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ALTERED GLYCOSYLATED PrP PROTEINS CAN HAVE DIFFERENT NEURONAL TRAFFICKING IN BRAIN BUT DO NOT ACQUIRE SCRAPIE-LIKE PROPERTIES*

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Running title: Glycosylation of PrP *in vivo*

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SUMMARY

N-linked glycans have been shown to have an important role in the cell biology of a variety of cell surface glycoproteins including PrP protein. It has been suggested that glycosylation of PrP can influence the susceptibility to transmissible spongiform encephalopathy (TSE) and determine the characteristics of the many different strains observed in this particular type of disease. To understand the role of carbohydrates in influencing the PrP maturation, stability and cell biology, we have produced and analyzed gene targeted murine models expressing differentially glycosylated PrP. Transgenic mice carrying the PrP substitution threonine for asparagine[180] (G1) or threonine for asparagine[196] (G2) or both mutations combined (G3), which eliminate the first, second and both glycosylation sites respectively, have been generated by double replacement gene targeting. An *in vivo* analysis of altered PrP has been carried out in transgenic mouse brains, and our data show that the lack of glycans does not influence PrP maturation and stability. The presence of one chain of sugar is sufficient for the trafficking to the cell membrane while the un-glycosylated PrP localization is mainly intracellular. However this altered cellular localization of PrP does not lead to any overt phenotype in the G3 transgenic mice. Most importantly, we found that, *in vivo,* un-glycosylated PrP does not acquire the characteristics of the aberrant pathogenic form (PrP^{Sc}) as was previously reported using *in vitro* models.

INTRODUCTION

Glycoproteins are subject to a number of post-translational modifications as they pass through the secretory pathway. During polypeptide chain synthesis N-glycosylation is initiated by the transfer of core glycans to target asparagines. Processing of core glycans into the complex type is then achieved in the endoplasmic reticulum (ER) and Golgi apparatus compartments (1). Protein-attached glycans have been shown to have a wide range of biological functions most notably stabilization of protein structure and cellular trafficking (2).

PrP is a glycoprotein attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (3-5). While its normal function has yet to be defined, expression of PrP is essential for the development of transmissible spongiform encephalopathy (TSE) or prion disease (6, 7). The TSEs are a group of fatal neurodegenerative diseases that can be sporadic, inherited, or acquired by infection. TSE diseases include scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and a number of human forms of the disease such as Creutzfeldt-Jackob disease (CJD), variant CJD (vCJD) linked with BSE, Gerstmann-Straussler-Scheinker syndrome (GSS), Kuru and fatal familial insomnia (FFI) (8-10). A central event in all prion diseases appears to be a conformational

modification of the normal cellular prion protein (PrP^{C}) from a soluble form with a predominant alpha-helical conformation to the pathogenic form (PrP^{Sc}) that is aggregated, rich in beta-sheets, partially resistant to proteinase-K digestion and insoluble in non-denaturing detergents (11).

PrP contains two N-glycan attachment sequences (N-X-T) at amino acids 180 and 196 in mice. These sites are variably glycosylated *in vivo* such that un-, mono- and di-glycosylated glycotypes are observed (12, 13). The biological significance of each of the glycotypes of PrP (un-, mono- and di-glycosylated) is unknown. Both N-glycosylation sites are conserved in the PrP gene (*Prnp*) from all species suggesting that N-glycans play an important role in the protein function (14).

A number of reports have shown that the lack of sugars can induce the PrP^{C} to PrP^{Sc} transition *in vitro*, suggesting that perturbations in glycosylation may contribute to the development of disease, destabilizing PrP^{C} structure and allowing it to acquire spontaneously PrP^{Sc}-like properties (15-19). It has also been reported that alterations in glycosylation may alter the intracellular trafficking of PrP (16, 17, 20-22).

To investigate the *in vivo* effect of glycosylation on PrP biochemical properties and its cellular biology we have developed a gene targeted transgenic model in which the host *Prnp* is replaced by a modified *Prnp* transgene in the correct genomic location (23). This model represents a valid tool to analyze the effect of mutations of the host *Prnp* in TSE susceptibility since the *Prnp* gene expression is controlled by the normal regulatory elements of endogenous PrP (24-28).

Three transgenic lines have been generated each containing a point mutation in the *Prnp* gene eliminating the first, second or both the glycosylation sites: N180T (G1); N196T (G2) and N180T-N196T (G3). Using these mice we have investigated whether the lack of glycans can alter the expression level of the PrP protein, its conformation and intracellular localization and its ability to acquire the biochemical characteristics of the pathogenic form.

We report here that while glycans appear to control the cellular location of PrP, the presence of sugars does not dramatically change the biology of PrP and there is no evidence of PrP^{Sc}-like properties in either mono- or un-glycosylated PrP.

The results reported here are important in determining the physiological function of PrP glycoforms and in understanding their role in the infectious and pathogenic process of TSEs.

EXPERIMENTAL PROCEDURES

Antibodies - Mouse monoclonal antibody 8H4, epitope (145-220) binding is independent of the N-linked glycosylation because it reacts with both recombinant PrP as well as all native glycoforms (29). 7A12 (epitope 90-140) is a mouse monoclonal anti PrP antibody (30). FH11 is a mouse monoclonal antibody that binds the N-terminal region of PrP and is used extensively in ELISA assays (31). AG4 is a mouse monoclonal antibody with epitope recognition between residues 31-51, with a further area of binding between amino acids 147-163. 1B3 and 1A8 are both rabbit polyclonal antibodies against PrP. Rat monoclonal antitubulin antibody (Abcam) has been used as loading control in Western blot experiments. Alexa Fluor® 488 and Alexa Fluor®568 are IgG labeled with fluorescent dye (Molecular Probes). Rabbit anti-cow glial fibrillary acidic protein (GFAP; DAKO) is an antibody recognizing a specific astrocytic marker. The endoplasmic reticulum marker antiERp60 is raised in rabbit against porcine ERp60 peptide PIIQEEKPKKKKKAQEDL in the C-terminus of the protein (32). The Golgi marker 23C Rat Monoclonal, IgG2c, clone #23c raised in rat against recombinant mouse TCP-1alpha, C-terminal half. (33).

Generation of targeting vectors for the $Prnp^{a180T}$ and $Prnp^{a196T}$ alleles - PrP codon 180 and 196 alterations were introduced into HM-1 ES cells. Briefly a gene targeting vector was constructed using isogenic 129/Ola $Prnp^{a}$ DNA from a HM-1 genomic library in λ DASH II (Stratagene). The PrP codon 180 and 196 alterations were introduced into a 1.1kb *Xma*III-*Eco*RI exon 3 fragment containing the open reading frame (ORF) by the Kunkel method (34). This was ligated with the 5' and 3' homologous sequences derived from a 7.8 kb *Bam*HI-*Eco*RV genomic clone spanning 129/Ola *Prnp* exon 3. A LoxP-neo/TK selectable cassette (provided by Alan Clarke, University of Cardiff, UK) was ligated into a unique *Sal*I site 1600 bases downstream

of exon 3 in the pBluescript plasmid (Stratagene). The pBluescript vector previously had its *Sal*I site removed, so this was a unique site in the targeting vector. The ORF encoding PrP in the targeting vector was sequenced, at each step in the cloning procedure to confirm the presence of the alterations and the absence of any other cloning artefacts.

Embryonic stem cell culture and gene targeting - Culture conditions for the embryonic stem cell (ES) line HM-1 have been described previously (35). HM-1 cells ($5x10^7$) were electroporated using a genepulser (Bio-Rad) at 800 volts and 3 mFd with 250 μg linearized targeting vector DNA in 0.8 ml Hepes-phosphate buffered saline pH 7.05. Cells were rested for 15 minutes and plated at $5x10^5$/ 10 cm plate. 24-48 hours after electroporation G418 selection medium was added. Medium was changed every 2-3 days, and colonies were selected for PCR screening 15 days after electroporation. 10^7 targeted HM-1 cells in 0.8 ml serum free growth media were electroporated with 25 μg of the plasmid pCre2 (provided by Alan Clarke, University of Cardiff, UK). Two pulses of 230V, 500μFd were given. Cells were rested for 15 minutes and plated at 10^4 cell/10 cm plate. On day 6 after electroporation 2 mM gancylovir was added to the growth media. Colonies were picked and screened on day 15.

PCR screening for targeting events - Half of the cells from surviving colonies were used to prepare DNA for all PCR analyses.

i) Detection of homologous recombination events. A 1600 bp PCR product was synthesized between the *neo*/*tk* cassette and a site outside the targeting vector. The reaction specific oligos are LoxP situated immediately upstream of the 3' loxP site: TCGATCGACTAGAGCTTGCGGA and 3'Map1 located 200 bases 3' to the *Eco*RV site: CTAAGTGACCTAGGCACATGTC. The cycle conditions were 3 minutes at 94°C, then 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C. Then 10 minutes at 72°C (GeneAmp 9700, Perkin Elmer). Those positive for the selection cassette were then analysed for the glycosylation mutation using the mismatch specific PCR reaction described below for genotyping.

ii) Removal of the selectable marker. The removal of the selectable marker left one LoxP site. This is screened for using oligonucleotides 5' and 3' to the PrP gene *Sal*I site NLTVitro creA: AGAACAGGTCTGACCACACTGGTT, and NLTVitro creB: AATGGTTAAACTTTCGTTAAGGAT. Wild type PrP alleles will give a PCR product of 242 bp, whilst those containing a loxP site will be 342 base pairs. Sites containing an unexcised *neo*/*tk* cassette would be over 5 Kb. The cycle conditions were 3 minutes at 94°C, then 30 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C. Then 10 minutes at 72°C.

Generation of gene targeted mice - Targeted ES cells were used to generate chimeric mice as described previously (25) to obtain G1 and G2 heterozygous mice expressing mono-and un-glycosylated PrP and G3 heterozygous mice expressing un-glycosylated PrP. Heterozygous mice were bred to produce inbred homozygous line. 129/Ola mice were used as wild type controls since the transgenics had been generated on a 129/Ola background. NPU $PrP^{-/-}$ mice (7) were used as negative controls in all the experiments performed.

