The Comprehensive Native Interactome of a Fully Functional Tagged Prion Protein

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Abstract

The enumeration of the interaction partners of the cellular prion protein, PrPC, may help clarifying its elusive molecular function. Here we added a carboxy proximal myc epitope tag to PrPC. When expressed in transgenic mice, PrPmyc carried a GPI anchor, was targeted to lipid rafts, and was glycosylated similarly to PrPC. PrPmyc antagonized the toxicity of truncated PrP, restored prion infectibility of PrPC-deficient mice, and was physically incorporated into PrPSc aggregates, indicating that it possessed all functional characteristics of genuine PrPC. We then immunopurified myc epitope-containing protein complexes from PrPmyc transgenic mouse brains. Gentle differential elution with epitope-mimetic decapeptides, or a scrambled version thereof, yielded 96 specifically released proteins. Quantitative mass spectrometry with isotope-coded tags identified seven proteins which co-eluted equimolarly with PrPC and may represent component of a multiprotein complex. Selected PrPSc interactors were validated using independent methods. Several of these proteins appear to exert functions in axomyelinic maintenance.

Introduction

The cellular prion protein, PrPC, is required for susceptibility to prion infections [1,2], for prion toxicity [3], and for prion transport within the body [4]. PrPC is a conserved glycoprotein that is anchored to the cell surface through a covalently attached glycosyl phosphatidyl inositol (GPI) residue [5]. PrPC undergoes a complex biogenesis encompassing co-translational secretion into the lumen of the endoplasmic reticulum, cleavage of an N-terminal signal peptide, addition of complex N-linked carbohydrate chains at two sites [6], addition of a preformed GPI anchor at its very C-terminus (Ser230), and removal of a C-terminal oligopeptide.

Despite the detailed chemical knowledge described above, the molecular details of the process by which PrPC is converted into a disease-associated homologue, PrPSc, are unclear [7]. Likewise, the chain of events emanating from prion infections and leading to neurodegenerative changes and clinical signs is unknown. Lastly, the physiological function of PrPC is unclear [8]. Most of the above processes may require interactions with proteins other than PrP, yet the nature of such interaction partners is largely unknown. The present study was initiated as an approach to discovering the functionally relevant interaction partners of PrPC.

Several diverse approaches have been used in the past to achieve the latter goals. In some instances, however, the techniques employed were not sufficiently sensitive or were fraught with other problems. Classical two-hybrid screens, in which fusion proteins leads to biological readouts in the cytosol of yeast, tend to produce when applied to membrane proteins like PrPC. The same holds true for cross-linking experiments, in which proteins resident in the same micro-environment may become linked together even if they do not functionally interact with each others.

In order to avoid the problems described above, and to minimize any interference with the conditions existing in vivo, we isolated native protein complexes containing PrPC and characterized them by mass spectrometry. The addition of epitope tags, for which high-affinity antibodies are available, has proven instrumental for the study of many supramolecular complexes. The engineering of appropriate tags into the proteins of choice yields “molecular handles” through which multi-component complexes can be immunoprecipitated and highly purified. PrPC lends itself to this approach as a particularly attractive bait, as its high-resolution structure is known [9] and thereby allows for the rational design of tags. If the precipitating antibodies are directed against linear, non-conformational epitopes within the tag, epitope-mimetic peptides can release the protein complexes in a highly specific way under non-denaturing conditions. The introduction of a tag is also a promising starting point for identifying functionally relevant complexes since it preserves
protein interactions that occur in the same region of an anti-PrP antibody.

GFP-PrP is a fusion protein that has proven useful for determining the subcellular distribution and trafficking of normal and mutated prion protein [10,11,12]. However, the suitability of GFP to the proteomic approach delineated above is limited. GFP is a bulky, highly structured and rigid tag whose molecular weight exceeds that of PrP. Therefore we reasoned that GFP may distort the composition of any native multiprotein complex that encompasses PrP.

In the present study, we have tagged the C-terminus of mouse PrP with the human “myc-tag”. The resulting chimaeric protein, termed PrPmyc, was used to immunoprecipitate and characterize the supramolecular complex containing the prion protein from transgenic mice. Using immunoprecipitation and mass spectrometry, we have identified a set of proteins associated with PrPmyc. Since the conversion of cellular prion protein PrP into the protease resistant isoform PrP is the central pathogenic process in prion diseases, we investigated whether PrPmyc can be converted into PrP. Our results indicate that C-terminally myc-tagged prions can contribute to prion infectivity and to neurotoxicity. Therefore, myc tagged PrP may also allow for identification of proteins interacting with PrP.

