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Expression and characterisation of fully posttranslationally modified cellular prion protein in *Pichia pastoris*

Abstract: Prion diseases are fatal neurodegenerative diseases which occur as sporadic, genetic, and transmissible disorders. A molecular hallmark of prion diseases is the conformational conversion of the host-encoded cellular form of the prion protein (PrP^{C}) into its misfolded pathogenic isoform (PrP^{Sc}). PrP^{Sc} is the main component of the pathological and infectious prion agent. The study of the conversion mechanism from PrP^{C} to PrP^{Sc} is a major field in prion research. PrP^{C} is glycosylated and attached to the plasma membrane via its glycosyl phosphatidyl inositol (GPI)-anchor. In this study we established and characterised the expression of fully posttranslationally modified mammalian Syrian golden hamster PrP^{C} in the yeast *Pichia pastoris* using native PrP^{C}-specific N- and C-terminal signal sequences. *In vivo* as well as *in vitro*-studies demonstrated that the signal sequences controlled posttranslational processing and trafficking of native PrP^{C}, resulting in PrP^{C} localised in the plasma membrane of *P. pastoris*. In addition, the glycosylation pattern of native PrP^{C} could be confirmed.

Keywords: eGFP-GPI; glycosyl phosphatidyl inositol (GPI)-anchor; neurodegenerative diseases; posttranslational protein modifications; prion protein signal sequences; prion protein trafficking.

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Introduction

Prion diseases, in human and animals, are fatal neurodegenerative diseases that occur spontaneously, genetically caused or by infection. A molecular hallmark within prion diseases is the conversion of the host-encoded cellular form of the prion protein (PrP^{C}) into its pathogenic isoform, PrP^{Sc}. PrP^{Sc} is the main component of infectious prions, the agent of prion diseases (Prusiner, 1998). The conformational transition from PrP^{C} to PrP^{Sc} changes the physicochemical properties of the protein and can be regarded as a posttranslational refolding process without any covalent modification (Cohen and Prusiner, 1998). Elucidating this mechanism on the molecular level is a major topic in prion research. To analyse this process *in vitro*, many studies utilise recombinant PrP (recPrP), expressed in *Escherichia coli* (Mehlhorn et al., 1996; Makarava and Baskakov, 2008; Stohr et al., 2008; Abskharon et al., 2012).

Native PrP^{C} undergoes several posttranslational modifications, including the addition of two N-linked glycosylations at amino acid residue (aa) position N181 and N197 (Endo et al., 1989; Prusiner, 1989; Weissmann, 1994) and a C-terminal glycosyl phosphatidyl inositol (GPI) anchor (Stahl et al., 1987), which do not occur in prokaryotic expression systems. All modifications are guided by an N-terminal endoplasmic reticulum import signal sequence (22 aa) and a C-terminal GPI-anchor attachment signal sequence (23 aa) (Hope et al., 1986; Bolton et al., 1987; Turk et al., 1988). Like many other GPI-anchored proteins, PrP^{C} is enriched in specific cellular membrane microdomains, so called lipid rafts (Naslavsky et al., 1997; Critchley et al., 2004; Gilch et al., 2006; Taylor and Hooper, 2006).

The conversion of non-infectious PrP^{C} into infectious PrP^{Sc} is suggested to take place at, or in close proximity

to the outer cell membrane (Vey et al., 1996). The crucial importance of membrane attachment via a GPI-anchor was demonstrated by a prevented conversion of PrP^{C} to PrP^{Sc} attached to the membrane via transmembrane segments. In contrast to these, GPI-anchored PrP^{C} could be successfully converted into PrP^{Sc} (Taraboulos et al., 1995; Kaneko et al., 1997; Resenberger et al., 2011).

In order to express fully posttranslationally modified PrP^{C}, some eukaryotic expression systems were used, e.g., Chinese hamster ovary cells (Blochberger et al., 1997) or N2a cells (Race et al., 1987; Butler et al., 1988). Moreover, posttranslational modifications were added *in vitro* after expression of recPrP in prokaryotic cells (Eberl et al., 2004; Hicks et al., 2006; Breydo et al., 2007; Olschewski et al., 2007; Chu and Becker, 2013). All of the previous expression systems, however, have distinct disadvantages, e.g. the systems are expensive, the protein yield of expressed PrP is low [for instance in Chinese hamster ovary cells 6–10 µg Chinese hamster ovary (CHO)-PrP^{C}/g CHO cells (Elfrink and Riesner, 2004)], or in case of bacterial cells, native posttranslational modifications are missing.

Over recent years, some studies were performed to express PrP^{C} in yeast systems like *Saccharomyces cerevisiae* (Ma and Lindquist, 1999; Schumacher et al., 2010) or *P. pastoris* (Weiss et al., 1995; Riley et al., 2002). The yeast *S. cerevisiae* was utilised for PrP^{C}-expression because of its ability to grow to high cell densities and to perform glycosylation, folding and disulfide bond formation (Higgins, 2001; Daly and Hearn, 2005; Cregg, 2007). Overcoming a drawback of *S. cerevisiae* – hyperglycosylation (Malissard et al., 1999) – the yeast *Pichia pastoris* was identified to form an even more human-related glycosylation pattern (Grinna and Tschopp, 1989; Daly and Hearn, 2005). Furthermore, *P. pastoris* utilises the tightly regulated alcohol oxidase (AOX) promoter (Ellis et al., 1985; Malissard et al., 1999; Daly and Hearn, 2005), resulting in up to 50–100 mg/l PrP^{C}-fusion-protein (Riley et al., 2002).

Within this study, we established the expression and posttranslational processing of Syrian golden hamster (SHa) PrP^{C} including its native N- and C-terminal signal sequences in the methylotrophic yeast *P. pastoris* under the control of an AOX promoter. We confirmed a localisation of glycosylated PrP^{C} in the plasma membrane, confirmed the posttranslational glycosylation pattern, and compared the influence of deviating N- and C-terminal signal sequences on posttranslational modification and cellular localisation. To support our results, we also utilised enhanced green fluorescent protein (eGFP) as a soluble model protein bearing the same N- and C-terminal signal sequences in order to confirm the recognition, posttranslational processing and trafficking within *P. pastoris* cells.

