



University of
Zurich^{UZH}

Zurich Open Repository and
Archive

University of Zurich
Main Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2008

Putative prion protein from Fugu (*Takifugu rubripes*)

Christen, Barbara ; Wüthrich, Kurt ; Hornemann, Simone

Abstract: Prion proteins (PrP) of mammals, birds, reptiles and amphibians have been successfully cloned, expressed and purified in sufficient yields to enable 3D structure determination by NMR spectroscopy in solution. More recently, PrP ortholog genes have also been identified in several fish species, based on sequence relationships with tetrapod PrPs. Even though the sequence homology of fish PrPs to tetrapod PrPs is below 25%, structure prediction programs indicate a similar organization of the 3D structure. In this study, we generated recombinant polypeptide constructs that were expected to include the C-terminal folded domain of Fugu-PrP1 and analyzed these proteins using biochemical and biophysical methods. Because soluble expression could not be achieved, and refolding from guanidine-HCl did not result in a properly folded protein, we co-expressed *Escherichia coli* chaperone proteins in order to obtain the protein in a soluble form. Although CD spectroscopy indicated the presence of some regular secondary structure in the protein thus obtained, there was no evidence for a globular 3D fold in the NMR spectra. We thus conclude that the polypeptide products of the fish genes annotated as corresponding to bona fide prnp genes in non-fish species cannot be prepared for structural studies when using procedures similar to those that were successfully used with PrPs from mammals, birds, reptiles and amphibians.

DOI: <https://doi.org/10.1111/j.1742-4658.2007.06196.x>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-191710>

Journal Article

Accepted Version



The following work is licensed under a Creative Commons: Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.

Originally published at:

Christen, Barbara; Wüthrich, Kurt; Hornemann, Simone (2008). Putative prion protein from Fugu (*Takifugu rubripes*). *FEBS Journal*, 275(2):263-270.

DOI: <https://doi.org/10.1111/j.1742-4658.2007.06196.x>

Putative prion protein from Fugu (*Takifugu rubripes*)

Barbara Christen, Kurt Wüthrich, and Simone Hornemann*

Institute of Molecular Biology and Biophysics, ETH Zurich, 8093 Zurich, Switzerland

Running title: Fugu Prion Proteins

*Corresponding author:

Simone Hornemann

Institute of Molecular Biology and Biophysics

Schafmattstrasse 20

ETH Zürich

CH-8093 Zürich, Switzerland

telephone number: +41 44 633 34 53

fax number: +41 44 633 14 84

e-mail: simone.hornemann@mol.biol.ethz.ch

www.mol.biol.ethz.ch/groups/wuthrich_group

Character count (including spaces): 24522

Abbreviations: GPI, glycosylphosphatidylinositol; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl- β -D-1-thiogalactopyranoside; PrP, prion protein; tr1-PrP, 6 x His-tagged Fugu-PrP1(298–423); mPrP, mouse prion protein; hPrP, human prion protein; SH3, Src homology domain 3; TSE, transmissible spongiform encephalopathy.

Keywords: Fish prion protein, *Takifugu rubripes*, chaperone co-expression, transmissible spongiform encephalopathy, NMR

Summary

Prion proteins (PrP) of mammals, birds, reptiles and amphibians have been successfully cloned, expressed and purified in sufficient yields to enable three-dimensional structure determination by nuclear magnetic resonance spectroscopy in solution. More recently, PrP orthologue genes have also been identified in several fish species, based on sequence relationships with tetrapod PrPs. Even though the sequence homology of fish PrPs to tetrapod PrPs is below 25%, structure prediction programs indicate a similar organization of the three-dimensional structure. In this work, we generated recombinant polypeptide constructs that were expected to include the C-terminal folded domain of Fugu-PrP1 and analyzed these proteins with biochemical and biophysical methods. Since soluble expression could not be achieved, and refolding from guanidine-HCl did not result in a properly folded protein, we co-expressed *E. coli* chaperone proteins in order to obtain the protein in a soluble form. Although circular dichroism spectroscopy indicated the presence of some regular secondary structure in the protein thus obtained, there was no evidence for a globular three-dimensional fold in the NMR spectra. We thus had to conclude that the polypeptide products of the fish genes annotated as corresponding to *bona fide prnp* genes in non-fish species cannot be prepared for structural studies when using similar procedures to those that were successfully used with PrP from mammals, birds, reptiles and amphibians. Although the outcome of this study represents a negative result we consider this result in respect of our long lasting experience in recombinant prion protein production as important enough to be published.