PCR genotyping of mouse tail DNA - G1 and G2 mutant alleles were detected using a mismatch PCR technique. An oligonucleotide mixture was used at 1 pmole that contained a forward oligo 9910: AACCTCAAGCATGTGGCAGGGGCTGCGG CAGCTGG, a reverse oligo 9912: TCAGTGCCAGGGGTATTAGCCTATGGGG GACACAG, and a mutant specific or wild type oligo (also in the reverse orientation); in a ratio of 20:1:20.

The reaction specific oligos are, G1-mut: GCTGCTTGATGGTGATA**G;** G1-WT; GCTGCTTGATGGTGATAT; G2-mut: CATCGGTCTCGGTGAAG**G**; G2-WT: CATCGGTCTCGGTGAAGT (mutated nucleotides are in bold). The cycle conditions were 3 minutes at 94°C, then 35 cycles of 50 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C. Then 10 minutes at 72°C. All other reaction components were those recommended by the suppliers (Invitrogen).

Southern blot analysis of Prnp - Genomic DNA was prepared using a Puregene Isolation

kit (Gentra Systems). DNA (15 mg/reaction) was digested with restriction enzymes and then separated on a 1% agarose gel and blotted to Hybond-N nylon membrane (Amersham). Hybridisation was performed using ULTAhyb solution (Ambion) using a 700 bp *Eco*RV-*Bam*HI fragment (3' probe) and an 884 bp PCR product as probes. Following stringent wash procedures (0.1×SSC at 65°C), the blots were exposed to X-ray film for 2 days.

Northern blot analysis of total mRNA from brains of transgenic mice - Total RNA was isolated using RNAzol^{TM} B (Biogenesis) based on the guanidinium thiocyanate–phenol–chloroform extraction method (36). A 20 μg aliquot of total RNA was separated on a 1.0% agarose–formaldehyde denaturing gel, transferred to Hybond N (Amersham Pharmacia Biotech) and probed with a ^{32}P-labelled 936 bp *Kpn*I–*Eco*RI fragment from exon 3 of *Prnp*.

Western blotting - Mice were killed by cervical dislocation and brains were removed, flash frozen in liquid nitrogen and then stored at -70°C until required. Half or whole brains were weighed and mechanically homogenized from frozen in nine volumes of ice cold NP40 lysis buffer (1% Nonidet 40, 0.5% Sodium deoxycholate, 150 mM NaCl, 50 mM Tris pH 7.5), with the addition of phenylmethysufonyl fluoride (PMSF) (final concentration 1 μM; Sigma) to prevent protein degradation by endogenous proteases. The homogenate was centrifuged at 8000 rpm for 10 minutes to remove debris. Total protein was denatured in 1X Novex Tris-Glycine SDS Sample Buffer (Invitrogen Life Technologies) and 1X NuPage Sample Reducing Agent (Invitrogen Life Technologies) for 30 minutes at 95 °C. Proteins were separated by electrophoresis at 125 V through a Novex Pre-cast Tris-Glycine gel (12% or 14% acrylamide, Tris-glycine; Invitrogen Life Technologies). Proteins in the acrylamide gel were transferred to polyvinylindene fluoride (PVDF) membrane at 25 V (125 A/gel) using a semi-dry transfer blotter (Biorad) in 1X Transfer Solution (48 mM Tris, 39 mM Glycine, 0.375 % sodium dodecyl sulphate (SDS), 20% methanol).

PNGaseF digestion - Total brain proteins (5% brain homogenate; NP-40 lysis buffer, 10 mM PMSF) were denatured in 1X glycoprotein denaturing buffer (0.5% SDS, 1% β-mercaptoethanol; New England Biolabs) at 100 °C for 10 minutes; prior to incubation with PNGaseF (30000 units/ml; New England Biolabs) in 1% Nonidet 40 (New England Biolabs) and 1X G7 Reaction Buffer (50 mM $NaPO_4$; New England Biolabs) at 37 °C for 2-4 hours. Reaction was terminated by freezing at -20 °C or SDS denaturation.

Proteinase K digestion - Mouse brain homogenates (10%) were prepared in ice-cold NP40 buffer. Each homogenate was then split into two aliquots one treated with proteinase K (PK; Roche Diagnostics, Mannheim) and one not. In order to assess the sensitivity to the enzyme digestion: wild type, G1, G2 and G3 brain homogenates were each treated with varying concentrations of PK: 20 μg/ml, 10 μg/ml, and 5 μg/ml at 37°C for 1 hour. A milder treatment was also carried out incubating the samples with PK (20 μg/ml) at 4°C for 1 hour. The samples were then analyzed by Western blotting using 8H4 or 7A12 monoclonal antibodies for PrP detection.

Delfia® analysis - The method is an adaptation of that of Barnard and colleagues (37). PrP^C was extracted from brain homogenate (10^{-1} tissue; NP-40 lysis buffer; 10 mM PMSF) by mechanical homogenization in 1 M guandine hydrocholoride (25 mM Tris; 1M Gnd HCl, Sigma; 0.5 % Triton X-100, Sigma). This was then diluted in DELFIA® Assay Buffer (TBS with bovine serum albumin (BSA), bovine gamma globulins, Tween 40, diethylenetiaminepentacetic acid (PerkinElmer Life Sciences), leading to final concentration equivalent to of 10 mg/ml of original tissue. Capture antibodies FH11 (1/200) or AG4, (1/200) were bound to 96 well plates by overnight incubation at 4 °C. Wells were blocked with 2% Bovine Serum Albumin (Roche Diagnostics) in sterile 1X PBS (Oxoid) with 3M NaN_3, for 1 hour shaking at room temperature. The plate was then incubated with samples and standards, shake at room temperature for one hour and then incubated with europium (Eu^{3+}) labeled detector antibodies 7A12 (FH11 or AG4 captures) or 8H4 (1/3000) (FH11 capture only).
DELFIA® Enhancement Solution was added to the samples to facilitate the formation of Eu-$(2\text{-NTA})_3(TOPO)_{2\text{-}3}$. After 5 minutes shaking

at room temperature Eu^{3+} emission (615 nm) was calculated using a time-resolved technique. Between each step, the plate was washed in 1X DELFIA® Wash Concentrate (TBST; PerkinElmer Life Sciences) using the DELFIA® automatic plate washer (Wallace). Program WorkOut was used to analyze absorbance from standard and samples and to produce the standard curve (based on a linear model of emission).

Detergent solubility assay – PrP^C was extracted from brain homogenate (10^{-1} tissue; NP-40 lysis buffer; 1 mM PMSF) by mechanical homogenization in 1 M guandine hydrocholoride (25 mM Tris; 1 M Gnd HCl, Sigma; 0.5 % Triton X-100, Sigma). This was then diluted in DELFIA® Assay Buffer leading to a final concentration equivalent to of 10 mg/ml of original tissue. Proteins insoluble in 1M Gnd HCl (PrP^{Sc}) were separated from those which were soluble (PrP^C) by centrifugation at 13000 rpm for 10 minutes. The resultant pellet was resuspended in 6 M Gnd HCl prior to dilution in DELFIA® Assay Buffer, to a concentration equivalent to 10 mg/ml original tissue. Measurement of the sample concentration was then performed as described above (Delfia® analysis).

Phospholipase C assay - Mouse brain homogenates (10%) were prepared by homogenizing in ice-cold PBS containing 10 mM PMSF. Each sample was centrifuged at 13000 rpm for 5 minutes at room temperature. The supernatant was collected and centrifuged at 25000 rpm for 10 minutes at 4°C. The pellets were resuspended in 500 µl of cold PBS and then each sample was split. One half was treated with phosphatidylinositol phospholipase C (0.5 U/ml, PIPLC; Sigma) whereas the second remained untreated. The samples were incubated for 10 hours at 4°C. Samples were then centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant (membrane-released fraction) and the pellet (membrane-associated fraction) were analyzed by Western blotting using the monoclonal antibody 8H4.

Aging experiment - A group of 10 homozygous G3 mice were monitored up to 850 days and compared with a group of wild type mice. The animals were age and sex matched. Animals were monitored constantly by a group of independent observers for any neurodegenerative signs. Between 800-900 days mice were culled. Brains were retained; half of the brain was fixed for standard lesion profiling and plaque analysis and the other half was flash frozen for biochemical analysis.

Lesion profiles - Brain Sections were haematoxylin and eosin stained and scored for vacuolar degeneration on a scale of 0 to 5 in nine standard grey matter areas and three standard white matter areas as described previously (38).

Immunocytochemical analysis - Sections were immunostained using standard procedures. Briefly, sections were blocked with normal goat serum and probed overnight with polyclonal antibody anti-GFAP at a dilution of 1:400. A parallel panel of sections was also probed with normal mouse serum as a control. Antibody binding was detected with biotinylated goat anti-rabbit secondary antibody (Jacksons) and the Vectastain Elite ABC Kit (Vector Laboratories). Reaction products were visualized with diaminobenzidine (DAB), and sections were lightly counterstained with haematoxylin. Pictures were taken using a Nikon Eclipse E800 microscope.