Results

Expression and cellular localisation of PrP^{C} in *P. pastoris*

P. pastoris cells were transformed with a plasmid containing the PRNP gene construct of the Syrian golden hamster including both the native N- and C-terminal signal sequences and a His-tag (PrP^{C}-GPI) under control of an AOX promoter [for details see the Materials and methods section (Figure 8)]. The expression was induced with methanol and its cellular localisation was analysed with immunofluorescence imaging using the monoclonal anti-PrP antibody SAF32 (Figure 1A–C). Any PrP-specific

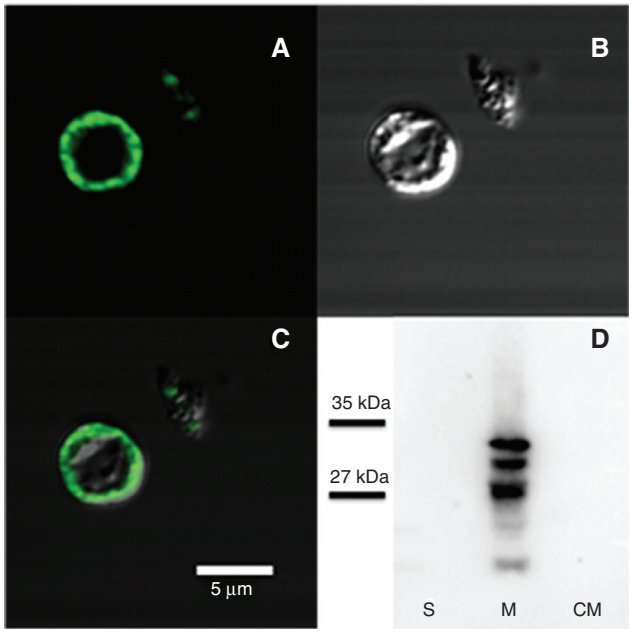

Figure 1 Expression and localisation of PrP^{C}-GPI in *Pichia pastoris*. (A) Fluorescence image for cellular localisation of PrP^{C}-GPI expressed in *P. pastoris* cells. Immunofluorescence cell analysis with anti-PrP antibody SAF32 and goat anti-mouse IgG H&L (Cy2) as secondary antibody. (B) Corresponding differential interference contrast (DIC) image. (C) Overlay of immunofluorescence and DIC images. Immunofluorescence cell analysis revealed the localisation of PrP^{C}-GPI at the outer cellular rim. (D) Distribution of PrP^{C}-GPI between soluble cytoplasm fraction (S), insoluble membrane fraction (M), and cell culture medium (CM) visualised by immunoblotting with the anti-PrP antibody SAF32. PrP-specific bands were detected in the insoluble membrane fraction.

fluorescence signal was confined to the outer cell rim, suggesting a localisation of PrP^C-GPI at the plasma membrane. To analyse the distribution of PrP^C-GPI between membrane fraction, cytoplasm, and the cell culture media, samples were subjected to subcellular fractionation by ultracentrifugation using a membrane preparation protocol (Lerner-Marmarosh et al., 1999) (see Materials and methods). PrP^C-GPI was neither detectable in the soluble cytoplasm fraction nor in the culture medium, but in the insoluble membrane fraction (Figure 1D). Moreover, the typical three-band pattern caused by the three PrP^C-isoforms, i.e. un-, mono- and diglycosylated PrP^C, were detected.

Glycosylation analysis of PrP^C-GPI

To confirm that the three-band pattern in Figure 1D was based on the presence of three differently glycosylated PrP^C-isoforms, PrP^C-GPI samples were digested with the enzyme peptide N-glycosidase F (PNGaseF), which specifically cleaves N-linked high mannose oligosaccharides. Whereas a three-band pattern is observed without PNGaseF treatment, both the supposedly di- and monoglycosylated PrP^C-GPI bands at 34 kDa and 31 kDa, respectively, were found to be digested after 1 h and completely vanished after 16 h of PNGaseF treatment (Figure 2). The additional increase in immunoreactivity of the 27 kDa PrP^C-GPI band (unglycosylated) in the digested sample confirms a native-like glycosylation of *P. pastoris* PrP^C-GPI before PNGaseF treatment. The slightly different mobility of PNGaseF treated samples within the gel could be explained with the increased protein amount in one band because of the additional protein amount of the digested mono- and diglycosylated PrP^C in the band of unglycosylated protein.

Glycosylation pattern and processing of PrP^C-GPI and $PrP^C\Delta SS$

In order to investigate the influence of N- and C-terminal signal sequences on processing and posttranslational modification of PrP^C in *P. pastoris*, we expressed a PRNP gene construct lacking both N- and C-terminal signal sequences ($PrP^C\Delta SS$), which are usually necessary for posttranslational processing, glycosylation, and attachment of the GPI-anchor. As already depicted in Figures 1D and 2, PrP^C-GPI is characterised by three glycoforms indicating a recognition of the N-terminal signal sequence, resulting in posttranslational processing of PrP^C and the localisation in the plasma membrane. In contrast, immunoblots of the $PrP^C\Delta SS$ construct showed only one protein band at a molecular weight of about 27 kDa (Figure 3), indicating the absence of any glycosylation.