Introduction

Prion diseases, such as scrapie in sheep, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) in deer, and Creutzfeldt-Jakob disease (CJD) in humans, are related to the conversion of the cellular form of the prion protein (PrP^{C}) to a protease-resistant β -sheet-rich form (PrP^{Sc}) [1]. Prion proteins from mammals, birds, reptiles and amphibians all possess the same molecular architecture, consisting of a flexibly extended 100-residue N-terminal tail and a globular C-terminal domain of similar size [2-7]. The C-terminal globular domain is preceded by a highly conserved hydrophobic polypeptide segment (figure 1). Its well-defined structure with three α -helices and an antiparallel β -sheet could be identified in all species studied so far [7]. Posttranslational modifications such as cleavage of N- and C-terminal signal sequences during the import into the endoplasmatic reticulum, formation of a disulfide bond that connects the helices $\alpha 2$ and $\alpha 3$, N-linked glycosylation in two sites, and addition of a C-terminal GPI anchor are present in all these species, which also contain putative SH3- and laminin- $\alpha 2$ -receptor binding sites [7, 8]. The physiological role in the healthy organisms and the evolutionary origin of PrPs remain controversial [9, 10].

Recently, genes coding for putative prion proteins in fish species such as Japanese pufferfish (*Fugu rubripes*) [11, 12], green spotted pufferfish (*Tetraodon nigroviridis*) [13], zebrafish (*Danio rerio*) [13, 14], atlantic salmon (*Salmo salar*) [12], rainbow trout (*Onchorhynchus mykiss*) [15], three-spine stickleback (*Gasterosteus aculeatus*) [8, 16], carp (*Cyprinus carpio*) [8], gilthead seabream (*Sparus aurata*) [17], Japanese medaka (*Oryzias latipes*) [GenBank: CAL64054], Japanese seabass (*Lateolabrax japonicus*) and Japanese flounder (*Paralichthys olivaceus*) [18] have been described and compared (for a sequence alignment, see Rivera-Milla *et al.* [8]). An early whole-genome duplication that

occurred in the evolution of ray-finned fish [19-23] resulted in the presence of two fish PrPs (PrP1 and PrP2), whereas only one PrP was identified in tetrapod species.

Comparison of biophysical and structural properties of tetrapod PrPs with fish PrPs might help to improve our understanding of PrP biology, such as structure–function relationships in healthy organisms, and species barriers in TSEs. In addition, new insights into the evolutionary development of PrPs might be obtained.

To begin of our work, we tried to express and purify the putative domains of Fugu (*Takifugu rubripes*) PrP2 (aa 215–404), Zebrafish (*Danio rerio*) PrP1 (aa 389–581) and PrP2 (aa 311–541) with the protocol used for other PrPs [4, 24]. However, we found that the proteins were only expressed at very low levels. We decided, therefore, to focus our work on the putative C-terminal domain of *Takifugu rubripes* PrP1, spanning residues 298–423, which could be obtained in sufficient quantities and, thus, appeared to us as the most promising candidate for further studies.

In a first approach, the protein was expressed in inclusion bodies followed by refolding attempts from guanidine-HCl by conventional Nickel-affinity chromatography. In a second approach, the protein was obtained in soluble oxidized form by co-expression with *E. coli* chaperone proteins [25-27] and purified without use of denaturants. The proteins thus obtained were studied with circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. The results presented in this study show that the putative C-terminal domain of *Takifugu rubripes* PrP1 does not exhibit a defined three-dimensional fold.

We are aware about the fact that we are reporting here a negative result. However, the finding that fish PrPs cannot be handled as other prion proteins appears to us as quite intriguing, especially in the light of our success and experience in expressing and purifying prion proteins from a wide variety of species. Regarding this aspect and that the

expression and purification of fish PrPs was a long-standing experiment we feel that we should make these intriguing results public.

Results and discussion

Identification of the putative C-terminal domain of *Takifugu rubripes* PrP1

An alignment of *Takifugu rubripes* PrP1 and PrP2 with murine PrP is shown in Figure 1. We determined the polypeptide segment of *Takifugu rubripes* PrP1 that should correspond to the C-terminal globular domain of tetrapod PrPs on the basis of recently published comparisons of fish and tetrapod PrP sequences [8, 12, 13, 18]. The N-terminus was defined at residue Val-298, which is in a hydrophobic segment that has high sequence homology to tetrapod PrPs. The C-terminus could not be identified unambiguously, since the sequence after the predicted α -helix 3 has no homology to non-fish PrPs. The GPI cleavage site could be either at Asn-424 or Ser-430 [28]. As no regular secondary structure was predicted for the region between residues 424 and 430, we decided to place the C-terminal end at residue Arg-423. In the remainder of this paper, the polypeptide fragment of residues 298–423 is referred to as tr1-PrP.