Confocal analysis - Mouse brains were fixed in periodate-lysine-paraformaldehyde (PLP) or in a paraformaldheyde-glutaraldehyde mix (4% PFA; 0.025% glutaraldheyde) for 4 hours. Brain sections were cut at a thickness of 70 µm using a vibrating microtome (Leica). The sections were permeabilized for 1 hour in PBS/ 0.1% triton at room temperature before blocking overnight at room temperature in PBS/0.5 % BSA in a humid chamber. Sections were blocked for a further hour at 37°C in Mouse On Mouse Ig blocking reagent/PBS when mouse monoclonal antibodies were used (Vector Laboratories). After blocking the tissues were incubated at 37°C with primary antibody (diluted in PBS/protein concentrate if mouse monoclonals used; Vector Laboratories) for 90 minutes (39). To get a specific signal, several anti-PrP antibodies were used at different concentrations: 8H4 (1/1000; 1/2000), AG4 (1/1000; 1/2000), 1B3 (1/800; 1/1000/; 1/2000), 1A8 (1/1000; 1/2000). After extensive washes in a Ca/Mg free PBS solution, the sections were incubated at 37°C with the secondary antibody

goat anti-mouse Alexa Fluor® 488 conjugate diluted 1/200 in PBS/BSA for 90 minutes. The samples were then washed with a Ca/Mg free PBS solution for 20 minutes and then stained with 4',6-diamidino-2-phenylindole nuclear marker (DAPI 1/10000; Molecular Probes) for 30 minutes at room temperature. After extensive washes in ultra-pure water the sections were mounted for microscopic analysis. Co-localization experiments were carried out using the same basic method as above with some modifications. For ERp60/8H4 (1/400 and 1/500) the primary antibodies were mixed in PBS/BSA so that the tissue was incubated with them simultaneously. Similarly the secondary antibodies Alexa 488 conjugated goat anti-mouse antibodies and Alexa 568 conjugated goat anti-rabbit were simultaneously incubated. Controls for cross reactivity were used and none was detected. For 23C/8H4 cross reactivity was detected; to eliminate it a sequential staining method was used. Sections were first incubated with 8H4 (1/500) diluted in PBS/Protein concentrate (Vector Laboratories), washed in PBS then incubated with Alexa 488 conjugated goat anti-mouse using a method identical to that for single 8H4 labelling. Sections were then washed 10 times in PBS before being blocked for one hour in MOM Ig blocking reagent (Vector Laboratories). Block was removed by washing for 10 minutes in PBS/Protein Concentrate before incubation of the section with 23C (1/50) in PBS/Protein concentrate. The sections were then washed and incubated with Alexa 568 conjugated goat anti-rat. The sections were washed, stained with DAPI and mounted for analysis.

Sections were imaged with a Leica TCS SP2 laser scanning confocal microscope.

RESULTS

Construction of gene-targeted mice with altered N-linked glycosylation of PrP - Using the Cre-loxP recombination and gene targeting approaches three inbred lines of transgenic mice with alterations in the N-linked glycosylation consensus sites Asn-Xxx-Thr were generated. Gene targeting was used to alter Asn residue to Thr at 180 (N180T), 196 (N196T) or both 180 and 196 (N180T-N196T) (Figure 1) in HM-1 embryonic stem cell line (ES). The positive ES clones were subsequently confirmed by DNA sequencing and Southern blot analysis (data not shown).

The characterized targeted ES cells were microinjected into blastocysts of C57Bl6 mice to obtain chimeric mice. Chimeric mice were identified by coat colour and mated with 129/Ola mice. ES cell derived offspring were recognized by eye and coat color and genotyped to identify gene-targeted transgenic mice. The heterozygous offspring were then inter-bred to obtain homozygous inbred lines carrying the mutated *Prnp* gene.

mRNA expression and protein levels of mono or un-glycosylated PrP are similar to wild type - The level of expression of the PrP gene from the different gene-targeted lines was assessed and compared with the wild-type gene. Northern blot analysis detected similar levels of PrP mRNA in mice with the mutant *Prnp* alleles (N180T; N196T and N180T-N196T) and in wild-type mice (Figure 2A). Thus the transgene expression levels in these new lines are the same as the wild type lines showing that neither the point mutation nor the presence of the LoxP site downstream of *Prnp* interfere with gene transcription.

Western blot analysis of brain homogenates of the transgenic mice demonstrates that both G1 and G2 lines lack di-glycosylated, but posses mono-glycosylated and un-glycosylated PrP, whilst G3 mice only exhibit un-glycosylated PrP, as confirmed by deglycosylation with PNGase F enzyme (Figure 2B).

Western blot analysis using different monoclonal PrP antibodies indicated that the steady-state level of the PrP protein in glycosylation-deficient transgenic mice is apparently lower than that in wild-type mice (Figure 2B). However accurate quantification of the difference in amount of PrP between the lines of mice has proved difficult by Western blot analysis, thus a more quantitative assay system has been utilized to address this question.

A DELFIA® assay was set up in order to quantify the total amount of PrP in brains from the three transgenic lines and compare it to that in wild type. Levels of PrP in brains from G1 and G2 transgenic lines were similar to that of wild type brains in the DELFIA® system. Un-glycosylated PrP amount is slightly lower (10% less) by this analysis. Since only a slight reduction of PrP levels was observed in G3 mice any biological effect is more likely to be due to lack of glycosylation rather than to this reduction in amount of protein (Figure 3). The

presence of such a high level of the un-glycosylated PrP was surprising since with the absence of sugars it was believed this protein would not mature and be rapidly eliminated by the cell. This data shows that the un-glycosylated PrP is considered to be a normal protein by the cellular quality control system, as it accumulates to significant levels *in vivo*.

Altered glycosylation - PrP does not acquire PrP^{Sc}-like properties - It is possible to distinguish between PrP^{Sc} and PrP^{C} conformers on the basis of biochemical properties: i) PK partial resistance; ii) insolubility in detergents; iii) resistance to the cleavage with PIPLC enzyme. Several studies performed in cell cultures (16; 17; 19) have shown un-glycosylated PrP spontaneously acquiring PrP^{Sc}-like properties. We aimed to investigate if these phenomena also occur *in vivo* with a normal level of PrP expression or if the effects observed in cell cultures may be due to transgene over-expression, ectopic expression or some other anomaly of the *in vitro* system.
First we analyzed the sensitivity to proteolytic action of PK enzyme in brain homogenates from all three lines of transgenic mice. PrP^{Sc} partial resistance to the proteolytic digestion of PK is detected by the presence of a 27-30 kDa fragment by Western blotting (11). Different enzyme concentrations (20 μg/ml; 10 μg/ml; 5 μg/ml) and reaction temperatures (4°C, 20 μg/ml) did not reveal any difference in PrP's PK resistance between wild type and transgenic forms, demonstrating that lack of glycans does not increase the resistance of PrP to PK (Figures 4A and 4B and data not shown).
To assess the solubility of mono- and un-glycosylated PrP a standard guanidine hydrochloride assay was employed. Brain homogenates were treated with guanidine hydrochloride at a low molarity (1 M) known to solubilize PrP^{C}; any material remaining insoluble at this concentration was then solubilized at a higher concentration (6 M) known to release PrP^{Sc} into solution. Treated brain fractions were then assayed in a DELFIA® assay. As in wild type animals, low guanidine hydrochloride insoluble PrP was not observed in any of the glycosylation transgenics, in contrast to the significant amount detected in the ME7 control (Figure 3).

PIPLC bacterial-derived enzyme cleaves the eukaryotic GPI anchor, releasing bound proteins from the membrane. Different sensitivities to the enzyme's action between PrP^{C} and PrP^{Sc} have been shown with PrP^{C} being sensitive whereas PrP^{Sc} is resistant to the enzyme (40). To investigate this, brain homogenates were treated with PIPLC and Western blot analysis was carried out. This assay revealed the same characteristics between wild type PrP and mono- or un-glycosylated PrP. The ability of mono- and un-glycosylated PrP to be cut by PIPLC enzyme is shown in figure 5A where bands of all the samples were detected in the supernatant fraction (enzyme released) migrated slower compared to the bands detected in the pellet (enzyme resistant). No shifted bands were detected in the supernatant fraction of all transgenics when brains had not been treated with the enzyme (Figure 5B). This assay suggests that all three mutants are GPI-anchored proteins still sensitive to PIPLC enzymatic digestion. Notably the presence of a GPI anchor in un-glycosylated PrP structure suggests that this protein is correctly processed since the attachment of the anchor is the last event in the protein maturation process in the ER. The observation that this step occurs without the presence of any sugar in PrP structure shows once again how the presence of un-glycosylated PrP is tolerated within the secretory pathway as well as di- and mono-glycosylated forms.

Aging analysis of transgenic mice - All glycosylation-deficient transgenic animals bred and aged normally and did not display any overt phenotype.
However to rule out any PrP^{C}/PrP^{Sc} pathological transition due to the absence of carbohydrates over a long term period, an ageing experiment of mice was set up. A group of un-infected G3 mice and wild type controls was monitored for up to 850 days. None of these mice developed clinical signs of TSE neurodegeneration. Moreover to fully exclude PrP^{Sc} presence, aged mice were culled at time points and then brain homogenates were digested with PK and analyzed by Western blotting. As shown in figure 6, no PK resistant PrP was detected in any of the animals confirming that the *in vivo* lack of sugars, when altered PrP is expressed at natural levels, is not sufficient to destabilize PrP^{C} structure

and produce a pathogenic form. Microscopic analysis carried out in brain sections did not reveal any abnormal deposition of PrP in all the aged mice ruling out any possibility of aggregation of un-glycosylated PrP (Supplementary figure 1).
Moreover no gliosis was observed in aged wild type and transgenic mice brains (Supplementary figure 2).