The absence of a C-terminal signal sequence and in turn the GPI-anchor in $PrP^C\Delta SS$ was expected to result in soluble PrP. As depicted in Figure 3, however, $PrP^C\Delta SS$ was found to reside in the insoluble membrane fraction. Immunofluorescence microscopy studies of $PrP^C\Delta SS$ showed a different distribution of PrP^C within the cell compared to PrP^C-GPI. Most of the $PrP^C\Delta SS$ seems to be accumulated at one specific region of the cell close to the outer cell membrane, resulting in a bright immunoreactive signal (Figure 4A–C). We therefore used a

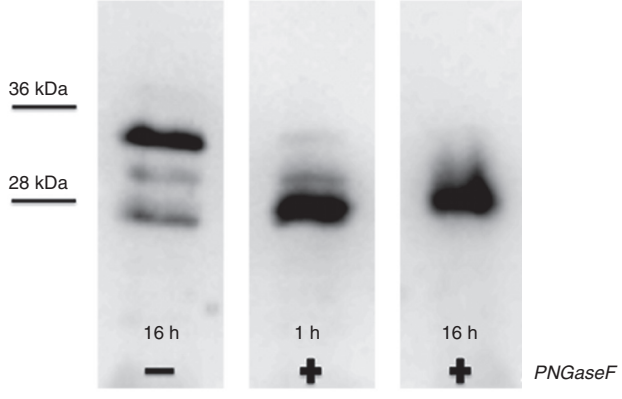

Figure 2 Glycosylation analysis of PrP^C-GPI expressed in *P. pastoris*.
Cell lysates were incubated without PNGaseF for 16 h or with PNGaseF for 1 h and 16 h, respectively, at 37°C to digest N-linked glycosylations. Samples were analysed by Western blot analysis using SAF32 as primary antibody.

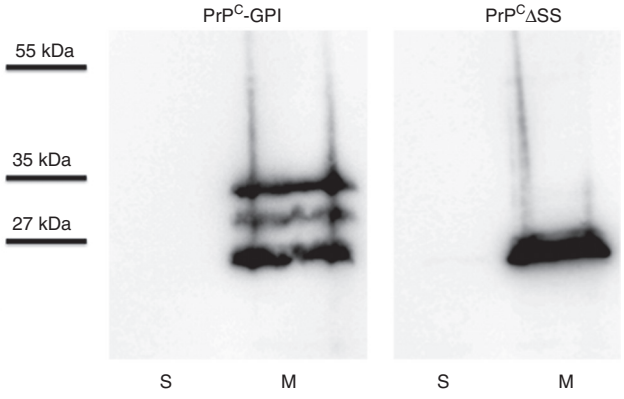

Figure 3 Comparison of glycosylation pattern and cellular distribution dependent of the signal sequences.
PrP^C-GPI and $PrP^C\Delta SS$ were compared in respect of glycosylation pattern and localisation in the cell fractions: soluble cytoplasm (S) and insoluble membrane (M) fraction. In contrast to PrP^C-GPI, $PrP^C\Delta SS$ showed only one band.

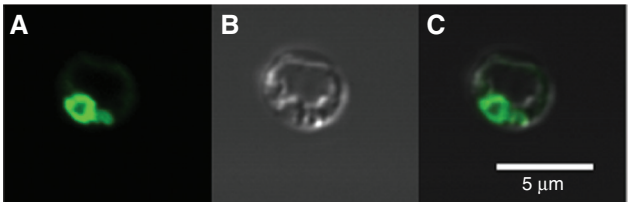

Figure 4 Expression and localisation of $PrP^{C}\Delta SS$ in *P. Pastoris*.
(A) Fluorescence image of cellular localisation of $PrP^{C}\Delta SS$ expressed in *P. pastoris* cells. Cells were analysed using anti-PrP antibody SAF32 and goat anti-mouse IgG H&L (Cy2) as secondary antibody. (B) Corresponding differential interference contrast (DIC) image. (C) Overlay of immunofluorescence and DIC images showed $PrP^{C}\Delta SS$ mainly accumulated at one specific cell compartment next to the cell membrane.

soluble model protein with the same PrP-specific signal sequences for a further proof for signal sequence processing and GPI-anchor attachment.

Cellular processing of eGFP-GPI and eGFPΔSS in *P. pastoris*

In order to prove that PrP^{C}-GPI is not enriched in the insoluble membrane fraction because of its hydrophobic character but the C-terminal attachment of a GPI-anchor, we analysed the cellular distribution of the highly soluble enhanced green fluorescent protein (eGFP). We designed constructs of eGFP with N- and C-terminal PrP-specific signal sequences (eGFP-GPI) or lacking these signal sequences (eGFPΔSS) and treated *P. pastoris* cells in the same way as described for PrP. For fluorescence imaging of the distribution of eGFP, no further fluorescence labelling was needed because of the inherent fluorescence character of GFP. Whereas eGFP-GPI was observed to be predominantly located at the outer rim of *P. pastoris* cells (Figure 5D–F), eGFPΔSS was uniformly distributed within cells (Figure 5A–C). The much higher fluorescence signal in eGFPΔSS compared to eGFP-GPI also indicates a higher expression level without signal sequences.

In agreement with PrP^{C}-GPI, eGFP-GPI was only located in the insoluble membrane fraction (Figure 6). In contrast to PrPΔSS however, eGFPΔSS was only detected in the soluble cytoplasm fraction. We therefore concluded that (i) both N- and C-terminal signal sequences guide the cellular location of PrP^{C} and eGFP, (ii) cell membrane attachment of eGFP and PrP^{C} is mediated by the presence of a C-terminal GPI-anchor, and (iii) PrPΔSS is enriched in the insoluble membrane fraction because of its propensity to aggregate.

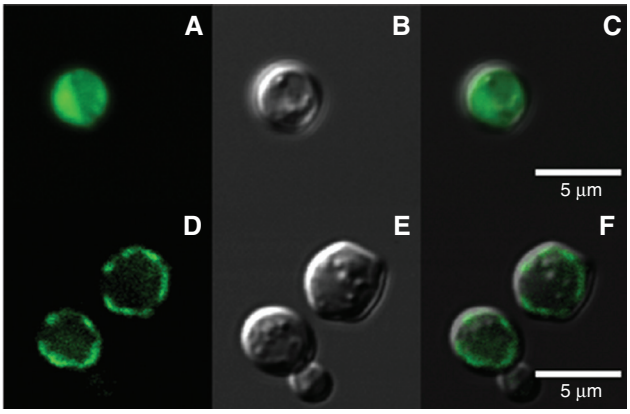

Figure 5 Cellular localisation of eGFP-GPI and eGFPΔSS in *Pichia pastoris*.
(A) Fluorescence image of cellular localisation of eGFPΔSS expressed in *P. pastoris* cells. (B) Corresponding differential interference contrast (DIC) image. (C) Overlay of fluorescence and DIC images. (D) Fluorescence image of cellular localisation of eGFP-GPI expressed in *P. pastoris* cells. (E) Corresponding differential interference contrast (DIC) image. (F) Overlay of fluorescence and DIC images. An analysis of the inherent eGFP-fluorescence revealed an even distribution of eGFPΔSS within the cytoplasm, whereas eGFP-GPI is mainly localised in the outer cellular membrane.