Expression and purification of tr1-PrP

The His-tagged protein was expressed and purified from inclusion bodies, using the same method [4, 24] that had been successfully applied to obtain protein samples for three-dimensional NMR structure determinations of a series of recombinant PrPs from mammals, birds, reptiles and amphibians [5, 7, 29]. Although the far-UV CD spectrum of tr1-PrP indicated the presence of some regular secondary structure, the $^1\text{H-NMR}$ spectrum revealed only small peak dispersion (data not shown), showing that the protein does not exhibit a globular fold and thus indicating possible improper refolding of the protein from the inclusion bodies. In additional experiments, the constructs Fugu-PrP1(298–450)[C426S] and Fugu-PrP1(355–450)[C426S], where Cys-426 was replaced

by serine, were tested for their folding properties. Fugu-PrP1(298–450)[C426S] was found to have a high tendency to aggregate during purification, whereas the behavior of Fugu-PrP1(355–450)[C426S] was similar to the one of tr1-PrP.

We next used an alternative expression strain with tr1-PrP, *E. coli* Origami BL21(DE3), which allows expression of proteins in oxidized soluble form in the cytoplasm of *E. coli*, and further enables variation of the IPTG concentration used to induce protein expression. In addition, chaperone systems such as Trigger Factor, GroEL/GroES and DnaJ/DnaK/GrpE were co-expressed to assist proper folding of the protein. Co-expression of Trigger Factor was found to yield the highest expression rate of soluble tr1-PrP and the lowest amount of co-purifying protein impurities (figure 2), whereas more impurities were observed with the GroEL/GroES system, and with the DnaJ/DnaK/GrpE system no expression of soluble tr1-PrP was obtained.

In small-scale experiments, the concentrations of the inductors arabinose and IPTG, the temperature and the expression time were adjusted so as to maximize the yield of soluble protein. In the final protocol, induction of chaperone pre-expression with 2 g (L)-(+)-arabinose per liter medium for 1 hour, a final IPTG concentration of 1 mM, an expression temperature of 25°C and an expression time of 15 hours were used (figure 2).

Soluble tr1-PrP was isolated from the cells by sonication and centrifugation in a buffer that does not contain any detergents or denaturants (see Experimental procedures). The protein was purified by Nickel-affinity chromatography, using a stepwise imidazole gradient to remove two co-purifying proteins that could be identified by Edman-Sequencing, MS and a database search as the ribosomal protein S15 and the ferric uptake regulation protein from *E. coli* (Swiss-Prot accession numbers P0ADZ4 and Q0TK00, respectively). With this protocol, 1.8 mg of soluble oxidized tr1-PrP was obtained per liter of bacterial

culture in rich medium, and in minimal medium, using ^{15}N -ammonium chloride as the sole nitrogen source, the yield was 0.4 mg per liter.

Characterization of tr1-PrP with CD and NMR spectroscopy

To compare the conformation of tr1-PrP with that of recombinant mammalian prion proteins, we used CD and NMR spectroscopy. In the far-UV CD spectra, there are indications that tr1-PrP and mPrP(121–231) both contain α -helical secondary structure, but the mean residue ellipticity of tr1-PrP is approximately one third less negative as the one of mPrP(121–231), indicating a lower content of residues located in regular secondary structure elements (figure 3).

In additional CD experiments, the thermal denaturation and the urea-induced unfolding transitions of tr1-PrP and mPrP(121–231) were compared (figure 4, A and B). Thermal denaturation and urea-induced unfolding of mPrP(121–231) is highly cooperative, as previously reported [30, 31], whereas tr1-PrP unfolds in a less-cooperative manner typical for proteins that have no compact globular fold.

NMR spectroscopy provided further evidence that no conformationally homogeneous sample of tr1-PrP was obtained in our experiments. The presence of peaks with variable line shape and intensity in the 2D [^{15}N , ^1H]-HSQC spectrum indicates that the protein is prone to aggregation (figure 5A). The absence of a globular fold is supported by the small dispersion of the amide proton chemical shifts (figure 5, A and B). In a 2D [^1H , ^1H]-NOESY spectrum, the region expected to contain NOE-peaks between methyl groups and aromatic rings in globular proteins is empty for tr1-PrP (figure 5B).

