^C is strongly overexpressed in human Merlin-deficient mesothelioma cell line TRA compared to human Merlin-positive mesothelioma cell line HIB (n=3). **(j and k)** Human Merlin-negative (Fish analysis of chromosome 22q loss (69)) meningioma grade I tissues (j, n=10) and primary cells (k, n=8) strongly overexpress PrP^C compared to normal Merlin-positive meningeal tissues and primary cells. Data are presented as $\pm$SEM (Ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.00$).

Figure 2.

The regulation of PrP^C expression. **(a-c)** Adenoviral reintroduction of the Merlin/NF2 gene into human Merlin-negative schwannoma (a, n=3), Merlin-negative TRA (b, n=3) and Merlin-negative meningioma grade I cells (c, n=3) significantly decreased PrP^C expression in schwannoma and TRA cells but not in meningioma cells. **(d)** $CRL4D^{CAF1\ shRNA}$ has no effect on PrP^C expression. **(e)** NFκB inhibitor SN50

significantly decreased PrP^C levels. Data are presented as ± SEM (Ns p>0.05, *p<0.05, **p<0.01, ***p<0.00).

Figure 3.

PrP^C is released from schwannoma cells via exosomes. (**a**) PrP^C is released significantly more from schwannoma cells (NF2-/-, dark grey bar) compared to Schwann cells (NF2+/+, light grey bar) detected by ELISA, n=3. (**b**) Schwannoma cells (NF2-/-) display strong expression of exosome/late endosome marker CD63 compared to Schwann cells (NF2+/+), n=3. (**c and d)** The majority of PrP^C released from schwannoma cells is released via exosomes (c, dark grey bar, n=3 and d second raw, n=5), only a very small amount is released as free peptide in the supernatant (c, black bar, n=3). (**e**) PrP^C co-localises to late endosome/exosome marker CD63 in schwannoma cells, n=3. (**f and g**) Treatment of schwannoma cells with PIPLC showed no change to the levels of PrP^C within cells (f, n=3) and PrP^C released from cells (g, n=3). **(h and i)** In schwannoma cells PrP^C cleavage and release is mediated by ADAM10 metalloproteinase. Cells were treated with ADAM10 inhibitor GI254023X (20μM) for 3 hours and PrP^C levels were detected by Western blotting in cell lysates (h, n=5) and by ELISA in culture media (i). E Cadherin is used as a control for ADAM10 inhibition (70). Phalloidin visualise actin. Data are presented as ±SEM (ns p>0.05, *p<0.05,**p<0.01, ***p<0.001). Multi-track, z-stack confocal microscopy was used for immunofluorescence (d and e).

Figure 4.

The role of PrP^C in schwannoma proliferation, survival and cell-matrix adhesion. (**a and i**) Number of Ki-67-positive nuclei reduces upon depletion of PrP^C using *PRNP* shRNA, n=5. (**b and j**) Number of Ki-67-positive nuclei reduces upon depletion of

PrP^C using anti-prion agent TCS, n=5. (**c and d**) levels of Cyclin D1 significantly reduce with *PRNP* shRNA (c, n=5) and TCS treatment (d, n=5). (**e and i**) Reducing PrP^C levels significantly increases expression of cleaved caspase-3 using *PRNP* shRNA, n=3. (**f and j**) Reducing PrP^C levels using TCS significantly increases expression of cleaved caspase-3, n=3. (**g and h**) Both *PRNP* shRNA (g, n=5) and TCS (h, n=5) reduce PrP^C levels and significantly increase expression of cleaved caspase-3**. (k, l and m)** PrP synthetic peptide (KTNMKHMAGAAAAGAC corresponding to amino acid residues 105 - 120 of the human prion protein, N-terminal) rescues schwannoma cells from H2O2-mediated cell death. Cells were pre-treated with 0.8μM of PrP peptide for 72 hours followed by addition of H2O2 (500μM) for additional 12 hours (n=3). Ki67 staining was used to detect proliferating cells (red) (k and l) and cleaved caspase 3 staining (green) for apoptotic cells (k and m). (**n and o**) Reducing PrP^C by shRNA (l, n=3) and TCS (m, n=3) reduces cell-matrix adhesion. Data are presented as ±SEM (ns $p>0.05$,*$p<0.05$,**$p<0.01$, ***$p<0.001$). In i, j and k total cell number was monitored by DAPI staining (blue).