PrP^{C}-GPI and $PrP^{C}\Delta SS$ proteolysis with protinase K

In order to analyse whether the localisation of $PrP^{C}\Delta SS$ is caused by some conformational change or a PrP^{Sc}-like structure, which was reported by Ma and Lindquist (1999) previously, we also subjected both PrP^{C}-GPI and $PrP^{C}\Delta SS$ to a proteinase K digestion. As shown in Figure 7, neither PrP^{C}-GPI nor $PrP^{C}\Delta SS$ show any resistance to proteinase K digestion, resulting in complete digestion of PrP^{C}.

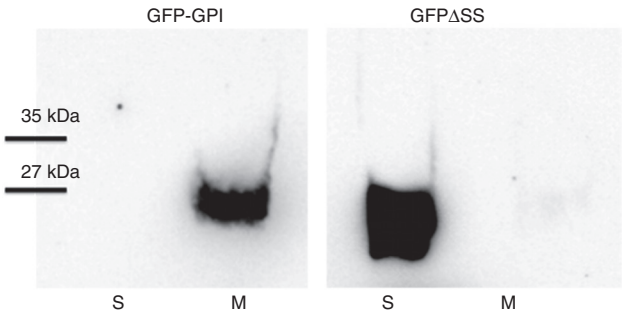

Figure 6 Comparison of the cellular distribution of eGFP-GPI and eGFPΔSS in soluble cytoplasm (S) and insoluble membrane (M) fractions.
Western blot analysis of eGFP-GPI and eGFPΔSS after differential centrifugation. eGFP-GPI has been shown to be mainly localised in the membrane fraction (M) whereas eGFPΔSS is localised in the cytoplasm fraction (S).

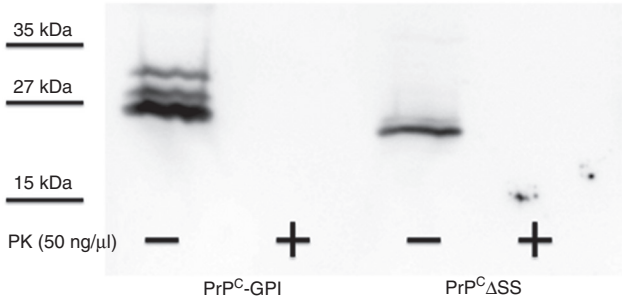

Figure 7 Proteinase K digestion of PrP^C-GPI and PrPΔSS. Proteinase K digestion using 50 ng/µl proteinase K for 1 h at 37°C resulted in a complete digestion of PrP^C-GPI and PrPΔSS and revealed the proteinase K sensivity of PrP^C expressed in *P. pastoris*.

Discussion

Several key questions in PrP^C metabolism still remain to be answered such as: (i) Are there structural differences between PrP^C glycoforms? (ii) How is the molecular mechanism of PrP^C-PrP^{Sc}-conversion? (iii) Which structural and/or mechanistic influence is caused by an attachment to the cell membrane? One major pending milestone to answer these questions on the molecular level is the production and purification of high amounts of fully posttranslationally modified PrP^C and its GPI-anchor-mediated attachment to the outer cell membrane.

Previously published methods analysing membrane-bound PrP relied on the recombinant expression of PrP in *E. coli* followed by covalent linkage to synthetically generated posttranslational modifications, i.e., the thiol-reactive lipid N-((2-pyridyldithio)-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Eberl et al., 2004; Hicks et al., 2006; Breydo et al., 2007; Olschewski et al., 2007; Chu and Becker, 2013). However, these approaches are afflicted with some challenges: (i) they are based on the presence of free thiol groups at the C-terminus of recPrP while the intrinsically disulfide bond needs to remain intact, (ii) chemical ligation reactions have to be performed in order to obtain chemically modified recPrP while recPrP needs to be prevented from aggregation, (iii) all of these modifications differ to the physiological, natively attached GPI-anchor.

In contrast, eukaryotic cell culture systems such as CHO cells or neuroblastoma (N2a) cells offer the advantage of native posttranslational modifications. However, they are time consuming, expensive, and suffer from low protein yields (Blochberger et al., 1997). An expression system able to overcome these limitations is the yeast system, e.g., *P. pastoris* and *S. cerevisiae*. Yeast cells are known for fast expression of high amounts of proteins because of the usage of strong promoters, e.g., the methanol-induceable AOX promotor. Utilising yeast expression systems also allow studying proteins comprising their native posttranslational modifications. In one study, Syrian golden hamster PrP^C was expressed and secreted by *P. pastoris* cells utilising an N-terminal signal sequence of acid phosphatase. No C-terminal GPI-anchor-specific signal sequence was used for attachment of a GPI-anchor and trafficking to the cell membrane. Furthermore, only yields below 0.1 mg/l cell culture medium were reported (Weiss et al., 1995). In order to transport nascent PrP^C into the endoplasmic reticulum for glycosylation, mouse PrP was expressed in *S. cerevisiae* with and without the N-terminal signal peptide sequence of Kar2 (Ma and Lindquist, 1999). Without the signal sequence, PrP was detected in an insoluble fraction. The insoluble PrP aggregates were described as PrP^{Sc}-like. With the signal sequence, glycosylated PrP was detected, together with unprocessed unglycosylated PrP, still comprising the signal sequence (Rose et al., 1989; Ma and Lindquist, 1999). Riley et al. exploited human PrP^C with specific N- and C-terminal signal sequences and expressed FLAG-tagged human PrP^C and human PrP^C dimers covalently connected by a FLAG peptide in *P. pastoris*. The FLAG peptide was used as an epitope tag for detection and purification. However, the FLAG peptide interrupted the PrP^C sequence at position 227 close to the C-terminal signal sequence resulting in a non-natural form of full length human PrP^C (Riley et al., 2002). Recently, human PrP was expressed in *S. cerevisiae* using the N-terminal signal sequence of Kre5p and the C-terminal signal sequence of Gas1p. Successful trafficking to the plasma membrane because of the attached GPI-anchor was shown using immunofluorescence microscopy (Schumacher et al., 2010).