Results

Transgenic mice expressing C-terminally tagged PrP

We tagged the murine prion protein by introducing a human myc epitope tag (EQLKLSNKEDL) at its C-terminus next to Ser230 and amino proximally to the C-terminal signal sequence for the GPI anchor (Fig. 1A). As the minimal myc epitope tag consists of only 10 amino acids, we reasoned that it might not interfere with the geometry and proper folding of PrP.2 and, with its function. The human myc epitope tag was detectable by both monoclonal and polyclonal anti-myc antibodies 9E10 and 4A6 [13]. To guarantee correct GPI linkage of this fusion protein, the sequence comprising Ser230 and its four immediately preceding N-proximal amino acids was duplicated after the tag. The resulting fusion molecule was termed PrPmyc.

Preliminary analyses of PrPmyc transfected cells indicate that the biosynthesis, processing, and trafficking of the resulting fusion protein were indistinguishable from those of endogenous PrP (data not shown).

To generate transgenic mice expressing C-terminally tagged PrP, PrPmyc was ligated into the ‘half-genomic’ phgPrP backbone, driven by the endogenous Prnp promoter [14]. Pronuclear injections of linearized purified DNA were performed into fertilized oocytes derived from a B6D2F1 in vivo progeny. Four founder mice were identified by PCR analysis using primers TAP 20 (5'-CCG ATG TGA AGA TGA TGG AGC) and myc 22 (5'-CCG TGC AGA TTC AGA TCC) specific for the myc-tag amplicon. The two highest-expressing lines, Tg940 and Tg941, were crossed twice to Prnp/o mice. Transgene expression in brain and spleen of these mice was analyzed by Western blotting using anti-PrP antibody POMI1 [15], and mouse monoclonal anti-myc antibody 9E10. Tg940 mice lacking PrP (henceforth termed Tg940 PrPmyc) expressed 1.6 fold more of PrPmyc protein in brain than wild-type mice (Fig. 1D), but had lower expression levels of the transgene in spleen (about 0.5 fold of PrPmyc, data not shown). Expression of PrPmyc in Tg941 PrPmyc was approximately 0.33 fold in brain and 2-fold in spleen of PrP expression in PrP (data not shown). Tg940 and Tg941 exhibited a three-banded pattern very similar to PrP glycoforms (37–25 kDa) in wild type mice (Fig. 1E).

PrPmyc is localized within detergent resistant membranes (DRMs)

We isolated DRMs from Tg940 brain tissue by gradient centrifugation [16]. A series of fifteen individual fractions was carefully removed from the tubes after centrifugation of typical DRM preparations from mouse cerebella of Tg940 PrPmyc, and analyzed by Western blotting. The quality of the preparations was monitored using the control proteins flavin 2 is known to reside in DRMs [17,18]. PrPmyc was found to reside in the same fractions as these proteins, confirming its localization in these specialized membrane domains (Fig. 1F). Therefore, the subcellular localization of PrPmyc was similar to that of endogenous PrP.

Testing the functionality of PrPmyc

Tg940 PrPmyc were crossed with the TgE55 line of mice expressing N-proximally truncated PrP, henceforth referred to as PrPoF. PrPoF mice suffer from degeneration of the cerebellar granular layer, leukoencephalopathy, and death at about 100 days of age [19,20,21]. This phenotype is dose-dependently counteracted by endogenous or transgenic co-expression of wild-type PrP. Presumably because of a competing activity supplied by PrP.

If the tagged protein PrPmyc is functional and appropriately localized, it should also rescue PrPoF mice from neurodegeneration. Indeed, Tg940 PrPmyc expressing PrPoF survived for 55±73 days (n=5; Fig. 1G) and maintained a normal weight throughout their lifetime. Mice were examined twice per week for neurological symptoms and scored as described [19], yet did not show clinical signs of CNS disease at any time. Furthermore, they did not develop histopathological changes in brain or other organs (data not shown), suggesting that PrPmyc is functional in vivo. Age and sex-matched PrPoF siblings died between 12 and 14 weeks of age (mean survival: 95±7 days, n=5; Fig. 1G).

In contrast, double-transgenic mice of the lower expressing line (Tg941) were not completely rescued and began to show first signs of illness around day 290. Some animals had to be sacrificed at the age of 12 months due to hind leg paresis (mean survival 391±57 days, n=9; Fig. 1G). As Tg941 PrPmyc mice express about one-third of the PrPmyc found in brains of Tg940 PrPmyc mice, this indicates that the action of PrPmyc like that of PrP, is dose-dependent.