Un-glycosylated PrP localization is mainly intracellular while mono-glycosylated PrP is localized in the external membrane - To establish if the glycosylation is important in determining trafficking of PrP inside neurons a confocal microscopy approach was developed using brain sections. Although highly expressed in the brain PrP^{C} is very difficult to detect. A number of monoclonal and polyclonal anti-PrP antibodies were tested: 1B3, 1A8, AG4 and 8H4 at different concentrations. Different types of tissue fixative methods were also considered (see experimental procedures). We observed the best results using 8H4 in 4% PFA/ 0.025% glutaraldehyde fixed tissues.
Labeling for PrP was seen throughout all the brain sections although it was less intense at the periphery of the brain. Analysis was focused on the hippocampus. In wild-type mice, PrP labeling was closely associated with the cell membrane, with some intracellular labeling also observed (Figure 7A). This observation is in accord with several results previously obtained both *in vitro* and *in vivo* with different detection techniques (41-48).
Clear membrane staining was also observed in G1 and G2 brains (Figures 7B and 7C); suggesting that the presence of just one sugar chain at either the first or the second site is sufficient for its trafficking through the secretory pathway to the external membrane. This differs from previous reports in transgenic mice over-expressing mono-glycosylated hamster PrP or in transfected cell cultures where altering the first glycosylation consensus site influenced the intracellular fate of PrP, blocking its trafficking to the cell membrane (16, 21, 49).
In G1 and G2 mice although PrP was mainly on the cell membrane there was a greater proportion of PrP protein located within the cell than in wild type mice. It is difficult to compare actual levels of labeling from one animal to the other, but it was clear that the ratio of membrane to cytoplasmic labeling was shifted towards the cytoplasm in the G1 and G2 mice. The more intense-intracellular signal detected in these transgenic mice suggests intracellular trafficking rate may be altered when the first or second glycosylation site has been ablated with a slower migration of these proteins toward the outer membrane.
Further more a clear and consistent increase in intracellular compared with membrane labeling for PrP was observed in G3 when compared to wild type, G1 and G2 brains (Figure 7D). However, this intracellular labeling was of a similar pattern to that observed in the wild type being tightly defined consistent with protein localization in sub-cellular compartments. Double labeling analysis using ER and Golgi markers revealed that mono- and un-glycosylated PrP are not blocked in the ER. No co-localization was observed between PrP and ERp60 ER marker suggesting that glycosylation deficient proteins are able to leave this compartment and traffic toward the cytoplasm and the cell membrane (Figures 7 A-D). This was further proven with a double staining using 8H4 and Golgi marker 23C. A significant proportion of the total intracellular anti-PrP was observed to be surrounded by anti-β-cop labelling indicating a localisation of PrP in the Golgi. The β-cop labelling appeared to surround a significant proportion but not all of the intracellular PrP in both wild type controls and the glycosylation-deficient transgenics (Figures 7 F-I). The non β-cop associated intracellular fraction is most likely contained in endosomes, lysosomes or similar endocytic compartments as previous work has demonstrated significant localisation of wild type PrP in these organelles (44, 46). It appears that un-glycosylated PrP therefore is able to enter in the secretory pathway but is then retained in the Golgi apparatus before it reaches the cell membrane. Importantly we have demonstrated un-glycosylated PrP is a GPI-anchored cytoplasmic protein and it is therefore likely to be bound to intra-cellular membranes.
To establish the specificity of the fluorescent signal, PrP knock-out mice brain sections have been treated with the same method. No signal was detected with both primary and secondary antibodies (Figures 7E and 7J) imaged with the same microscope settings as used for the wild type mice.

The microscopic analysis described here clearly shows how sugars can be important in determining the location of PrP protein in the cell.

DISCUSSION

PrP glycosylation may represent the key factor in understanding not only PrP function but also TSE infectious process and the existence of a number of strains of agent in TSE disease (50, 51).

We report here that un- or mono-glycosylated PrP is expressed at physiological levels in the central nervous system. Un-glycosylated PrP does not acquire the PrP^{Sc} characteristics such as PK partial resistance or detergent insolubility. Thus it is unlikely that lack of glycans can destabilize the entire protein structure facilitating the onset of a TSE phenotype.

To date the role of carbohydrates in PrP cell biology had been addressed using *in vitro* transfection studies or over-expressing transgenic mouse models *in vivo* which have produced contradictory results. PrP over-expression represents a major problem because it is now clear that different results may be obtained when PrP expression levels are altered in both cell cultures and transgenic mice (23, 52). Some experiments performed in cell culture models have shown that the lack of carbohydrates can in some way destabilize PrP^{C} structure thus allowing it to acquire all the PrP^{Sc} hallmarks (16, 17, 19). However, recently Neuendorf and colleagues (22) have shown that altered glycosylated PrPs display just some of the pathogenic protein characteristics such as detergent insolubility while maintaining the PK sensitivity of wild type PrP. Moreover in the case of a mono-glycosylated PrP they also observed a resistance to PIPLC whereas other mutants can be released by the enzyme. This discrepancy of results obtained may be a result of a combination of factors: different point mutations introduced in the PrP gene, different constructs, distinct cell lines, random integration of the transgene and different levels of *Prnp* expression or in some cases use of drugs to prevent glycosylation that can cause intracellular stress. Moreover experiments which introduced the T182A mutation to abolish the attachment of sugars at the first site (16, 20, 21) may be misleading since this mutated PrP can cause familial TSE disease (49, 53) in a glycosylation-independent manner (54).

A number of transgenic lines have been developed by our group using the gene targeting technique whereby altered PrP has been introduced in the correct genomic location (23). This system is important not only for understanding the contribution to the disease by mutated endogenous PrP but also to study the biology of these proteins. Gene-targeted transgenic mice may indeed represent the best comparison for wild type animals as any alteration observed can be directly related to the mutated PrP protein expressed. Importantly this system allows a direct comparison not only between transgenic lines and wild-type mice but also between different transgenic lines.

All three glycosylation mutants we have generated retain the same biochemical characteristics of wild type PrP. Surprisingly the amounts of un-glycosylated protein in brain were comparable to wild type and mono-glycosylated PrP levels. In theory one of the functions of the sugars is to stabilize the glycoproteins and facilitate folding (1, 2). If this does not happen the protein can be considered non-mature and it is eliminated by the ubiquitin/proteasome system (UPS) (55). The lack of sugars has been previously reported to influence the degradation by the UPS of PrP decreasing its half life in cell cultures and it is generally believed that PrP, without sugars is a non-mature protein (22, 56). Here we report that un-glycosylated PrP is apparently not considered by the UPS to be non-mature and an unfolded protein because its amount in the brain is similar to that of wild type. The additional observation that sugar-deficient PrP has a GPI anchor shows once again that during its synthesis and translocation in the ER the protein is not blocked as would normally happen with unfolded proteins. Since the un-glycosylated form of PrP exists *in vivo*, the cell does not need to eliminate it faster suggesting this un-glycosylated protein is functional.

Using our models we have also investigated the contribution of carbohydrates to directing PrP intracellular trafficking and localization inside neurons of the central nervous system. Several earlier cell cultures studies investigated the possible role of sugars in PrP localization and different results have been

reported. In general un-glycosylated PrP is not detected at the cell surface, remaining trapped in the cytoplasm (16, 20, 21). However Korth *et al*. (17) and later Neuendorf *et al*. (22) detected un-glycosylated PrP on the cell surface of different cell lines when transfected with some but not all glycosylation mutants, suggesting that the mutation of an amino acid rather than the lack of sugars can influence the intracellular fate of PrP. However it has been also observed that PrP without complex-type glycans after treatment with geldanamycin localizes to the cell surface despite any alteration in *Prnp* (19), suggesting that PrP can traffic independently of the presence of mature sugars or different amino acids. Our results show that the presence of just one sugar chain is sufficient for the protein to leave the intracellular compartments of the endoplasmic reticulum and Golgi apparatus and traffic to the cell surface. Contrastingly un-glycosylated PrP has a mainly intracellular localization. Using a double staining approach we were able to establish that PrP without carbohydrates is not held in the ER but its main localization is associated with Golgi apparatus. This protein is therefore considered mature by the cell and, leaving the ER, enters in the secretory pathway like di- and mono-glycosylated forms. The lack of sugars causes PrP trafficking to stall and the protein to remain in the cytoplasm. Interestingly this protein is still GPI-anchored, probably attached to intracellular membranes where it may have a physiological role. Alternatively di- and mono-glycosylated proteins can leave the Golgi apparatus and traffic to the cell membrane with a small amount remaining in the cytoplasm. In our model all PrP is expressed under the endogenous regulatory sequences at physiological levels and is subjected to the cellular control machinery without any possibility of artifacts arising from over-expression. Moreover mono-glycosylated PrP proteins produced here have shown similar biochemical characteristics and cellular localization to that of wild type PrP.

While we cannot rule out that the differences in localization are due to the point mutations introduced rather than differences in glycosylation we would suggest that the results presented here point to differences in glycosylation being the most likely determinant of cellular localization.

Some reports have described cytoplasmic PrP in rodent brains (44, 46). A more recent study suggested that intracellular localization of PrP is probably due to retro-translocation of the protein from the cell membrane to the proteasome system (57). Here we propose that the presence of a certain amount of intracellular PrP is physiological for neurons and probably is due to the functional folded un-glycosylated isoform.

The observed intracellular accumulation of PrP in G3 mice will also assist in understanding the potential neurodegenerative role of intracellular PrP. It has been shown that accumulation of un-glycosylated PrP can be neurotoxic for transgenic mice that develop a severe ataxia, with cerebellar degeneration and gliosis (58). This is not the case in our transgenics as the mice with accumulation of intracellular PrP did not develop any type of neurodegeneration even after a prolonged period. However our intracellular PrP is GPI-anchored while that described previously (58) is soluble thus suggesting that accumulation of un-anchored PrP may lead to neurodegeneration. If this is the case, the absence of the anchor rather then the lack of sugars may be the real signal for PrP to be considered an unfolded protein that should retained in the ER.

In summary using gene targeted animal models we have reported that mono- and un-glycosylated PrP retains the same biochemical characteristics of di-glycosylated PrP, suggesting no influence of sugars in determining spontaneous PrP^{C} to PrP^{Sc} transition. However glycosylation can influence PrP cellular localization in neurons of the central nervous system balancing its presence between the cytoplasm and the cell membrane. These observations lead us to suggest that PrP may only be partially dependent on glycosylation.

We have also reported here that presence of intracellular PrP is a normal event for the cell determined by the un-glycosylated form attached to membranes within the cell and its accumulation is not apparently toxic to the cell.

We now aim, therefore, to use these models to define the role of PrP in the infectious process.

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FOOTNOTES

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[1]The abbreviations used are: BSA, bovine serum albumin; DAB, diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole nuclear marker; ER, endoplasmic reticulum; ES, embryonic stem cells; FFI, fatal familial insomnia; GFAP, glial fibrillary acidic protein; Gnd HCl, guandine hydrocholoride; GPI, glycosylphosphatidylinositol; CJD, Creutzfeldt-Jakob disease; GSS, Gerstmann-Straussler-Scheinker syndrome; neo/TK, neomycin/thymidine kinase; PBS, phosphate buffered saline; PFA, paraformaldheyde; PIPLC, phosphatidylinositol phospholipase C; PK, proteinase K; PLP, periodate-lysine-paraformaldehyde; PMSF, phenylmethysufonyl fluoride; *Prnp*, PrP protein gene; PrP^{C}, cellular form of PrP protein; PrP^{Sc}, disease-associated form of PrP protein; PVDF, polyvinylindene flouoride; SDS, sodium dodecyl sulphate; TBS, tris buffered saline; TSE, transmissible spongiform encephalopathy; UPS, ubiquitin/proteasome system; vCJD, variant Creutzfeldt-Jakob disease.