Within this study, we established the expression of Syrian golden hamster PrP^C in the yeast *P. pastoris*, exploiting native Syrian golden hamster PrP^C-specific signal sequences in order to obtain native-like PrP^C. In presence of native N- and C-terminal signal sequences, PrP^C was demonstrated to contain both N-linked glycosylations and localisation at the cell membrane. The presence of all three PrP^C glycosylation isoforms, i.e., di-, mono-, and unglycosylated PrP^C, was confirmed by PNGaseF-digestion, supported by the observation that a PrP construct lacking N- and C-terminal signal sequences does not show any glycosylations. The presence of a covalently linked GPI-anchor was deduced from the localisation at the cell membrane. A protein construct comprising the soluble eGFP flanked by the same N- and C-terminal signal sequences of PrP^C was demonstrated to be located at the cell membrane and to be enriched in the insoluble membrane fraction

after differential centrifugation. In contrast, in the absence of N- and C-terminal signal sequences, eGFP was confined to the soluble cytoplasm fraction and showed an even distribution within *P. pastoris* cells.

Like Ma et al. we also observed an insolubility of $PrP^{C}\Delta SS$ but we explained the localisation of $PrP^{C}\Delta SS$ in the membrane fraction with the high natural aggregation tendency of PrP, especially at high concentrations in consequence of AOX promoter-driven expression. This assumption is also affirmed by immunofluorescence results where $PrP^{C}\Delta SS$ seems to be accumulated within a distinct cell area in the cell. In contrast to Ma et al., using the yeast *S. cerevisae* we observed a proteinase K sensitivity of PrP^{C}, leading to the conclusion that PrP expressed in *P. pastoris* did not show any PrP^{Sc}-like character.

In summary, we conclude that only the presence of both N- and C-terminal signal sequences results in a native posttranslational processing of PrP^{C} in *P. pastoris* including glycosylation and covalent linkage of a GPI-anchor and consequently to an attachment to the outer cell membrane.

Our results provide a simple, fast, and powerful tool for analysing PrP trafficking as well as interaction studies of PrP^{C} in a close natural membrane environment. It also can serve for expression and subsequent purification of fully posttranslational modified PrP^{C}.

Materials and methods

Expression vectors

The nucleotide sequence coding for Syrian golden hamster prion protein (SHaPrP) was amplified via PCR. A plasmid containing the nucleotide sequence coding for PrP aa 23-231, which served for PrP expression in *E. coli*, was used as a template. N- and C-terminal signal sequences were added using the following oligonucleotides:

- Forward: 5′ ATT GAA TTC ATG GCG AAC CTT AGC TAC TGG CTG CTG GCA CTC TTT GTG GCT ATG TGG ACT GAT GTT GGC CTC TGC CAT CAT CAT CAT CAT CAT CAC GGA AAG AAG CGG CCA AAG CCT GGA GGG TGG AAC AC 3′
- Reverse: 5′ GAT CGG GCC CTC ATC CCA CCA TCA GGA AGA TGA GAA AGG AAA TGA GGA GGA TCA CAG GAG GGG AGG AGA ACA GCA CCG CGC TGG ACC TTC TTC CAT CGT AGT AGG CCT GGG ACT CCT TC 3′

The PCR product was cloned into the *P. pastoris* expression vector pPICzA (Zeocin resistance, AOX1 promoter) (Invitrogen, Carlsbad, USA) using the restriction sites *Eco*RI/*Apa*I, resulting in pPICzA-PrP. The sequence was verified by Sanger sequencing. For construction of Syrian golden hamster prion protein construct, PrP aa 23-231 lacking N- and C-terminal signal sequences (PrPΔSS) the plasmid pPICzA-PrP served as template. The sequence was amplified using the primers:

- Forward: 5′ GAT TGA ATT CAT GCA TCA TCA TCA TCA TCA TCA CGG AAA GAA GCG GCC AAA GCC TGG AGG GTG GAA CAC 3′
- Reverse: 5′ GAT CGG GCC CTT AGG ACC TTC TTC CAT CGT AGT AGG CC 3′

The PCR product was cloned into the expression vector pPICzA using the same restriction sites resulting in pPICZA-PrPΔSS. Primers were synthesised by MWG eurofins, Ebersberg, Germany. Restrictions enzymes were ordered by Fisher Scientific, Schwerte, Germany.

The enhanced GFP (eGFP) gene including Syrian golden hamster prion protein N- and C-terminal signal sequences was synthesised by GeneArt life technologies (Invitrogen, Darmstadt, Germany). The eGFP gene sequence was cloned directly out of the synthesised vector into the expression vector pPICzA using the restriction sites *Eco*RI and *Apa*I resulting in pPICzA-eGFP-native.

For the eGFPΔSS construct the plasmid pPICzA-eGFP-native served as a template. Using the primers 5′ GAT CGA ATT CAT GGT TTC CAA GGG TGA AGA GTT 3′ and 5′ GAT CGG GCC CTT ACT TGT ACA ACT CGT CCA TAC CCA AAG TGA TA 3′ the new sequence was amplified via PCR and cloned into the vector pPICzA using the same restriction sites. All sequences were verified by Sanger sequencing. The resulting constructs are shown schematically in Figure 8.