Conclusions

Our presently reported investigations indicate that the gene coding for tr1-PrP, which has been annotated as the fish gene corresponding to *prnp* in mammals [11, 12], does not encode a protein that can be isolated and purified with the biochemical methods used for other PrPs. This might be due to the fact that the identification of fish *prnp* genes was based on the coincidence with characteristic features that had previously been identified in *bona fide* PrPs, i.e., the N-terminal signal sequence, the Gly–Pro-rich region, the hydrophobic region and the presence of two cysteine residues, two glycosylation sites and the putative C-terminal GPI-anchor site (figure 1). The overall sequence homology of the globular C-terminal domain with different tetrapod PrPs is actually only between 15–25% [11, 12]. Furthermore, the sequence identity is largely concentrated in the segment 114–154 (numeration according to mPrP), which covers a hydrophobic stretch preceding the globular domain, and the regular secondary structures β 1 and α 1 (figure 1). In the remaining part of the putative globular domain with helices α 2 and α 3, the homology is essentially limited to the alignment of the two Cys residues (figure 1).

On grounds of principle, one cannot *a priori* exclude that alternative constructs with variable lengths would lead to a folded protein, especially since previous studies with mammalian PrPs showed that deletions at the N-terminal as well as the C-terminal end of the globular domain resulted in destabilization of the three-dimensional structures ([32], unpublished data). However, since the N-terminal part of the presently studied fish prion protein constructs includes the highly homologous hydrophobic stretch (figure 1), which is unstructured in *bona fide* prion proteins, it seems unlikely that N-terminal elongation would result in a folded protein. The C-terminal end of the presently used tr1-PrP construct was chosen at the proposed GPI-anchor site, and an alternative construct including the natural

stop-codon (tr1-PrP(298–450)[C426S]) yielded no folded protein either. It thus appears that the absence of a globular domain cannot be rationalized by inappropriate truncation of the tr1-PrP constructs used.

Overall, we conclude from our data that the Fugu-PrP1 gene annotated as corresponding to *bona fide prnp* genes in all non-fish species studied so far, does not encode a protein that forms a typical prion protein three-dimensional structure when isolated with the same purification and refolding methods that were successful with the other species. Considering that the sequence homology among fish species is 60% within PrP1s, 50% within PrP2s, and 40% between PrP1s and PrP2s [8], one is tempted to hypothesize that all fish PrPs might behave differently from tetrapod PrPs.

Experimental procedures

Cloning of the proteins

The plasmid containing the genes for Zebrafish PrP1, PrP2 and for Fugu-PrP1 were kindly provided by Dr. E. Málaga-Trillo (University of Konstanz, Germany). All protein fragments were cloned into the vector pRSET-A (Invitrogen, Carlsbad, CA, USA), which contains an N-terminal hexa-histidine tag (6 x His) and a thrombin cleavage site [4].

Expression, purification and refolding of tr1-PrP from inclusion bodies

Recombinant tr1-PrP was expressed, purified and refolded from inclusion bodies without removing the 6 x His tag, as described by Zahn *et al.* [4, 24].

Expression and purification of soluble tr1-PrP

Tr1-PrP was expressed in *E. coli* Origami B cells (Novagen, Darmstadt, Germany), which have the ability to form disulfide bonds in the cytoplasm and allow variation of the IPTG concentration used to induce protein expression. Cells containing two plasmids, one coding for a co-expressing chaperone protein (Takara Bio Inc., Otsu, Japan) and one for the expression of recombinant tr1-PrP, were grown at 37°C either in rich medium or in minimal medium containing $^{15}\text{NH}_4\text{Cl}$ (1 g/l) as the sole nitrogen source under selective conditions (ampicillin 100 mg/l, kanamycin sulfate 15 mg/l, chloramphenicol 35 mg/l, tetracycline 12.5 mg/l). At an OD_{600} of 0.6, L-(+)-arabinose (1–4 g/l) was added to induce chaperone expression for 1–4 hours before the expression of tr1-PrP was induced by addition of IPTG. To optimize the expression yield, various temperatures in the range 20–30°C and IPTG concentrations in the range 10 μM to 1 mM were tested.

In the final expression protocol, pre-expression of the chaperone proteins was carried out for 1 hour at 25°C, with an arabinose concentration of 2 g/liter, and after addition of 1 mM IPTG, both proteins were expressed for 15 hours.