FIGURES LEGENDS

Figure 1 **Schematic representation of the double-step gene targeting replacement of wild type *Prnp* allele with mutated *Prnp* allele in embryonic stem cells**. After electroporation of embryonic stem cells (ES), 180T, 196T or 180T/196T PrP gene with marker for selection replaced wild type *Prnp* allele by homologous recombination. ES cells were put in selection medium and after screening the selection cassette was removed. PCR analysis was performed to screen positives ES clones that then were then used to generate transgenic mice.

Figure 2 **Expression of glycosylation deficient PrP**. (**A**) Northern blot analysis of PrP mRNA in the brain. Total brain RNA was probed with a 936 bp *Kpn*I-*Eco*RI mouse PrP exon 3 DNA probe to demonstrate that PrP mRNA production levels in G1, G2 and G3 mice were comparable to wild type ones with no over- or under-expression phenomena. (**B**) Western blot of brain homogenates from mice expressing wild type PrP and gene targeted PrP. Samples were treated with PNGase F enzyme (+) or not (-) and then resolved on SDS-PAGE and transferred to PVDF membrane. PrP was detected using mouse monoclonal antibody 8H4. $PrP^{-/-}$ (null mice) were used as negative controls. Altered N-glycosylation has been successfully achieved introducing the N180T or/and T196T point mutations in *Prnp*. Band between 64 and 50 kDa represents tubulin used as loading control

Figure 3 **PrP quantification and solubility analysis in wild type and transgenic mouse brains**. The total amount of PrP was tested using a DELFIA® immunoabsorbent assay. 96 well plates were coated using capture antibodies FH11 or AG4. Brain homogenates from wild type, G1, G2, G3, null and ME7 infected mice were diluted in the appropriate buffer and added to the pre-treated wells. PrP was detected using detector antibodies 7A12 or 8H4 shaking at room temperature for 1 hour and basing on europhium emission (615 nm) using a time-resolved technique. Total PrP amount in transgenic mice brains was comparable to wild type with a slight reduction in G3 brains.
All PrP was recovered in the 1 M guanidine hydrocholoride fraction (black bars) where PrP^{C} is soluble. PrP was detected in 6 M fraction (gray bars) only in the ME7 infected control brain.

Figure 4 **PK resistance Western blot analysis**. Brain homogenates from wild type, transgenics, null and ME7 infected mice were treated (+) or not (-) with PK at different dilutions (20 μg/ml, figure A or 10 μg/ml, figure B). PrP was detected using monoclonal antibody 8H4. No bands were detected in wild type and in transgenic brains when PK treated, while PK resistant PrP was detected in the ME7 infected brain at both enzyme concentrations. Band between 64 and 50 kDa represents tubulin used as loading control.

Figure 5 **Membrane attached PrP in wild type and transgenic mice**. **A)** Brain homogenates from wild type and transgenic mice were treated with PIPLC enzyme and incubated for 10 hours at 4°C. The supernatant (**s**; membrane-released fraction) and the pellet (**p**; membrane-associated fraction) were separated by centrifugation at 13000 rpm for 15 minutes at 4°C and then analyzed for the presence of PrP by Western blotting using the monoclonal antibody 8H4. All glycosylation altered PrPs are associated with membranes as for wild type since a slower migrating band was detected in the s lane in all samples following enzymatic digestion.
B) When samples were not treated with PIPLC no slower migrating bands were detected in the supernatant fractions

Figure 6 **PK resistance in aged wild type and transgenic mouse brains**. Brain homogenates from wild type and transgenic mice aged for up to 850 days and culled at different time points were treated (+) or not (-) with PK enzyme (2 0μg/ml) for 1 hour at 37°C. Null and ME7 infected mouse brain homogenates were also analyzed as controls. PrP detection was carried out by Western blot analysis using monoclonal antibody 8H4. No bands were detected in wild type and in transgenic

brains when PK treated, while PK resistant PrP was detected in the ME7 infected brain. Band between 64 and 50 kDa represents tubulin used as loading control. Wt (1) 866 days old; Wt (2) 865 days old; Wt (3) 865 days old; G3 (1) 807 days old; G3 (2) 813 days old; G3 (3) 809 days old.

Figure 7 **Colocalization of PrP and markers of the ER and Golgi in mice brain sections using confocal analysis.** PrP was detected using the mouse monoclonal 8H4 and an Alexa 488 conjugated anti-mouse (**A-E**) or Alexa 488 conjugated anti-mouse IgG1 secondary antibody (green) (**F-J**). The ratio of intracellular to extracellular PrP is higher in G3 brains (**D** & **I**) compared to wild type controls (**A** & **F**). No colocalization of PrP with Endoplasmic Reticulum resident protein ERp60 was detected in either the wild type control (**A**) nor the G1 (**B**), G2 (**C**) and G3 (**D**) transgenics, using a rabbit polyclonal antibody raised against an ERp60 C-terminus peptide and an Alexa 568 conjugated anti-rabbit secondary antibody (red). Localisation of PrP in the Golgi apparatus was determined using an rat monoclonal which reacts with the β-cop subunit of coatamer and an Alexa 568 conjugated anti-rat secondary antibody (red) (**F-J**). In the wild type control a proportion of intracellular PrP (green) colocalizes with the Golgi apparatus (**F**). A similar pattern of Golgi localization is seen in the G1 (**G**), G2 (**H**) and G3 (**I**) brain sections. The specificity of 8H4 staining is shown by the use of PrP null sections (**E** and **J**).

Supplementary Figure 1 **Microscopic analysis of PrP deposition in aged mice brain sections**. Sections were haematoxylin and eosin stained and then analyzed for presence of plaques in different areas of the brain using a Nikon Eclipse E800 microscope with a 10X objective. (**A**) Wild type brain cerebellum area showing no deposition. (**B**) Wild type cortex with no deposition. (**C**; **E**; **G**) G3 transgenic mice cerebellum showing no deposition as in wild type brain. (**D**; **F**; **H**) G3 transgenic mice cortex showing no deposition as in wild type brain.

Supplementary Figure 2 **Immunohistochemical analysis of astrocyte proliferation in aged mice brain sections.** Sections were blocked with normal goat serum and probed overnight with polyclonal antibody anti-GFAP and then with biotinylated goat anti-rabbit secondary antibody. Pictures were taken using a Nikon Eclipse E800 microscope with a 20X objective. (**A**; **B**) Cerebellum and cortex area of a terminally ill ME7 infected mouse brain showing high gliosis. (**C**; **D**) Cerebellum and cortex area of 866 days old wild type mouse brain showing normal astrocytes presence. (**E**; **F**) Cerebellum and cortex area of 807 days old G3 mouse brain with normal astrocyte levels as in wild type brains. (**G**; **H**) Cerebellum and cortex area of 813 days old G3 mouse brain with normal astrocyte levels as in wild type brains.