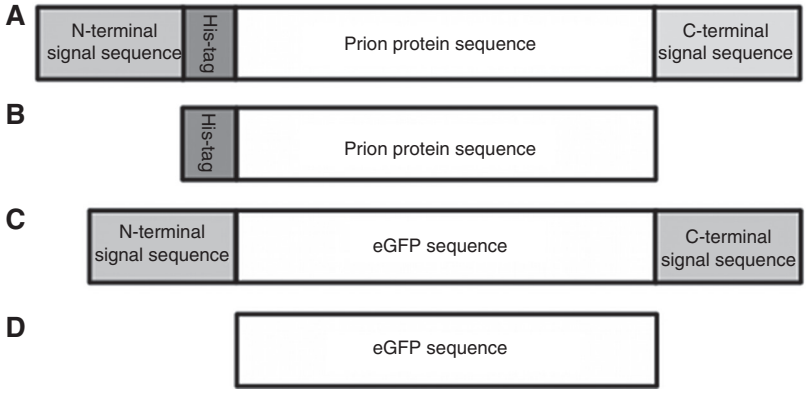

Figure 8 Scheme of PrP/eGFP constructs.
(A) PrP^{C}-GPI, Syrian hamster prion protein construct with N- and C-terminal native hamster PrP signal sequences (1-254), auxiliary a His7-tag. (B) $PrP^{C}\Delta SS$, Syrian hamster prion protein construct without N- and C-terminal native hamster PrP signal sequences (23-231), auxiliary a His7-tag. (C) eGFP-GPI, Enhanced green fluorescent protein construct with N- and C-terminal native hamster PrP signal sequences. (D) eGFPΔSS, enhanced green fluorescent protein construct without native hamster PrP signal sequences.

Transformation in *P. pastoris*

Plasmid DNA (10 µg) was transformed into freshly prepared electrocompetent *P. pastoris* X-33 cells (Invitrogen) following the manufacturer's instructions. Transformed cells were plated on yeast extract peptone dextrose medium (YPD) (1% yeast extract, 2% peptone, 1% dextrose) plates supplemented with 1 M sorbitol and 200 µg/ml Zeocin (Invitrogen) and incubated at 30°C for 3–7 days until colonies grew. Ten clones were selected for small-scale test expression

Small-scale test expression in *P. pastoris*

Twenty milliliter YPD medium supplemented with 200 µg/ml Zeocin was incubated overnight at 30°C and 200 rpm (Infors HT Multitron Standard). After 12–24 h, cells were pelleted by centrifugation at 2000 *g* for 10 min at 4°C and resuspended in 20 ml minimal expression medium (MMY) (1.34% YNB; 4×10^{-5}% biotin; 0.5% methanol) and further incubated for 24 h.

Large-scale expression

Fifty milliliter YPD including 200 µg/ml Zeocin was inoculated with a single colony from a plate and incubated over night at 30°C and 200 rpm (Infors HT Multitron Standard). The desired volume of YPG (1% yeast extract, 2% peptone, 1% glycerol) medium was inoculated to an $OD_{600\ nm}$=1.0 and incubated for 48 h at 30°C and 200 rpm in baffled flasks. Cells were harvested and resuspended with MMY and incubated at 30°C and 200 rpm for further 24 h (Villatte et al., 2001). Cells were harvested at 5000 *g* for 5 min at 4°C, washed once in phosphate buffered saline (PBS), centrifuged again and cell pellets were snap frozen in liquid nitrogen and stored at -80°C until use.

Cell lysis and isolation of yeast whole cell membranes

Cell pellets of 50 ml cultures were resuspended in 6 ml ice-cold breaking buffer (50 mM NaH_2PO_4, 1 mM EDTA, 5% glycerol, pH 7.4). Glass beads were added and cells were disrupted using a bead beater (FastPrep 24; MP Biomedicals, Eschwege, Germany) five times for 30 s at 4°C. The cell lysate was centrifuged at 3000 *g* for 5 min at 4°C. One milliliter of supernatant was centrifuged at 10 000 *g* for 20 min at 4°C. Five hundred microliter of this supernatant was used for the membrane preparation by separating insoluble and soluble fraction by a 100 000 *g* spin for 1 h at 4°C using a Beckman Optima™ ultracentrifuge. The resulting pellet contained the insoluble membrane fraction whereas the cytosolic, soluble fraction remains in the supernatant (Wang et al., 2006). The membrane pellet was solved in SDS sample buffer (70 mM Tris/HCl pH 6.8; 0.05% bromphenolblue; 5% glycerol; 2% SDS; 5% β-mercaptoethanol) and analysed by SDS-PAGE (12% polyacrylamide gel) and Western blotting.

Glycosylation analysis

To elucidate the glycosylation pattern of PrP expressed in *P. pastoris*, an enzymatic digestion with amidase PNGaseF (New England Biolabs, Ipswich, USA) was used. Directly after the 10 000 *g* spin, supernatant was transferred to one new tube. From this stock solution for each reaction sample, 9 µl cell suspension were incubated with 1 µl PNGaseF (500 U/µl) referring to the manufacturers protocol (37°C) or without PNGaseF for the negative control. Reaction was stopped after 1 h or 16 h by adding SDS sample buffer. Analysis was performed using SDS-PAGE and Western blotting.

Proteinase K digestion

To analyse whether PrP^C expressed in *P. pastoris* shows any proteinase K resistance, PrP^C-GPI and $PrP^C\Delta SS$ was subjected to a proteinase K digestion (proteinase K, recombinant PCR grade, Roche Diagnostics GmbH, Mannheim, Germany). After cell disruption and the 10 000 *g* spin, supernatant was transferred to one new tube. From this stock solution for each reaction, samples of 20 µl cell suspension were adjusted to a final concentration of 0.002 M $MgCl_2$ and 1 µl Benzonase (250 U/µl, Merck, Darmstadt) was added. Finally, proteinase K was added to a final concentration of 50 ng/µl. The samples were incubated at 37°C, 300 rpm for 1 h. Reaction was stopped by adding SDS sample buffer and boiling for 10 min at 99°C. The samples were analysed by SDS-PAGE and Western blotting.