After cell harvesting, the protein was resuspended in 100 ml buffer A (100 mM sodium phosphate, 5 mM Tris/HCl, 10 mM imidazole, 0.1 mg/ml lysozyme, 1 mg DNase, pH = 8.0), sonicated for 30 minutes and centrifuged (19'000 rpm, 4°C, 1 hour). The supernatant was added to 20 ml of nickel-nitrilotriacetic acid (NTA) agarose resin (Qiagen, Valencia, CA, USA) and stirred for 1 hour. The agarose was first washed with buffer B (100 mM sodium phosphate buffer, 5 mM Tris/HCl, 10 mM imidazole, pH = 8.0) before the protein was eluted by a stepwise imidazole gradient of 50 mM, 150 mM and 500 mM imidazole in buffer C (100 mM sodium phosphate buffer, 5 mM Tris/HCl, pH = 8.0). Fractions containing tr1-PrP were pooled and dialyzed against 10 mM sodium acetate buffer at pH 4.5, using a Spectrapor membrane (Rancho Dominguez, CA, USA) with MWCO 3500, and concentrated. The N-terminus of the protein was analyzed by Edman sequencing, and its mass was verified by ESI (calculated mass: 16'701.5 Da, measured mass: 16'701.8 Da). The Ellman assay showed absence of free thiols after unfolding, indicating that the purified tr1-PrP was completely oxidized [33]. Protein concentrations were measured by the absorbance at 280 nm, using a molar extinction coefficient of 20'590 M⁻¹cm⁻¹.

CD spectroscopy

All measurements were performed in 10 mM sodium acetate pH 4.5 on a Jasco J710 CD spectropolarimeter at 20°C. The sample of denatured tr1-PrP additionally contained 8 M urea. The CD spectra were recorded in 0.1 cm cuvettes at protein concentrations of 13–19 µM. All spectra were corrected for the presence of the buffer.

Thermal unfolding transitions were monitored by following the mean residue ellipticity, $[\Theta]_{MRW}$, at 222 nm between 20 and 90°C at a constant heating rate of 1°C/min and protein concentrations of 27 μ M tr1-PrP and 19 μ M mPrP(121–231), respectively.

To study the urea-induced unfolding transitions, the mean residue ellipticities at 222 nm were recorded in the presence of different urea concentrations at protein concentrations of 22 μ M for tr1-PrP and 33 μ M for mPrP, respectively. The mean residue ellipticity was recorded for 30 s and averaged. The data for mPrP(121–231) were analyzed according to a two-state model of folding by using a six-parameter fit [34].

NMR experiments

All measurements were performed at 20°C on Bruker DRX750 and Avance900 spectrometers. The samples were measured in 10 mM $[d_4]$ -sodium acetate buffer at pH 4.5, containing 90% H₂O/10% D₂O. The 2D [¹H,¹H]-NOESY spectrum was recorded with a mixing time of 60 ms, using a 600 μ M protein sample.

Acknowledgements

This work was supported by the Swiss National Science Foundation and the Federal Institute of Technology Zürich through the National Center of Competence in Research (NCCR) "Structural Biology".

References

1. Prusiner SB (1998) Prions. *Proc Natl Acad Sci USA* 95, 13363–13383.
2. Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R & Wüthrich K (1996) NMR structure of the mouse prion protein domain PrP(121–231). *Nature* 382, 180–182.
3. López-García F, Zahn R, Riek R & Wüthrich K (2000) NMR structure of the bovine prion protein. *Proc Natl Acad Sci USA* 97, 8334–8339.
4. Zahn R, Liu A, Lührs T, Riek R, von Schroetter C, López-García F, Billeter M, Calzolari L, Wider G & Wüthrich K (2000) NMR solution structure of the human prion protein. *Proc Natl Acad Sci USA* 97, 145–150.
5. Lysek DA, Schorn C, Nivon LG, Esteve-Moya V, Christen B, Calzolari L, von Schroetter C, Fiorito F, Herrmann T, Güntert P & Wüthrich K (2005) Prion protein NMR structures of cats, dogs, pigs, and sheep. *Proc Natl Acad Sci USA* 102, 640–645.
6. Gossert AD, Bonjour S, Lysek DA, Fiorito F & Wüthrich K (2005) Prion protein NMR structures of elk and of mouse/elk hybrids. *Proc Natl Acad Sci U S A* 102, 646–650.
7. Calzolari L, Lysek DA, Perez DR, Güntert P & Wüthrich K (2005) Prion protein NMR structures of chickens, turtles, and frogs. *Proc Natl Acad Sci USA* 102, 651–655.
8. Rivera-Milla E, Oidtmann B, Panagiotidis CH, Baier M, Sklaviadis T, Hoffmann R, Zhou Y, Solis GP, Stürmer CA & Málaga-Trillo E (2006) Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. *FASEB J* 20, 317–319.