Figure 1

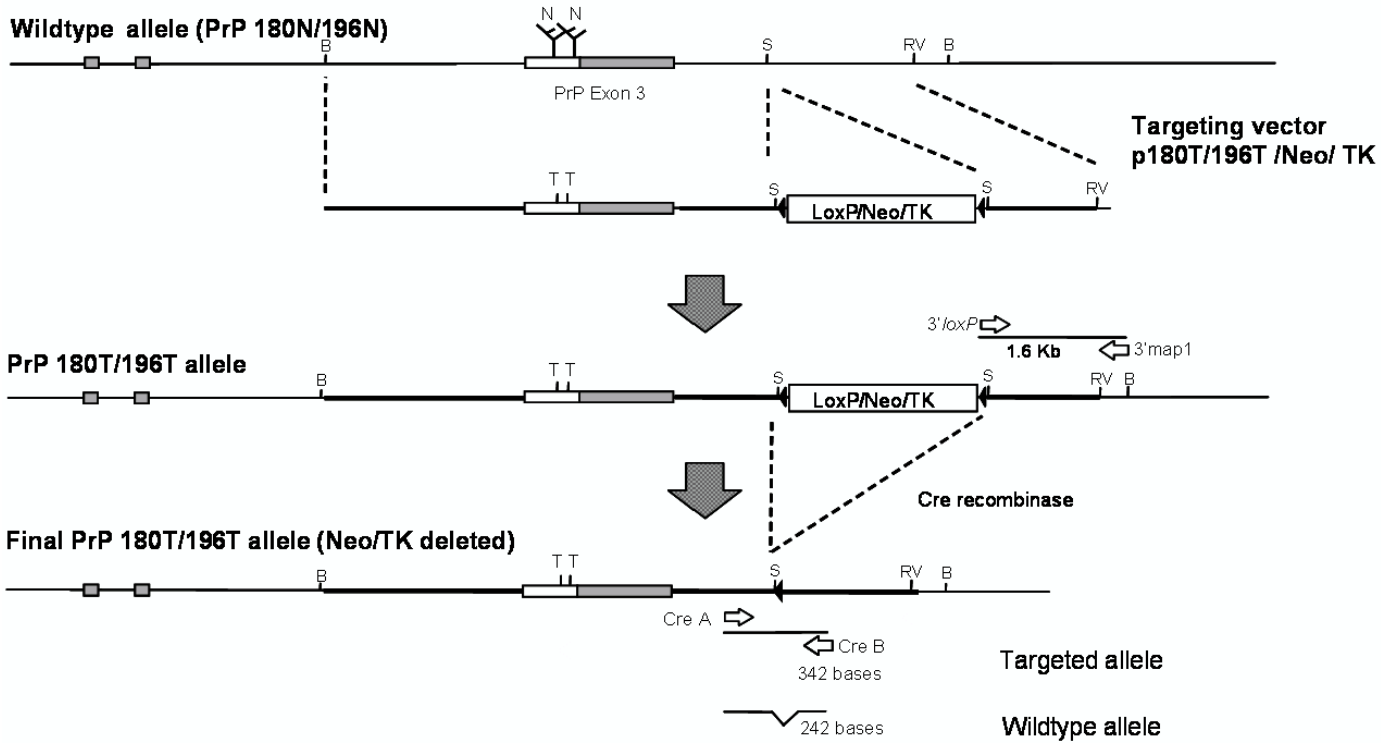


Figure 2

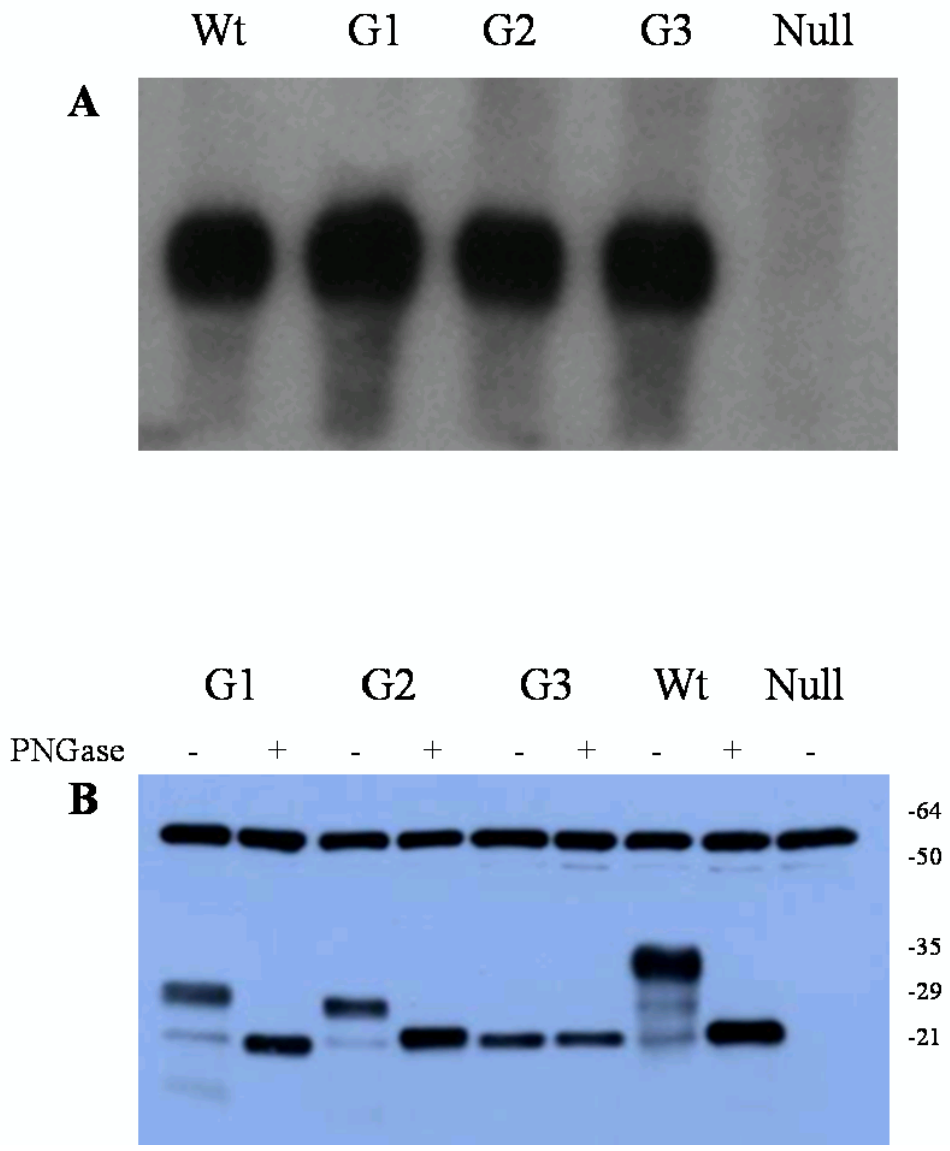


Figure 3

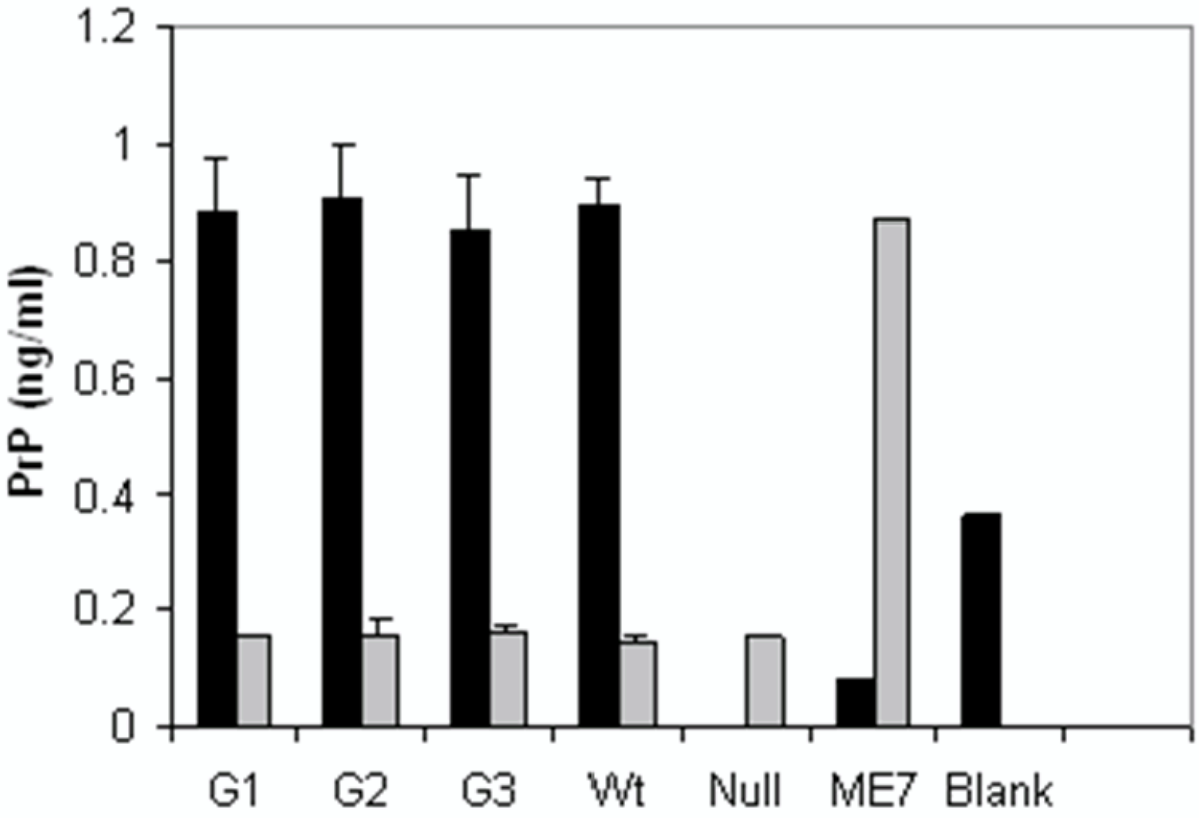