Immunofluorescence microscopy

PrP was expressed in *P. pastoris* as described above. Para-formaldehyde was added to a concentration of 4% to 500 µl of cell culture and incubated for 1 h at 30°C. Cells were pelleted for 5 min at 3000 *g* and resuspended in 1 ml S-buffer (50 mM HEPES, 1.2 M sorbitol, pH 7.5) and pelleted again. After resuspension in 500 µl S-buffer, 20 µl Zymolyase 100T (2.5 mg/ml) (MP Biomedicals) were added for cell wall digestion and cells were incubated at 30°C for 45 min. After cell wall digestion, cells were washed twice in S-buffer and finally resuspended in 100 µl S-buffer. Twenty-five microliter were applied to poly-L-lysine-coated cover slips and incubated for 10 min, followed by two washing steps with PBS. Blocking was performed with 2% milk powder in PBS for 30 min. After another washing step the anti-PrP antibody SAF32 was added for 1 h (BIONITY, SpiBio, Montigny-le-Bretonneux, France) [5 µg/ml in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween20)]. Cover slips were washed and the fluorescent labelled secondary goat anti-mouse antibody IgG H&L (Cy2®) (Abcam, ab6944, 10 µg/ml) was added for 1 h. After three additional washing steps the cover slips were inverted on a glass slide containing ProLong Gold Antifade Reagent (Invitrogen, P36934) and stored at 4°C.

eGFP was expressed in *P. pastoris* as described above. Para-formaldehyde was added to a concentration of 4% to 500 µl of cell culture and incubated for 1 h at 30°C. Cells were pelleted for 5 min at 3000 *g* and resuspended in 1 ml S-buffer, washed and pelleted again. Cells were directly resuspended in 100 µl ProLong Gold Antifade Reagent, 50 µl applied on a glass slide, covered by a cover slip and stored at 4°C (Ellinger et al., 2013).

Fluorescent imaging was performed using the confocal laser-scanning microscope Olympus FV1000 equipped with a 60× UPL-

SAPO objective N.A. 1.35. The fluorescent labelled antibodies were excited at 488 nm and emission detected at 500–600 nm.

SDS-PAGE and Western blot analysis

Samples were analysed on a 12% SDS-PAGE and subsequently transferred onto a PVDF membrane (PALL Life Sciences) using the TE70X semi-dry transfer unit (Hoefer). The blot membrane was blocked for 1 h with 5% milk powder in PBST, washed three times with PBST, incubated with either anti-PrP antibody (SAF32, 0.1 µg/ml), anti-GFP antibody (mouse anti-GFP, Invitrogen, 1.5 µg/ml) or with anti-PrP antibody 6H4 (mouse anti-PrP, Prionics, Wolfratshausen, Germany, 0.15 µg/ml) for the proteinase K experiment for 1 h. After three washing steps the membrane was incubated for 1 h with the secondary antibody goat anti-mouse HRP conjugate (Jackson, Immuno Research Inc., 0.1 µg/ml). Protein detection was performed, after three additional washing steps with PBST, using a SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualised with a LAS 4000 (Fuji Film).

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References

Abskharon, R.N., Ramboarina, S., El Hassan, H., Gad, W., Apostol, M.I., Giachin, G., Legname, G., Steyaert, J., Messens, J., Soror, S.H., et al. (2012). A novel expression system for production of soluble prion proteins in *E. coli*. Microb. Cell. Fact. *11*, 6.

Blochberger, T.C., Cooper, C., Peretz, D., Tatzelt, J., Griffith, O.H., Baldwin, M.A., and Prusiner, S.B. (1997). Prion protein expression in Chinese hamster ovary cells using a glutamine synthetase selection and amplification system. Protein Eng. *10*, 1465–1473.

Bolton, D.C., Bendheim, P.E., Marmorstein, A.D., and Potempska, A. (1987). Isolation and structural studies of the intact scrapie agent protein. Arch. Biochem. Biophys. *258*, 579–590.

Breydo, L., Sun, Y., Makarava, N., Lee, C.I., Novitskaia, V., Bocharova, O., Kao, J.P., and Baskakov, I.V. (2007). Nonpolar substitution at the C-terminus of the prion protein, a mimic of the glycosylphosphatidylinositol anchor, partially impairs amyloid fibril formation. Biochemistry *46*, 852–861.

Butler, D.A., Scott, M.R., Bockman, J.M., Borchelt, D.R., Taraboulos, A., Hsiao, K.K., Kingsbury, D.T., and Prusiner, S.B. (1988). Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. J. Virol. *62*, 1558–1564.

Chu, N.K. and Becker, C.F. (2013). Recombinant expression of soluble murine prion protein for C-terminal modification. FEBS Lett. *587*, 430–435.

Cohen, F.E. and Prusiner, S.B. (1998). Pathologic conformations of prion proteins. Annu. Rev. Biochem. *67*, 793–819.

Cregg, J.M. (2007). Introduction: distinctions between Pichia pastoris and other expression systems. Methods Mol. Biol. *389*, 1–10.

Critchley, P., Kazlauskaite, J., Eason, R., and Pinheiro, T.J. (2004). Binding of prion proteins to lipid membranes. Biochem. Biophys. Res. Commun. *313*, 559–567.

Daly, R. and Hearn, M.T. (2005). Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. J. Mol. Recognit. *18*, 119–138.

Eberl, H., Tittmann, P., and Glockshuber, R. (2004). Characterization of recombinant, membrane-attached full-length prion protein. J. Biol. Chem. *279*, 25058–25065.

Elfrink, K. and Riesner, D. (2004). Purification of PrPC. In: Methods and Tools in Biosciences and Medicine: Techniques in Prion Research (Basel: Birkhäuser Verlag).

Ellinger, P., Kluth, M., Stindt, J., Smits, S.H., and Schmitt, L. (2013). Detergent screening and purification of the human liver ABC transporters BSEP (ABCB11) and MDR3 (ABCB4) expressed in the yeast *Pichia pastoris*. PLoS One *8*, e60620.

Ellis, S.B., Brust, P.F., Koutz, P.J., Waters, A.F., Harpold, M.M., and Gingeras, T.R. (1985). Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. Mol. Cell. Biol. *5*, 1111–1121.

Endo, T., Groth, D., Prusiner, S.B., and Kobata, A. (1989). Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. Biochemistry *28*, 8380–8388.

Gilch, S., Kehler, C., and Schatzl, H.M. (2006). The prion protein requires cholesterol for cell surface localization. Mol. Cell. Neurosci. *31*, 346–353.

Grinna, L.S. and Tschopp, J.F. (1989). Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. Yeast *5*, 107–115.