Neuropathology in inoculated PrPmyc mice

To assess whether PrPmyc can be converted into myc-tagged protease-resistant PrP, PrPmyc, PrPmyc and PrPmyc mice from lines...
Figure 1. Molecular characterization of the PrP<sub>myc</sub> transgenic mouse lines Tg940 and Tg941. (A) Scheme of the PrP<sub>myc</sub> transgene. SP: secretory signal peptide, cleaved after sorting of the precursor to endoplasmic reticulum; repeats: five repeats of eight amino acids; CC: charge cluster; HC: hydrophobic core; H1, H2, H3: a-helices of the globular carboxy-proximal domain; MYC: human myc epitope tag (EQLLISEEDL); MA: membrane anchor of precursor protein, replaced during maturation with glycosyl phosphatidyl inositol anchor. (B) Southern blot analysis of lines Tg940 PrP<sub>myc</sub> (lanes 1, 2, 6) and Tg941 PrP<sub>myc</sub> (lanes 3, 5, 7). Lane 4: Tg941 PrP<sub>myc</sub><sup>ΔF</sup> mouse co-expressing N-proximally truncated PrP<sub>myc</sub>. Lane 8: PrP<sub>AS</sub> mouse. The bands diagnostic for PrP<sub>myc</sub> and PrP<sub>AS</sub> were 3039 and 2709 bp, respectively. Numbers of transgenic copies per haploid genome, as determined by quantitation of Southern blot signals against the respective PrP<sub>myc</sub> genomic band, revealed higher copy numbers in Tg940 PrP<sub>myc</sub> (6/6) than in Tg941 PrP<sub>myc</sub> mice (5/6). (C) Northern blot analysis of individual Tg940 PrP<sub>myc</sub> and Tg941 PrP<sub>myc</sub> brains using a PrP probe. Mice homozygous for the transgenic allele PrP<sub>myc</sub> (lanes 2, 3, 4 from Tg940 and lanes 8, 9, 10 from Tg941) showed higher levels of PrP<sub>myc</sub> mRNA than hemizygous mice (lanes 1 and 5 from Tg940 and lanes 6, 7, 10 from Tg941). An actin probe was used as a loading control (lower panel). (D) Similar expression levels of transgenic protein from Tg940 PrP<sub>myc</sub> and full-length PrP from 1295/2/SvPas wild-type mice, analyzed by Western blotting of total brain homogenate using anti-PrP antibody POM1. (E) Similar glycosylation pattern of full-length PrP from 1295/2/SvPas wild-type and PrP<sub>myc</sub> from Tg940 PrP<sub>myc</sub> mice. Brain homogenates were subjected to PNGase F treatment as indicated, and analyzed by Western blotting using POM1 antibody to PrP. (F) Detergent-resistant membrane preparations from cerebella of Tg940 PrP<sub>myc</sub> transgenic mice showed PrP<sub>myc</sub> in lipid rafts. PrP<sub>myc</sub> was detectable by Western blotting in fractions with 5–30% Optiprep. PrP<sub>myc</sub> resided in the same fractions as flotillin (48 kDa) confirming its localization in DRMs. (G) A genetic in vivo assay for the function of the PrP<sub>myc</sub> protein. Survival curves of mice expressing PrP<sub>AS</sub> in absence of full length PrP<sub>sc</sub> in presence of PrP<sub>myc</sub> from two transgenic lines. Toxicity of PrP<sub>AS</sub> was counteracted by PrP<sub>myc</sub>, leading to a longer survival and suggesting that PrP<sub>myc</sub> has retained at least some of the function of PrP<sub>sc</sub>. Line PrP<sub>SR</sub> Tg940 and Tg941 consisted of 5, 5, and 9 individuals, respectively.

doi:10.1371/journal.pone.0004446.g001

Tg940 and Tg941 were inoculated with mouse-adapted sheep prions (RML strain, passage 5). After low dose intraperitoneal (ip) inoculation with 10<sup>5</sup> IU or intracerebral (ic) inoculation with 300 IU of RML5 brain homogenate, Tg940 PrP<sub>myc</sub><sup>o</sup> mice showed signs of CNS dysfunction at 250±92 (n = 5/3) and 236±76 (n = 6/6) days post inoculation (dpi), respectively (Fig. 2A and B). Mice expressing less PrP<sub>myc</sub> in brain (Tg941) developed signs of CNS dysfunction and terminal scarpie-like disease more slowly, at 316±20 (n = 4/4) days after low-dose intracerebral inoculation (Fig. 2B and Table S1).

Brain homogenates prepared from terminally sick Tg940 PrP<sub>myc</sub><sup>o</sup> mice were inoculated into gtu20 mice overexpressing PrP<sub>sc</sub> [14] to test for infectivity in an in-vivo mouse assay. All of the gtu20 mice developed neurological signs of terminal scarpie at around 80 dpi (Table S1). Prion infection was confirmed by immunohistochemical and histopathological analysis in all terminally sick mice. PrP<sub>myc</sub><sup>o</sup> mice developed neurological dysfunction and terminal disease significantly earlier than Prnp<sup>o</sup> mice: the mean incubation time was 276±9 days for Prnp<sup>o</sup> (n = 6) and 226±13 days for Tg940 PrP<sub>myc</sub><sup>o</sup> mice (n = 8) after high dose ic inoculation (Fig. 2C and Table S1). Therefore, PrP<sub>myc</sub> contributes to, rather than interfering with, prion pathogenesis in Prnp<sup>o</sup> mice.