Figure 4

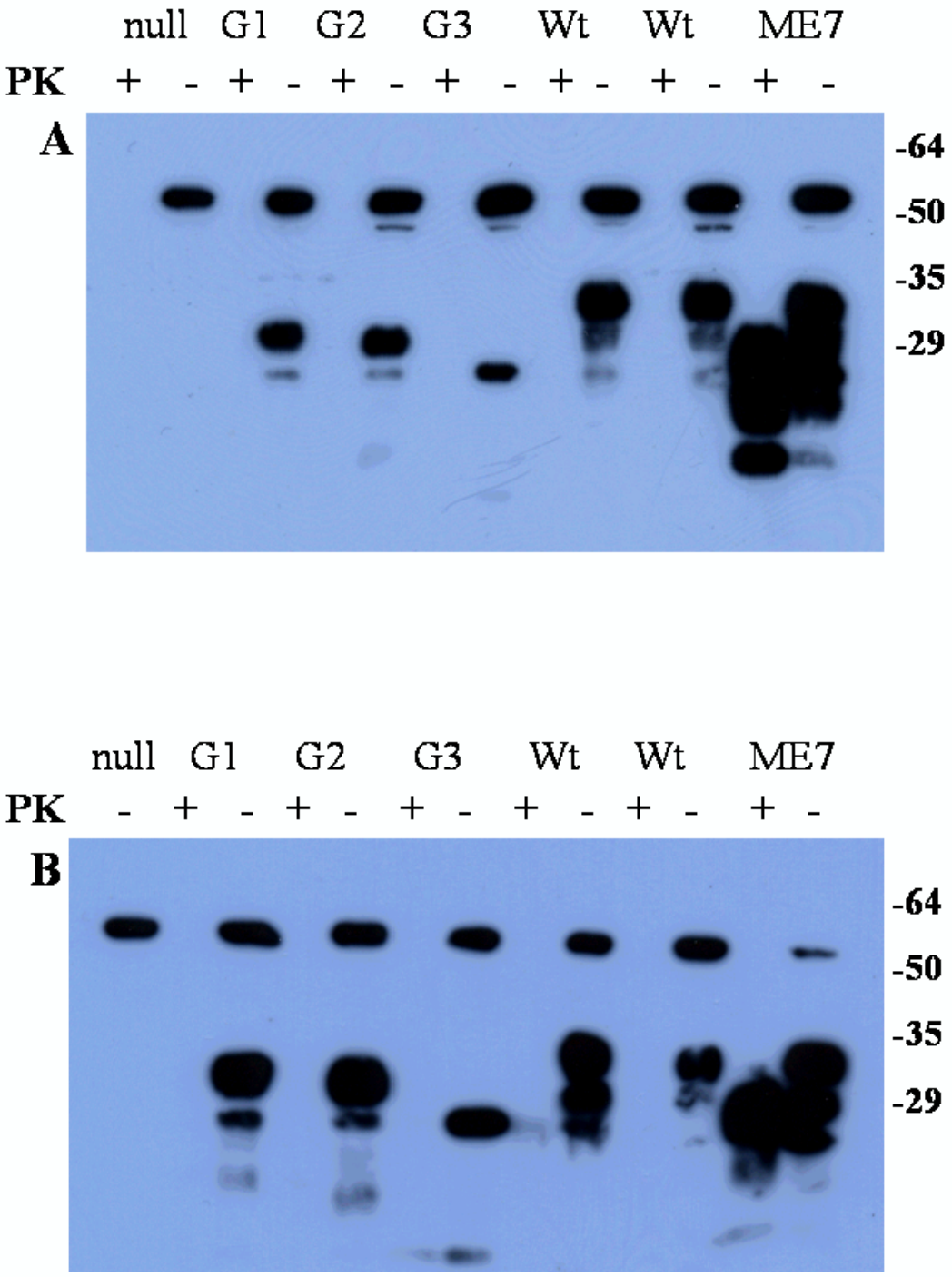


Fig. 5

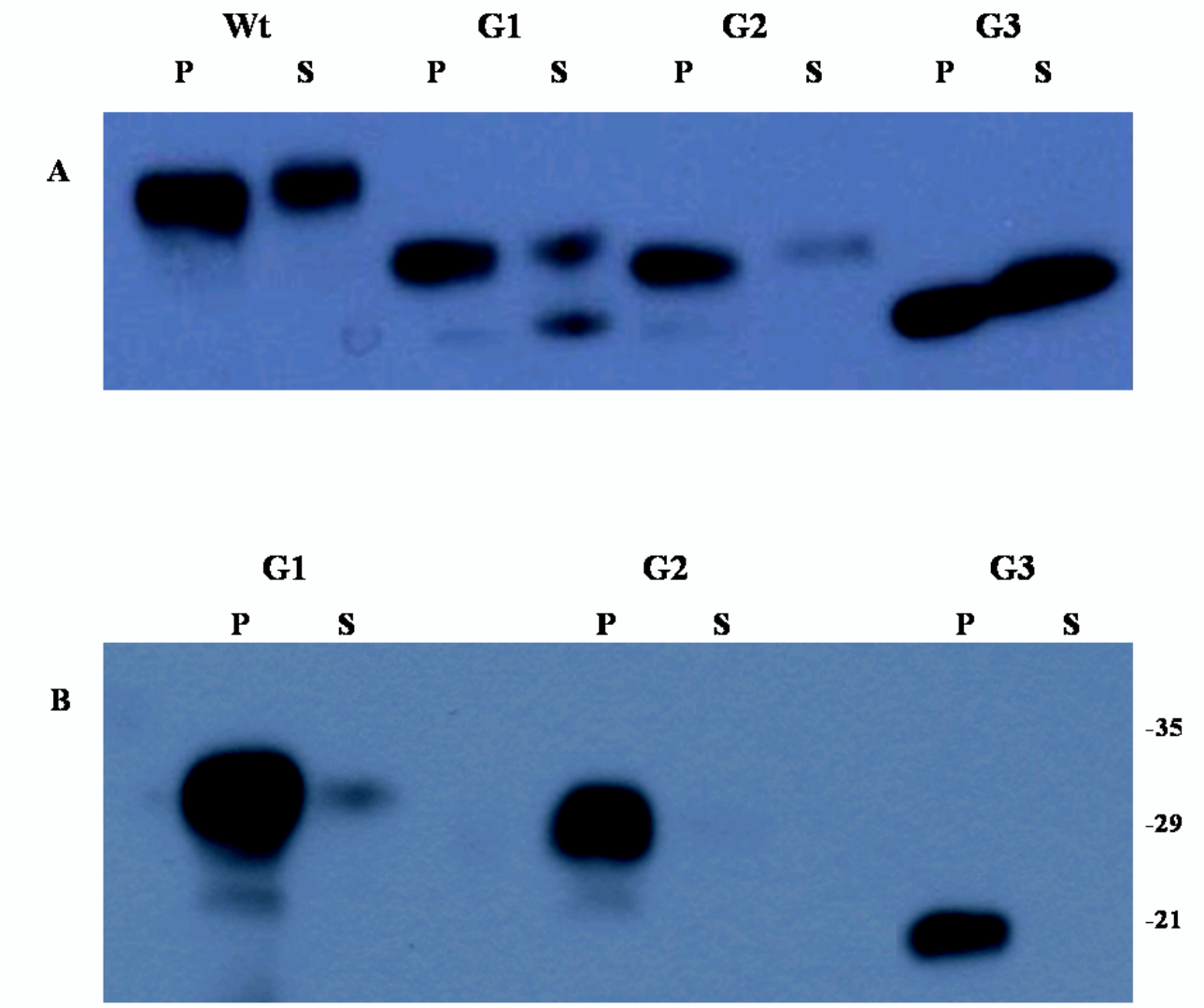
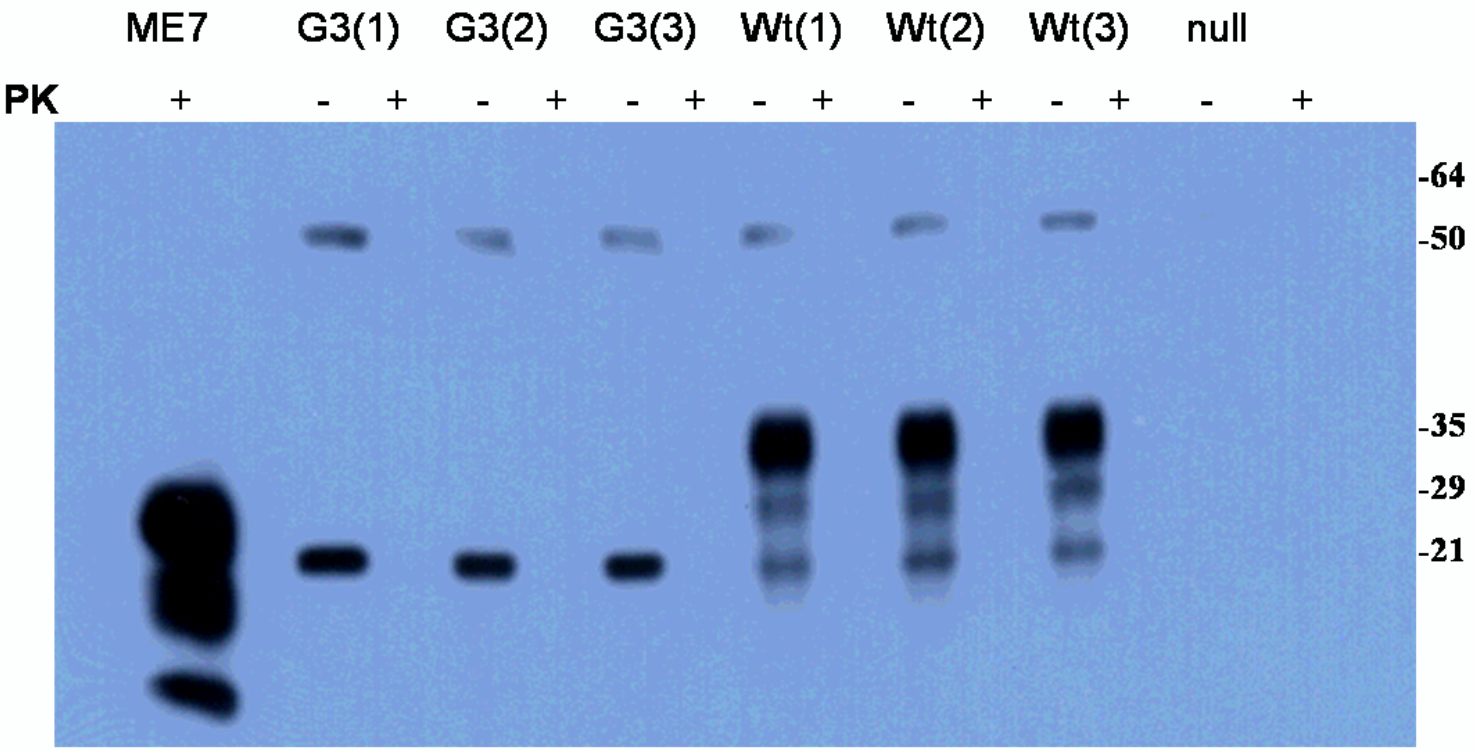