Hicks, M.R., Gill, A.C., Bath, I.K., Rullay, A.K., Sylvester, I.D., Crout, D.H., and Pinheiro, T.J. (2006). Synthesis and structural characterization of a mimetic membrane-anchored prion protein. FEBS J. *273*, 1285–1299.

Higgins, D.R. (2001). Overview of protein expression in *Pichia pastoris*. Curr. Protoc. Protein Sci. Chapter 5, Unit5.7.

Hope, J., Morton, L.J., Farquhar, C.F., Multhaup, G., Beyreuther, K., and Kimberlin, R.H. (1986). The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). EMBO J. *5*, 2591–2597.

Kaneko, K., Vey, M., Scott, M., Pilkuhn, S., Cohen, F.E., and Prusiner, S.B. (1997). COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. Proc. Natl. Acad. Sci. USA *94*, 2333–2338.

Lerner-Marmarosh, N., Gimi, K., Urbatsch, I.L., Gros, P., and Senior, A.E. (1999). Large scale purification of detergent-soluble P-glycoprotein from *Pichia pastoris* cells and characterization of nucleotide binding properties of wild-type, Walker A, and Walker B mutant proteins. J. Biol. Chem. *274*, 34711–34718.

Ma, J. and Lindquist, S. (1999). De novo generation of a PrPSc-like conformation in living cells. Nat. Cell Biol. *1*, 358–361.

Makarava, N. and Baskakov, I.V. (2008). Expression and purification of full-length recombinant PrP of high purity. Methods Mol. Biol. *459*, 131–143.

Malissard, M., Zeng, S., and Berger, E.G. (1999). The yeast expression system for recombinant glycosyltransferases. Glycoconj. J. *16*, 125–139.

Mehlhorn, I., Groth, D., Stockel, J., Moffat, B., Reilly, D., Yansura, D., Willett, W.S., Baldwin, M., Fletterick, R., Cohen, F.E., et al. (1996). High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. Biochemistry *35*, 5528–5537.

Naslavsky, N., Stein, R., Yanai, A., Friedlander, G., and Taraboulos, A. (1997). Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. J. Biol. Chem. *272*, 6324–6331.

Olschewski, D., Seidel, R., Miesbauer, M., Rambold, A.S., Oesterhelt, D., Winklhofer, K.F., Tatzelt, J., Engelhard, M., and Becker, C.F. (2007). Semisynthetic murine prion protein equipped with a GPI anchor mimic incorporates into cellular membranes. Chem. Biol. *14*, 994–1006.

Prusiner, S.B. (1989). Scrapie prions. Annu. Rev. Microbiol. *43*, 345–374.

Prusiner, S.B. (1998). Prions. Proc. Natl. Acad. Sci. USA *95*, 13363–13383.

Race, R.E., Fadness, L.H., and Chesebro, B. (1987). Characterization of scrapie infection in mouse neuroblastoma cells. J. Gen. Virol. *68* (Pt 5), 1391–1399.

Resenberger, U.K., Harmeier, A., Woerner, A.C., Goodman, J.L., Muller, V., Krishnan, R., Vabulas, R.M., Kretzschmar, H.A., Lindquist, S., Hartl, F.U., et al. (2011). The cellular prion protein mediates neurotoxic signalling of b-sheet-rich conformers independent of prion replication. EMBO J. *30*, 2057–2070.

Riley, M.L., Leucht, C., Gauczynski, S., Hundt, C., Brecelj, M., Dodson, G., and Weiss, S. (2002). High-level expression and characterization of a glycosylated covalently linked dimer of the prion protein. Protein Eng. *15*, 529–536.

Rose, M.D., Misra, L.M., and Vogel, J.P. (1989). KAR2, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. Cell *57*, 1211–1221.

Schumacher, M.C., Resenberger, U., Seidel, R.P., Becker, C.F., Winklhofer, K.F., Oesterhelt, D., Tatzelt, J., and Engelhard, M. (2010). Synthesis of a GPI anchor module suitable for protein post-translational modification. Biopolymers *94*, 457–464.

Stahl, N., Borchelt, D.R., Hsiao, K., and Prusiner, S.B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell *51*, 229–240.

Stohr, J., Weinmann, N., Wille, H., Kaimann, T., Nagel-Steger, L., Birkmann, E., Panza, G., Prusiner, S.B., Eigen, M., and Riesner, D. (2008). Mechanisms of prion protein assembly into amyloid. Proc. Natl. Acad. Sci. USA *105*, 2409–2414.

Taraboulos, A., Scott, M., Semenov, A., Avrahami, D., Laszlo, L., and Prusiner, S.B. (1995). Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. J. Cell Biol. *129*, 121–132.

Taylor, D.R. and Hooper, N.M. (2006). The prion protein and lipid rafts. Mol. Membr. Biol. *23*, 89–99.

Turk, E., Teplow, D.B., Hood, L.E., and Prusiner, S.B. (1988). Purification and properties of the cellular and scrapie hamster prion proteins. Eur. J. Biochem. *176*, 21–30.

Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S.J., Smart, E.J., Anderson, R.G., Taraboulos, A., and Prusiner, S.B. (1996). Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc. Natl. Acad. Sci. USA *93*, 14945–14949.

Villatte, F., Hussein, A.S., Bachmann, T.T., and Schmid, R.D. (2001). Expression level of heterologous proteins in *Pichia pastoris* is influenced by flask design. Appl. Microbiol. Biotechnol. *55*, 463–465.

Wang, Z., Stalcup, L.D., Harvey, B.J., Weber, J., Chloupkova, M., Dumont, M.E., Dean, M., and Urbatsch, I.L. (2006). Purification and ATP hydrolysis of the putative cholesterol transporters ABCG5 and ABCG8. Biochemistry *45*, 9929–9939.

Weiss, S., Famulok, M., Edenhofer, F., Wang, Y.H., Jones, I.M., Groschup, M., and Winnacker, E.L. (1995). Overexpression of active Syrian golden hamster prion protein PrPc as a glutathione S-transferase fusion in heterologous systems. J. Virol. *69*, 4776–4783.

Weissmann, C. (1994). Molecular biology of prion diseases. Trends Cell Biol. *4*, 10–14.