In all terminally sick PrP<sub>myc</sub><sup>o</sup> mice tested we detected proteinase K (PK) resistant material in brain and spleen after ic or ip inoculation with RML prions. To distinguish between wild-type PrP<sub>sc</sub> and PrP<sub>myc</sub><sup>o</sup> we stained Western blots of brain homogenates of Tg940 PrP<sub>myc</sub><sup>o</sup> mice with anti-PrP (POM1) and anti-myc (4A6) antibodies, we could specifically detect PK-resistant PrP in Tg940 PrP<sub>myc</sub><sup>o</sup> mice which accumulated proteinase K resistant PrP<sub>myc</sub><sup>o</sup> in brain and spleen of Tg940 PrP<sub>myc</sub><sup>o</sup> mice at 50 to 100 days after ic or ip inoculation, yet 8 of 34 (23%) PrP<sub>myc</sub><sup>o</sup> mice eventually developed a progressive neurological syndrome clinically indistinguishable from scrapie after RML inoculation (Table S2). Brain homogenate from these sick mice was then inoculated to a second generation of Tg940 PrP<sub>myc</sub><sup>o</sup> mice. Western blot analysis of brain homogenate from these second-passage ic-inoculated Tg940 PrP<sub>myc</sub><sup>o</sup> mice revealed PK-resistant PrP; these mice had clinical signs of scrapie and developed vacuolation in the neuropil, intense astrogliosis, and abundant PrP aggregates (Fig. 3A–C). For control, Tg940 PrP<sub>myc</sub><sup>o</sup> mice were inoculated with non-infectious brain homogenate. These mice showed no evidence of vacuolar degeneration or neuro cell loss, and only mild astrogliosis when aged (Fig. 3D–F).

As an additional method to distinguish between PrP<sub>sc</sub> derived from wild-type PrP and PrP<sub>myc</sub>, we performed histoblot analysis of cryosections of terminal Tg940 PrP<sub>myc</sub><sup>o</sup> mice and Tg940 PrP<sub>myc</sub><sup>z</sup> mice (Fig. 3G–I). Using anti-PrP (POM1) and anti-myc (4A6) antibodies, we could specifically detect PK-resistant PrP in terminal C57BL/6 mice. Tg940 PrP<sub>myc</sub><sup>o</sup> and Tg940 PrP<sub>myc</sub><sup>z</sup> mice. This technique allowed us to map the distribution of PrP<sub>sc</sub> in different transgenic mice. We then investigated whether PrP<sub>myc</sub> infectivity would increase upon serial transmission, as frequently observed in strain adaptation [22]. Brain homogenate derived from RML-inoculated Tg940 PrP<sub>myc</sub><sup>o</sup> mice was passed into Tg940 PrP<sub>myc</sub><sup>z</sup> mice which all got sick after 390±56 days (n = 3) (Table S3). One of these second-passage mice was used as a source for a third passage into 5 Tg940 PrP<sub>myc</sub><sup>z</sup> mice. All of them show similar neurological signs as in the second passage, but with a shorter incubation period of 367±38 (n = 5), which is suggestive of strain adaptation (Table S3).

We then tested whether deposition of PrP<sub>myc</sub> accompanies prion replication, defined as increase in prion infectivity. Samples from Tg940 PrP<sub>myc</sub><sup>o</sup> mice after the second passage were used to infect the Pk1 subclone of N2a neuroblastoma cells in the Scapril cell assay in endpoint format (SCEPA) [23]. As shown in the Fig. 3 J the titers for the PrP<sub>myc</sub> is the same as the standard RML.

Identification of PrP<sub>myc</sub>-containing protein complexes

Crude brain homogenates from Tg940 PrP<sub>myc</sub><sup>o</sup> mice were subjected to immunoprecipitation (IP) experiments with paramagnetic microbeads coupled to mouse monoclonal anti-myc antibody (4A6, Upstate, USA). Release of myc-containing protein complexes from beads was carried out by exposing the beads to an excess of the synthetic epitope-mimicking myc peptide described above. Control experiments were carried out to verify the specificity of the eluted proteins, and included (1) incubation of beads with 12932/SiPas wild-type brains followed by elution with the myc peptide, as well as (2) incubation of beads with Tg940 PrP<sub>myc</sub><sup>o</sup> homogenate followed by elution with a scrambled version of the myc peptide. In the eluates from 4A6-coupled beads
Figure 2. Survival and neuropathology of PrP<sup>myc</sup> mice after prion inoculation. (A) Survival curves of Tg940 PrP<sup>myc</sup> mice and Prnp<sup>+/o</sup> mice low dose ip inoculated with RML5 prions. Groups Tg940 PrP<sup>myc</sup> and Prnp<sup>+/o</sup> consisted of 5 and 2 individuals, respectively. (B) Survival curves of Tg940 PrP<sup>myc</sup>, Tg941 PrP<sup>myc</sup>, and Prnp<sup>+/o</sup> mice inoculated low dose ic with RML5 prions. Group Tg940 PrP<sup>myc</sup> comprises 6, group Tg941 PrP<sup>myc</sup> 4, and Prnp<sup>+/o</sup> 3 individuals, respectively. (C) Survival curves of Tg940 PrP<sup>myc</sup> mice and Prnp<sup>+/o</sup> mice high dose ic inoculated with RML5 prions. Line Tg940 PrP<sup>myc</sup> comprises of 8 and line Prnp<sup>+/o</sup> of 6 individuals, respectively. (D) PrP<sub>myc</sub> was converted into myc-tagged proteinase K-resistant PrP<sub>Sc</sub> in presence of a wild-type PrP allele. Western blot analysis using brain homogenate from an inoculated, terminally sick PrP<sub>myc</sub> mouse. Antibodies
incubated with 129S2/SvPas wild-type brain homogenates, PrP<sup>C</sup> was not detected, whereas only traces of PrP<sup>Sc</sup> were detected in the scrambled-peptide eluate from IPs of Tg<sup>940 PrP<sup>Sc</sup>/o</sup> brain homogenates (Fig. 4A).