Figure 6



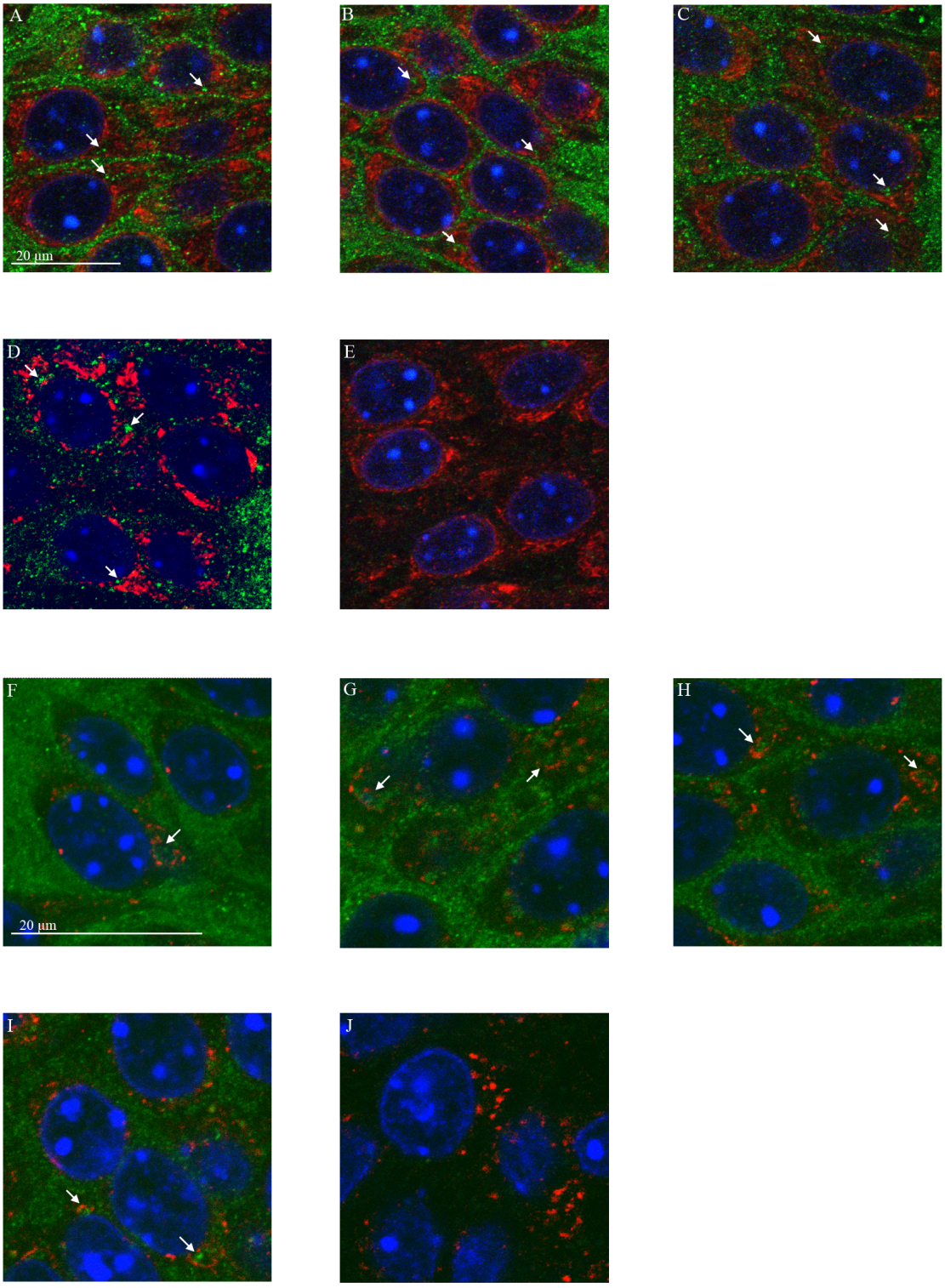
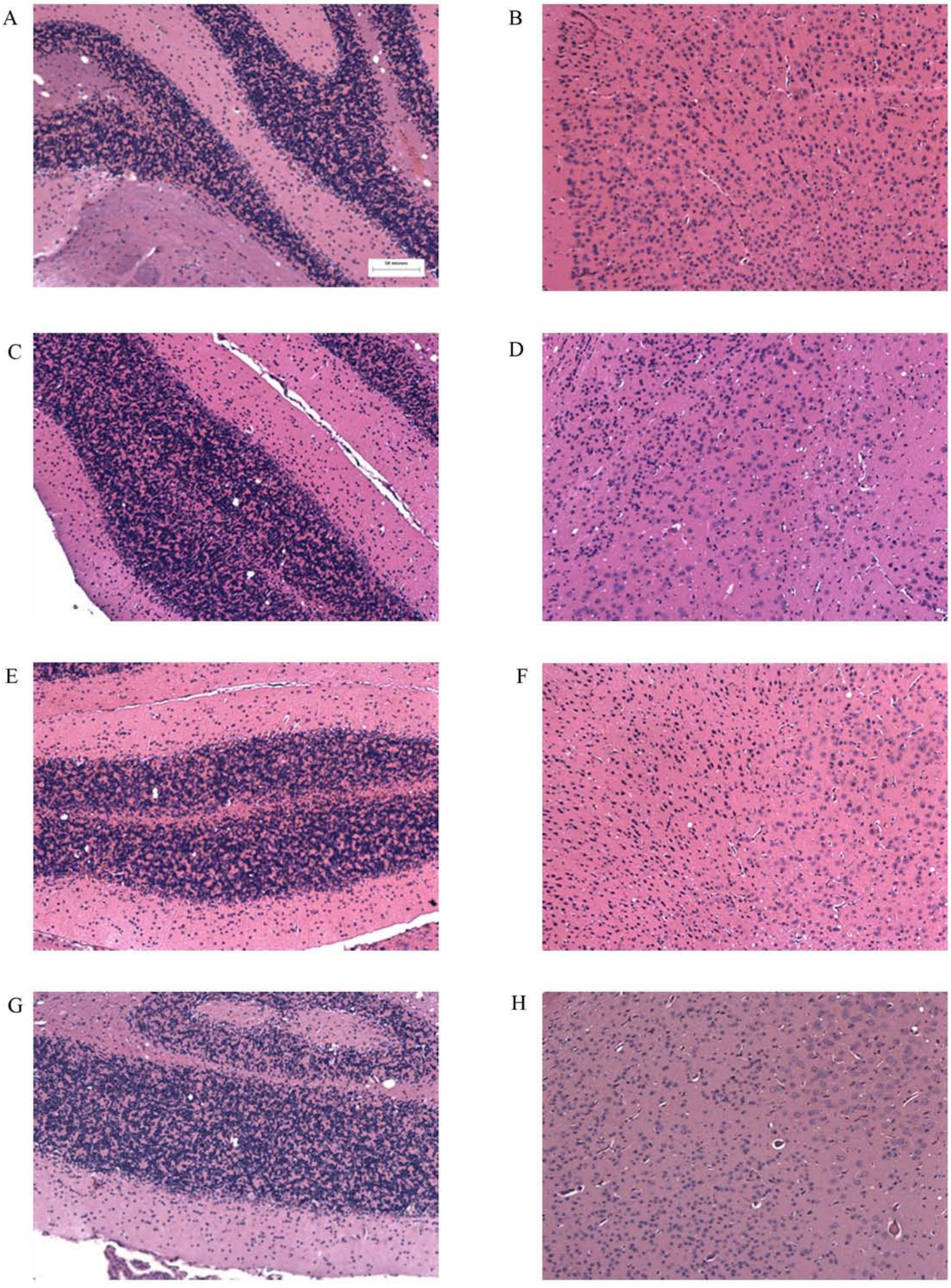
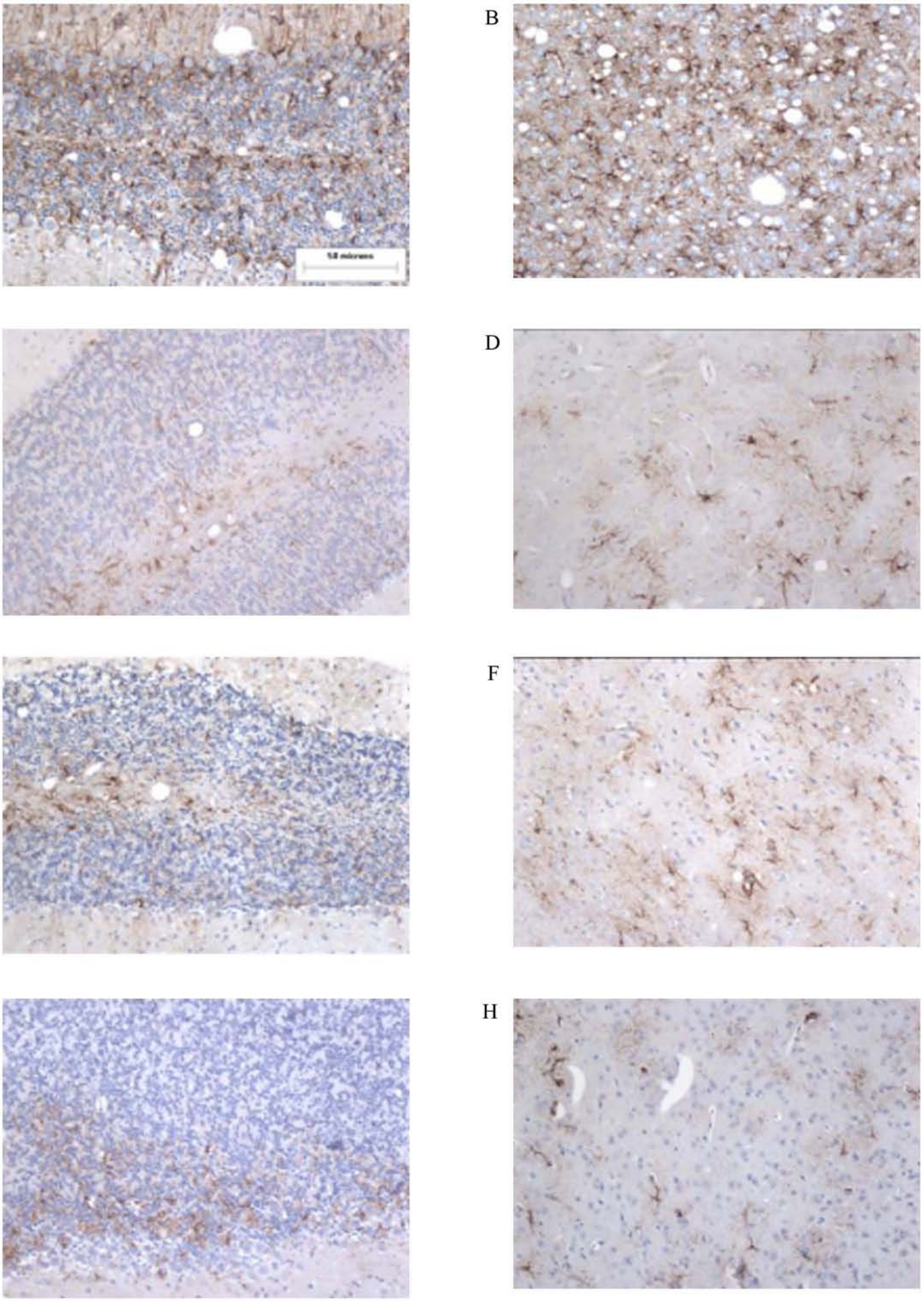


Figure 7



Supplement Fig.1



Supplement Fig.2

Altered glycosylated PrP proteins can have different neuronal trafficking in brain but do not acquire scrapie-like properties

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