9. Vana K, Zuber C, Nikles D & Weiss S (2007) Novel Aspects of Prions, Their Receptor Molecules, and Innovative Approaches for TSE Therapy. *Cell Mol Neurobiol* 27, 107–128.
10. Westergard L, Christensen HM & Harris DA (2007) The cellular prion protein (PrP^C): Its physiological function and role in disease. *Biochim Biophys Acta* 1772, 629–644.
11. Rivera-Milla E, Stürmer CA & Málaga-Trillo E (2003) An evolutionary basis for scrapie disease: identification of a fish prion mRNA. *Trends Genet* 19, 72–75.
12. Oidtmann B, Simon D, Holtkamp N, Hoffmann R & Baier M (2003) Identification of cDNAs from Japanese pufferfish (*Fugu rubripes*) and Atlantic salmon (*Salmo salar*) coding for homologues to tetrapod prion proteins. *FEBS Lett* 538, 96–100.
13. Premzl M, Gready JE, Jermiin LS, Simonic T & Marshall Graves JA (2004) Evolution of vertebrate genes related to prion and Shadoo proteins - clues from comparative genomic analysis. *Mol Biol Evol* 21, 2210–2231.
14. Cotto E, Andre M, Forgue J, Fleury HJ & Babin PJ (2005) Molecular characterization, phylogenetic relationships, and developmental expression patterns of prion genes in zebrafish (*Danio rerio*). *FEBS J* 272, 500–513.
15. Maddison BC, Patel S, James RF, Conlon HE, Oidtmann B, Baier M, Whitlam GC & Gough KC (2005) Generation and characterisation of monoclonal antibodies to Rainbow trout (*Oncorhynchus mykiss*) prion protein. *J Immunol Methods* 306, 202–210.
16. Premzl M & Gamulin V (2007) Comparative genomic analysis of prion genes. *BMC Genomics* 8, 1.

17. Favre-Krey L, Theodoridou M, Boukouvala E, Panagiotidis CH, Papadopoulos AI, Sklaviadis T & Krey G (2007) Molecular characterization of a cDNA from the gilthead sea bream (*Sparus aurata*) encoding a fish prion protein. *Comp Biochem Physiol B Biochem Mol Biol* 147, 566–573.
18. Liao M, Zhang Z, Yang G, Sun X, Zou G, Wei Q & Wang D (2005) Cloning and characterization of prion protein coding genes of Japanese seabass (*Lateolabrax japonicus*) and Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* 249, 47–53.
19. Aparicio S, Chapman J, Stupka E, Putnam N, Chia JM, Dehal P, Christoffels A, Rash S, Hoon S, Smit A, Gelpke MD, Roach J, Oh T, Ho IY, Wong M, Detter C, Verhoef F, Predki P, Tay A, Lucas S, Richardson P, Smith SF, Clark MS, Edwards YJ, Doggett N, Zharkikh A, Tavtigian SV, Pruss D, Barnstead M, Evans C, Baden H, Powell J, Glusman G, Rowen L, Hood L, Tan YH, Elgar G, Hawkins T, Venkatesh B, Rokhsar D & Brenner S (2002) Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science* 297, 1301–1310.
20. Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard V, Jubin C, Castelli V, Katinka M, Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, De Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau JP, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff JN, Guigo R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach J & Roest Crollius H (2004) Genome duplication in the

teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* 431, 946–957.

21. Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S & Venkatesh B (2004) Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Mol Biol Evol* 21, 1146–1151.
22. Vandepoele K, De Vos W, Taylor JS, Meyer A & Van de Peer Y (2004) Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. *Proc Natl Acad Sci USA* 101, 1638–1643.
23. Brunet FG, Crollius HR, Paris M, Aury JM, Gibert P, Jaillon O, Laudet V & Robinson-Rechavi M (2006) Gene loss and evolutionary rates following whole-genome duplication in teleost fishes. *Mol Biol Evol* 23, 1808–1816.
24. Lysek DA & Wüthrich K (2004) Prion protein interaction with the C-terminal SH3 domain of Grb2 studied using NMR and optical spectroscopy. *Biochemistry* 43, 10393–10399.
25. Nishihara K, Kanemori M, Kitagawa M, Yanagi H & Yura T (1998) Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. *Appl Environ Microbiol* 64, 1694–1699.
26. Nishihara K, Kanemori M, Yanagi H & Yura T (2000) Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Appl Environ Microbiol* 66, 884–889.