Inspection of silver-stained gels revealed more protein bands in the specific than in the unspecific elution fraction (Fig. 4B), in particular the PrP<sub>myc</sub> band exclusively present in the myc-specific elution fractions by using cleavable isotope-coded affinity tags (cICAT) approach the two labeled fractions contain the interacting proteins in the specific and unspecific peptide eluate.

Were uniquely present in the nonspecific eluate, 96 proteins were precipitated from wild-type brains and unspecific elution under the same conditions. While 442 individual proteins were detected in both the specific and the nonspecific eluates, and 277 proteins were uniquely present in the nonspecific eluate, 96 proteins were present in the specific eluate but absent from the nonspecific eluate.

We then sought to determine the relative abundance of PrP and the interacting proteins in the specific and unspecific peptide elution fractions by using cleavable isotope-coded affinity tags (cICAT) as a quantitative mass spectrometrical technique. In the classical cICAT approach the two labeled fractions contain the same amount of protein. Since this is not the case for the specific and unspecific IP elution fractions, we could only determine the relative ratio of PrP between the specific and the unspecific elution fractions.

The two elution fractions derived from immunoprecipitations of PrP<sub>myc</sub> and wild-type brains were labeled with the “heavy” (cICAT-13C9) and “light” (cICAT-12C9) cICAT tags, mixed, and mass/charge (m/z) elution profiles were determined by mass spectrometry. Sequest [24], PeptideProphet [25] and Xpress were used to identify the proteins and to access the cICAT ratios (Fig. 4C, Table S5). Of the 157 peptide pairs that could be assigned to a heavy/light ratio between 0.1 and 100, seven proteins were found to have a comparable ratio to PrP and, at the same time, were identified as specific proteins by the gel-based approach (Table 1). Any ratios below 1 are indicative of proteins more abundant in the scrambled elution than in the myc-specific elution. Proteins displaying a similar abundance in both samples would yield a ratio of 1, which most probably indicates nonspecific binding to and elution from the beads. The ratio for PrP was about 14, and the proteins listed in Table 1 represent values between 4 and 15.

We then sought to confirm the results of mass spectrometric analyses by immunochenical analyses of selected proteins. Indeed, the identity of PrP, 2',3'-cyclic nucleotide 3'-phosphodiesterase, M6a and Neurofascin was unambiguously confirmed by Western blot analysis. Fig. 4D shows the characteristic double band of CNPase after myc-peptide elution and a low-intensity band for the scrambled-peptide elution. Western blot analysis with antibodies to Neurofascin 155 and M6a revealed specific bands for the specific-peptide elution but in none of the negative controls (Fig. 4E–F). The signal for M6a from the specific elution shows two strong bands most probably originating from alternative splicing. For both Neurofascin and M6a, the protein expression level in wt and Tg<sup>940 PrP<sup>Sc</sup>/o</sup> brain were approximately the same as illustrated in Fig. 4E–F.

**Discussion**

Our understanding of the function of PrP<sup>C</sup> and its conversion into PrP<sup>Sc</sup> continues to be sketchy. Genetic experiments have helped defining the domains of PrP<sup>C</sup> necessary for prion propagation [21] and, with some limitations, for PrP<sup>Sc</sup> function [19,26,27,28], yet, we have identified that further proteins that may be required for this process. However, the field may crucially benefit from enumerating and/or manipulating the PrP-interacting proteome. Towards the latter goals, we have studied the biogenesis, localization, and terminally myc-tagged version of PrP<sup>C</sup> (PrP<sub>myc</sub>). Since the physiological function of PrP<sup>C</sup> is unknown, we used a well-established approach of reverse genetics [14] to assay the biological activity of PrP<sub>myc</sub>. This approach is so far the most proximal surrogate to study the function of PrP. We found PrP<sub>myc</sub> to be fully functional and substitute dosage-dependently for endogenous PrP in rescuing the neurodegenerative phenotype induced by PrP<sup>AF</sup>.