Figure 5.

Signalling pathways activated by PrP^C in schwannoma. (**a, c and e**) Reducing levels of PrP^C using *PRNP* shRNA significantly decreased levels of both total (a, dark grey bars, n=5) and pERK1/2 (a, light grey bar, n=5), $pAKT^{S473}$ (c, light grey bars, n=5) but not total AKT (c, dark grey bars, n=5) and both total (e, dark grey bars, n=5) and $pFAK^{Y397}$ (e, light grey bars, n=5). (**b, d and f**) Reducing levels of PrPC using TCS significantly decreased levels of both total (b, dark grey bars, n=5) and pERK1/2 (b, light grey bar, n=5), $pAKT^{S473}$ (d, light grey bars, n=5) but not total AKT (d, dark grey bars, n=5) and both total (f, dark grey bars, n=5) and $pFAK^{Y397}$ (f, light grey bars, n=5). Data are presented as ±SEM (ns $p>0.05$,*$p<0.05$,**$p<0.01$,***$p<0.001$). *PRNP*

shRNA from both Santa Cruz biotechnologies and GE healthcare were used and the results were merged (Supplementary figure 3ai and 3aii).

Figure 6.

Increasing PrP^C levels in normal Schwann cells (NF2+/+) leads them to obtain schwannoma-like properties. **(a-b**) Schwann cells treated with PrP (105 – 120) synthetic peptide for six days showed an increase in the number of Ki67-positive (a, n=3) and c-Jun-positive cells (b, n=3). **(c)** PrP synthetic peptide (105 – 120) altered cell morphology of Schwann cells over the course of six days and, even more so, after 2 weeks (c, phalloidin green, n=3). Cell area was measured using ImageJ software in n=52 cells (6 days) and in n=50 cells (2 weeks) (c right panel). (**d**) 6 days treatment with PrP (105 – 120) synthetic peptide the PrP in Schwann cells was still sensitive to proteinase K degradation (d, n=3). (**e-g**) overexpression of PrP^C with a clone of the *PRNP* gene significantly increases expression of Cyclin D1 (e and g, n=3) and c-Jun (f and g, n=3). Data are presented as ±SEM (*p<0.05,**p<0.01, ***p<0.001).

Figure 7.

PrP^C interacts with and signals via the 37/67kDa non-integrin laminin receptor protein (LR). **(a and b**) PrP^C co-localises with LR in schwannoma throughout the cytosol and in the membrane of permeabalised cells (a, n=3) and in discreet membrane ruffles of non-permeabalised cells (b, n=3). (**c and d**) Co-immunoprecipitation (co-IP) of LR in schwannoma cell lysates pulls down PrP^C (c, n=5) and co-IP of PrP^C in schwannoma cell lysates pulls down LR (d, n=8). (**e-h**) Treatment of schwannoma cells with PrP (105 – 120) synthetic peptide increases levels of cyclin D1 (e light grey bars, n=5), pERK1/2 (f light grey bars, n=5), $pAKT^{S473}$

(g light grey bars, n=5) and pFAK^{Y397} (h light grey bars, n=5). LR knockdown using shRNA reduces the ability of PrP peptide to increase levels of cyclin D1 (e, dark grey bars, n=5), pERK1/2 (f dark grey bars, n=5), pAKT^{S473} (g dark grey bars, n=5) and pFAK^{Y397} (h dark grey bars, n=5). (**i**) Western blot pictures corresponding to (e-h) graphs. Data are presented as ±SEM (ns $p>0.05$*, $p<0.05$,**$p<0.01$, ***$p<0.001$). Multi-track confocal microscopy z-stacks were used for co-localisation of immunofluorescence (a and b).

Supplementary Figure 1.