27. Georgiou G & Valax P (1996) Expression of correctly folded proteins in *Escherichia coli*. *Curr Opin Biotechnol* 7, 190–197.
28. Eisenhaber B, Bork P & Eisenhaber F (1998) Sequence properties of GPI-anchored proteins near the omega-site: constraints for the polypeptide binding site of the putative transamidase. *Protein Eng* 11, 1155–1161.
29. Zahn R, von Schroetter C & Wüthrich K (1997) Human prion proteins expressed in *Escherichia coli* and purified by high-affinity column refolding. *FEBS Lett* 417, 400–404.
30. Hornemann S & Glockshuber R (1998) A scrapie-like unfolding intermediate of the prion protein domain PrP(121–231) induced by acidic pH. *Proc Natl Acad Sci USA* 95, 6010–6014.
31. Apetri AC & Surewicz WK (2003) Atypical effect of salts on the thermodynamic stability of human prion protein. *J Biol Chem* 278, 22187–22192.
32. Eberl H & Glockshuber R (2002) Folding and intrinsic stability of deletion variants of PrP(121–231), the folded C-terminal domain of the prion protein. *Biophys Chem* 96, 293-303.
33. Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* 82, 70–77.
34. Santoro MM & Bolen DW (1988) Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry* 27, 8063–8068.

Figure Legends

Figure 1: Amino acid sequence alignment of the putative fugu PrPs with mouse PrP.

Mouse PrP [GenBank:NP_035300] (residues 108–254), Fugu-PrP1 [GenBank:AAN38988] (residues 286–450) and Fugu-PrP2 [GenBank:AAR99478] (residues 203–425) have been aligned using the EMBL ClustalW program (<http://www.ebi.ac.uk/clustalw/>). The residues in the box represent a pronouncedly hydrophobic region of the proteins. For the globular C-terminal domain of mouse PrP, the regular secondary structure elements are indicated above its sequence. Residues with a black background indicate identical amino acids in all three species, the ones in grey show the residues that are conserved in Fugu-PrP1 and PrP2.

Figure 2: Expression and purification of tr1-PrP.

A 16% Coomassie Blue-stained SDS-PAGE shows tr1-PrP (band at 16.7 kDa, marked with B) in the presence of the co-expressing chaperone trigger factor (band at 48 kDa, marked with A). Lane M: marker; lane 1: cell extract before arabinose induction; lane 2: cell extract 1 hour after arabinose-induction (2 g/liter culture); lane 3: cell extract after IPTG-induction (final concentration 1 mM) and protein expression for 15 hours; lane 4: purified tr1-PrP.

Figure 3: Comparison of the CD-spectra of tr1-PrP and mPrP(121–231).

The spectra of native (solid line) and urea-denatured (dotted line) tr1-PrP, and of mPrP(121–231) (broken line) were measured at pH 4.5. $[\Theta]_{MRW}$ is the mean residue ellipticity in deg/cm²/dmol.

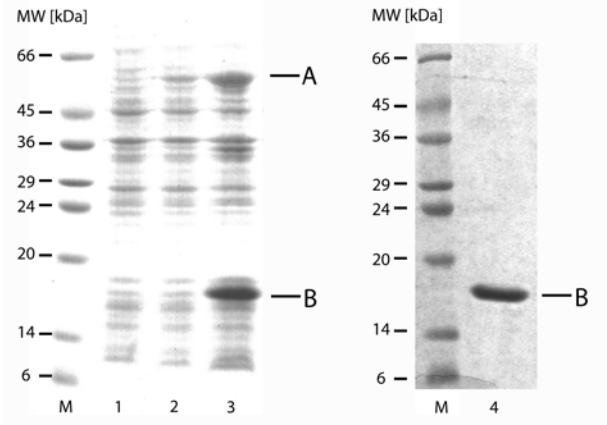
Figure 4: Thermal denaturation and chemical unfolding of tr1-PrP and mPrP(121–231).