Conversion of cellular prion protein PrP<sup>C</sup> into the disease-causing isoform PrP<sup>Sc</sup> is the central pathogenic process in prion diseases [29]. Therefore, any claim of the biological authenticity of a modified PrP protein should be substantiated by its ability to sustain prion replication. We approached this important question in a variety of paradigms. Whereas direct intracerebral inoculation of PrP<sub>myc</sub> transgenic mice with prions rarely induced scrapie, we found that in the presence of a wild-type P<sub>np</sub> allele PrP<sub>myc</sub> is converted into a P<sub>K</sub>-resistant isoform (PrP<sup>SC</sup><sub>myc</sub>). The disease of prion-infected PrP<sup>myc</sup>/o mice was transmissible by ic inoculation of brain homogenates to wild-type mice and also, importantly, to PrP<sub>myc</sub>/o mice. Since it is known, that PrP<sup>Sc</sup> levels do not necessarily correlate with infectivity titers, we decide to evaluate the infectivity titers by SCEPA and compare to RML, and also in that paradigm PrP<sub>myc</sub> behave as normal RML. The latter finding establishes beyond any doubt that PrP<sub>myc</sub> supports prion replication and scrapie pathogenesis.

In many paradigms, expression of heterologous PrP molecules which differ from the endogenous PrP by as little as one amino acid can profoundly interfere with the overall accumulation of PrP<sup>Sc</sup> [30,31], suggesting that precise homotypic interactions between PrP molecules are important for PrP<sup>Sc</sup> accumulation [31,32]. However, when inoculated with the same dose of prions, PrP<sup>myc</sup>/o mice developed disease faster than P<sub>np</sub> mice, implying that PrP<sub>myc</sub> cooperates, rather than interfering, with PrP<sup>Sc</sup> in disease pathogenesis. This was unexpected in view of the many instances of interference that have documented to occur even between naturally occurring PrP alleles [12]. If one accepts that interference is brought about by disturbances of the replicative interface of prions, one might speculate that the carboxy terminus of PrP<sup>C</sup> does not participate to such an interface.
Interactome of Myc-Tagged PrP

G. Mock inoculated

| C57BL/6 | Tg940 PrP^{o/o} | Tg940 PrP^{+/-} |
|---------|----------------|----------------|
| untreated | POM1  | 4A6   | POM1  | 4A6   |
| PK treated | POM1  | 4A6   | PK treated | POM1  | 4A6   |

H. RML inoculated

| Tg940 PrP^{o/o} myc | Tg940 PrP^{+/-} myc |
|---------------------|---------------------|
| untreated | POM1  | 4A6   | POM1  | 4A6   |
| PK treated | POM1  | 4A6   | PK treated | POM1  | 4A6   |

I. RML inoculated

| Pmp^{+/-} | Tg940 PrP^{+/-} myc | Tg940 PrP^{o/o} myc |
|-----------|---------------------|---------------------|
| untreated | 4A6    | 4A6    | 4A6    |
| PK treated | 4A6  | 4A6    | 4A6    |

J. Scrapie cell assay

Scrapie cell assay graph showing log TCI (U/mL) against RML and PrP^{myc}.
The latter conclusion, however, is tempered by another observation. When PrP<sup>PO/o</sup> mice were inoculated with RML prions, only a few animals developed clinical signs of scrapie. This suggests that the C-terminally modified prion protein presents a “prion transmission barrier” to mouse-adapted scrapie prions, analogously to the species barrier seen in many natural and experimental prion diseases [33].

The specificity of these interactions was validated by comparison to wild-type brain eluates and elution with a scrambled peptide. Some of the PrP-interacting proteins described before and summarized in recent reviews [36,37], including for instance Tubulin, Hsp60 and Laminin, were detected in the specific as well as unspecific elution fraction of our approach and therefore not included into the list of possible candidates.

We utilized a quantitative MS technique, isotope-coded affinity tagging (ICAT), to determine the relative abundance of PrP and other proteins in the various samples, so to identify proteins that might exist in an equimolar complex with PrP<sup>Fc</sup>. Such PrP<sub>myc</sub>-interacting proteins would display an ICAT ratio of specific/unspecific signals similar to that of PrP<sup>Fc</sup>. Based on this mass spectrometric approach, we found a small number of protein candidates equimolarly associated with PrP<sub>myc</sub> in native brain homogenates.

Some candidate proteins are shown to be mutually exclusive in culture units per ml of brain homogenates. The remaining hits detailed in Table S4 should not be dismissed because of their non-equimolar ICAT ratios.