Western blot demonstrating **(a, left panel)** Merlin/NF2 status in human primary schwannoma cells (n=12 different patients) and (a, right panel) PrP expression and Merlin/NF2 status in human schwannoma tissues (n=7). **(b)** S100 staining on Schwann cell cultures. In (a, left) Merlin reintroduction was performed using recombinant adenovirus AdMerlin/NF2. GAPDH was used as a loading control.

Supplementary Figure 2.

Inhibition of NFκB (green) translocation into the nucleus by NFκB inhibitor SN50 (permeable inhibitory peptide). Schwannoma primary cells were treated with 8μg/ml SN50 for 72 hours and NFκB localisation monitored by immunocytochemistry. DAPI is a nuclear marker (Blue).**Supplementary Figure 3.**

Reduction of PrP^C using *PRNP* shRNA and anti-prion agent TCS.. (**a**) Treatment of schwannoma cells with a pool of five different *PRNP* shRNA hairpins (Santa Cruz) consistently reduced PrP^C expression (ai) as did homemade TRC lentivirus *PRNP*_88 (GE Dharmacon, aii). *PRNP* shRNA from both companies were equally effective in the decreasing of Cyclin D1 and pERK1/2 expression (ai, n=10 and aii, n=6; bottom panels). (**b**) Upon treatment with TCS at 20μM there was a tendency for PrP^C levels to be reduced although this was not significant, at 100μM and 500μM PrP^C expression in schwannoma was significantly reduced, n=10. Data are presented as ±SEM (ns $p>0.05$, * $p<0.05$,** $p<0.01$, *** $p<0.001$).

Supplementary Figure 4.

Reduction of PrP^C decreases total cell number and the percentage of alive cells. **(a-d**) Upon reduction of PrP^C in schwannoma cells using both *PRNP* shRNA (a, n=6) and almost all concentrations of anti-prion agent TCS.(b, n=5), the total cell number, judged using DAPI, was reduced. Both *PRNP* shRNA (c, n=6) and high levels of anti-prion agent TCS (d, n=4) reduced the number of alive schwannoma cells. (e, n=3) PrP synthetic peptide (KTNMKHMAGAAAAGAC corresponding to amino acid residues 105 - 120 of the human prion protein, N-terminal) rescues schwannoma cells from H2O2-mediated cell death. Cells were pre-treated with 0.8μM of PrP peptide for 72 hours followed by addition of H2O2 (500μM) for additional 12 hours (n=3, MTS test). Number of alive cells was calculated by subtracting the number of condensed or fragmented nuclei from the total cell number. Data are presented as ± SEM ($p>0.05$, $*p<0.05$,$**p<0.01$, $***p<0.001$).

Supplementary Figure 5.

Structure of the pLenti6.2/V5-DEST-PRNP overexpressing vector used to create *PRNP* overexpressing lentiviral particles. *PRNP* overexpressing clone was made using DNA corresponding to the coding region of the *PRNP* gene which was PCR cloned and inserted into pENTR11 entry vector before being gateway cloned into pLenti6.2/V5-DEST destination vector just after the CMV promoter region. Lentiviral particles containing the pLenti6.2/V5-DEST-PRNP vector were manufactured in HEK293FT cells before being collected and purified from the cell culture medium. Image courtesy of SnapGene®.

Supplementary Figure 6.

LR/37/67kDa (LR) expression. (**a**) LR is expressed both in Schwann cells (NF2+/+) and schwannoma cells (NF2-/-), there is no difference in LR expression between the two cell types, n=4. (**b**) Treatment of schwannoma cells with PrP (105-120) synthetic peptide but not a scramble mock peptide increases pAKT, showing specificity for the PrP (105-120) synthetic peptide, n=4. Data are presented as ± SEM ($p>0.05$, $*p<0.05$,$**p<0.01$, $***p<0.001$).

Supplementary Figure 7.

The overexpression of PrP^C significantly increases levels of both Cyclin D1 and c-Jun in Schwann cells compared to the GFP-containing control. DNA from *PRNP* coding region (NCBI Reference Sequence: NG_009087.1) from O/E2B transformation starter colonies was used.