Thermal (A) and urea-induced unfolding (B) of tr1-PrP (●) and mPrP(121–231) (○) were monitored by the mean residue ellipticity at 222 nm. For this comparison, the previously reported unfolding curves for mPrP(121–231) [30] have been re-measured at identical conditions to those for tr1-PrP. The pH-value was 4.5, and the urea-denaturation was pursued at 20°C. For mPrP(121–231), continuous lines represent a fit of the data according to a two-state transition. $[\Theta]_{MRW}$ is the mean residue ellipticity in deg/cm²/dmol.

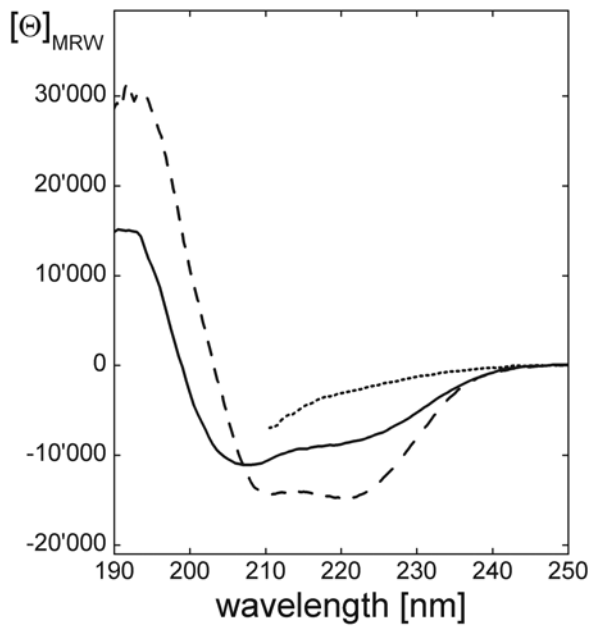
Figure 5: NMR-experiments with tr1-PrP.

(A) 2D [¹⁵N,¹H]-HSQC spectrum of the uniformly ¹⁵N-labeled protein. (B) 2D [¹H,¹H]-NOESY spectrum of unlabeled tr1-PrP. The box in (B) marks the region where NOEs between aromatic protons and side chain methyl protons are typically observed in globular proteins.

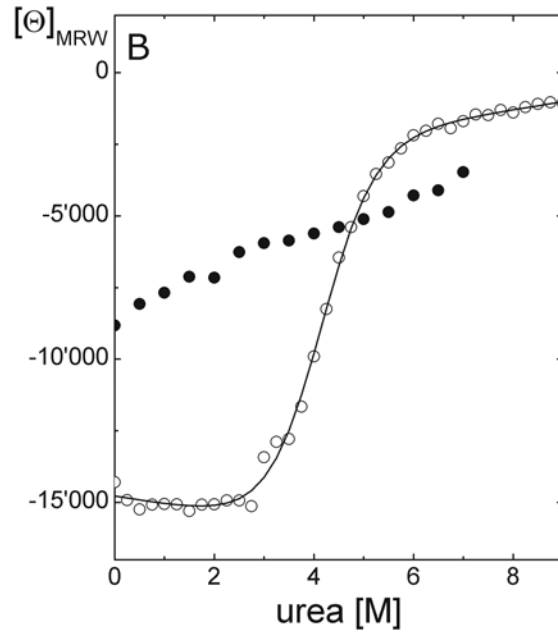
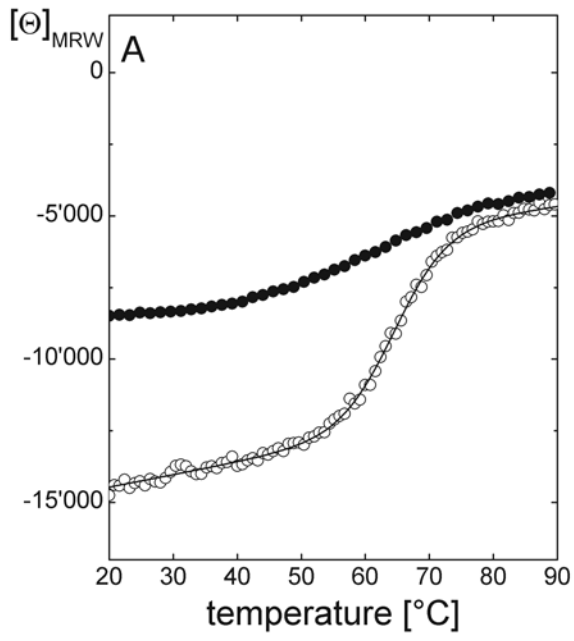
Christen *et al*, Figure 2



Christen *et al*, Figure 3



Christen *et al*, Figure 4



Christen *et al*, Figure 5