Two of the latter seven proteins (Q8U89 clathrin linked; Q01853 translational ER ATPase) are not well-characterized and no antibodies to them appear to be available. Several PrP <sup>myc</sup> interactors belong to the families of neuronal glycoproteins and myelin-associated proteins. These include the neuronal membrane glycoprotein M6-a, Neurofascin, and 2<sup>α</sup>,3<sup>α</sup>-cyclic nucleotide 3-phosphodiesterase (CNP), P0 glycoprotein of compact PNS myelin, myelin-associated glycoprotein (MAG), and others have well-defined roles in the formation, maintenance and degeneration of myelin sheaths [40]. Myelin proteins also appear to mediate signals between the myelin-forming cell and the axon [41]. Recent research suggests that CNP is required for maintenance of axon-glial interactions at the nodes of Ranvier in the CNS [42]. The interaction between PrP and CNP may underlie the myelin damage observed in old PrP<sup>myc</sup> mice [43] and in various transgenic PrP deletion mutants age [19,20,21]. In support of this hypothesis, recent studies suggest that myelin integrity may be maintained by a constitutively active neurotrophic protein complex involving PrP<sup>Fc</sup> [19].

A possible functional relation between neurofascin and PrP<sup>Fc</sup> is particularly intriguing in view of the lethal phenotype of transgenic mice expressing PrP deletion mutants, which display extensive central and peripheral myelin degeneration [19]. Neurofascin 186 (NF186) is expressed prenatally on dorsal root ganglia neurons and it may modulate their adhesive interactions with Schwann cells, which express NF155 postsynaptically and require it for development of axon-glial paranodal junctions. The major isoform of NF186 inhibits cell adhesion, and this activity may be important in formation of the node of Ranvier [44].

Another enticing candidate for functionally relevant interactions is M6-a, a membrane glycoprotein involved in neuronal differentiation as part of a Ca<sup>2+</sup> channel [45]. The lack of the cellular prion protein was shown to affect Ca<sup>2+</sup> homeostasis in neurons [46], and therefore it is thinkable that PrP<sup>Fc</sup> and M6-a are involved in a complex possessing an ion channel-like function.

In addition to identifying the interactors described above, the tools introduced here may allow for studying supramolecular complexes containing the disease-associated prion protein PrP<sup>Sc</sup>. The biophysical properties and aggregational state of PrP<sup>Sc</sup> are vastly different from those of PrP<sup>Fc</sup>, and there is reason to hypothesize that the PrP<sup>Sc</sup>-interactor will only partially overlap with that of PrP<sup>Fc</sup>. Since most prion strains are both neurotropic and lytrophic [47,48], and inflammatory conditions specify for example the infectivity in the brain, these pathological expression may be mediated by inflammatory pathways.
the tropism of prions [49,50], the interactome of PrP<sup>C</sup> and PrP<sup>Sc</sup> in lymphoid organs will also be of interest. The inoculation of wild-type animals with myc-tagged prions may help elucidating the initial events that occur during infection of an animal with prions. Finally, the successful conversion of PrP<sub>myc</sub> into a protease-resistant moiety may allow for the purification of native PrP<sup>Sc</sup>-containing complexes using the techniques described above for PrP<sup>C</sup>. The latter studies may lead to the identification of the elusive chaperones involved in prion propagation, strain barriers and strain adaptation, as well as the crossing of prion species barriers.

**Materials and Methods**

**Generation of myc-tagged PrP<sup>C</sup>**

PCRs were performed in 50 μl volumes containing 10 ng of template DNA phgPrP [14], 200 μM of each dNTP, 20 pmol of
each primer (Pnl: 5'-TTT TTT TTC AGC TGT GGA TGC TTG CAG) and (ClaI: 5'-TTT TTT TTA TCG ATC TTC TCC TCC CCT CCT GTC ATC-3'), Xma: 5'-TTT TTT TTC CGG AGG GAA GCC CTG GAG GCA ACC GTT-3', ClaI: 5'-TTT TTT TTA TCG ATC TTC TCC TCC CCT CCT GTC ATC-3'). The final insert of pGEM-PrP( ClaI site of pGEM-PrP(ClaI) generating pGEM-PrPmyc(ClaI) consists of a mutated PrP cDNA fragment as the 'readthrough' RNA from the disrupted Prnp locus [51].

Southern blot analyses were performed using a 640 bp DNA probe synthesized by incorporation of digoxigenin-11-dUTP (Roche, Switzerland) during PCR using PrP-specific primers and hybridization was performed following established protocols [52]. For the actin control the Northern blot was probed with an in-house generated mouse beta-actin probe cloned from full-length cDNA.

The phgPrP-myC plasmid, driven by the endogenous Prnp promoter in the context of the PrP "half-genomic" construct (phgPrP) [14], was digested with NcoI and SalI to remove its prokaryotic backbone. Promiscuous injections were performed into fertilized oocytes derived from a B6D2F1 x B6;129S5-Prnp+/o/o genotype the presence of the endogenous Prnp allele was tested by PCR analysis using primers Prnp intron 2 (5'-ATA CTG GGC ACT GAT ACC TTG TTC CTC AT) and P10rev (reverse complementary of P10 5'-GCT GGT TTT GTC TGA CTA TGT ATT AGT GGT AGC) amplifying a 532 bp product for the Prnp wild-type allele but no PCR product for the Prnp allele.

For Northern blot analyses, RNA was extracted using Trizol (Invitrogen). A randomly 32P-labeled (Rediprime II Random Prime Labelling System, Amersham Biosciences) restriction fragment encompassing all of exons 1 and 2, all of the ORF and a part of exon 3 (XbaI fragment) was used as a PrP probe. This probe hybridizes with all wild-type and tagged PrP mRNAs as well as the "readthrough" RNA from the disrupted Prnp locus [51].

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Rescue of Shmerling's disease
PrPmyC mice were crossed with PrPAF [19,21] mice to obtain double transgenic animals with Prnp+/+/o/o genotype needed for the experiment described in Fig. 1. Animals were examined twice each week for symptoms of cerebellar dysfunction, including ataxia [53], tremor, weight loss, rough hair coat, and kyphosis. Scoring of neurological signs was performed according to a four-degree clinical score system [19] and mice were euthanized within 3 days of reaching a score of 3.5.

Western blot analyses
Homogenates of noninfectious brain and spleen (10% w/v) were prepared in sterile PBS/0.3% Nonidet P-40 and protease inhibitors (Complete; Roche, Switzerland) by repeated extrusion through syringe needles of successively smaller size. Homogenates of infectious brains were generated using a rhybolyzer in a
biosafety level 3 laboratory. After centrifugation for 10 min at 2,400 rpm at 4°C, supernatant was loaded onto 12% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Germany) by wet blotting, and first exposed to mouse monoclonal anti-PrP antibody POM-1 [15], 1:10'000 or mouse monoclonal anti-myc antibody 4A6 (1:1000, Upstate, USA), then to peroxidase-labeled rabbit anti-mouse antibody (1:10000; Zymed, CA, USA) and developed using the ECL detection system (Pierce, USA). Antibody incubations were performed in 1% Top Block (FLUKA, Switzerland) in PBS-Tween for 1 hour at room temperature or overnight at 4°C. The same protocol was applied to generate Western blots shown in Fig. 4 D–F using anti-M6-7 antibody (kindly provided by C. Lagenaur) diluted 1:5000, anti-CNase antibody (Abcam, Cambridge, UK) diluted 1:500 and anti-Neurodacsin 155 antibody (Chemicon) diluted 1:3000.

Preparation of DRMs

Brain homogenates were extracted for 1 hour on ice in 1% Triton X-100/25 mM MES/5 mM DTT/2 mM EDTA at pH 7.0 [16] and protease inhibitors. Extracts (500 μg protein/ml buffer) were mixed with 60% OptiPrep™ (Nycomed, Denmark) to reach a final concentration of 40% and overlaid in a SW40 centrifugation tube (Beckman, CA, USA) with a step density of 30% OptiPrep™ in MES-buffer. After centrifugation at 35’000 rpm (12 hrs), 9 fractions were collected starting from the top. The raft fraction was obtained from the interphase 5-30% OptiPrep™. Mouse monoclonal anti-PrP antibodies (POM-1) and mouse monoclonal anti-flotillin 2 (BD Transduction, USA) were used to characterize the OptiPrep™ fractions by Western blot.

Histopathology and Immunohistochemistry

Organs were fixed in 4% formaldehyde in PBS (pH 7.5) and paraﬁn-embedded. Two μm brain sections were stained with hematoxylin–eosin (HE). Immunohistochemistry was performed for glial ﬁbrillary acidic protein (activated astrocytes) using a GFAP monoclonal antibody (DAKO, Carpinteria, CA, USA). PrPSc aggregates were detected on paraﬁn sections using monoclonal antibody SAF-84. For histological analyses anatomic brain regions were selected according to standard strain-typing protocols (Bruce, 1991, Fraser, 1960). Spongiosis was evaluated on a scale of 0–5 (not detectable, mild, moderate, severe, and status spongiosus). Gliosis and PrP immunoreactivity were scored on a four-degree scale (undetectable, mild, moderate, severe). Histological analyses were performed by investigators blinded to animal identification.

Histoblot analysis

Cryosections were transferred to a nitrocellulose membrane and digested for 4 h with 20 μg/ml of proteinase K at 37°C. Blocking of the sections was done in 5% TopBlock, incubation with primary (POM1: 1:10’000, 4A6: 1:1000) and secondary antibodies (Dako D0486, AP goat anti mouse, 1:1000) were done in 1% TopBlock, respectively. The blots were incubated in BCIP/NBT in B3 buffer (100 mM Tris, 100 mM NaCl, 100 mM MgCl2, pH 9.5 plus tablets and levamisole) for 45–60 min.

Scrapie cell assay in endpoint format (SCEPA)

Prion-susceptible neuroblastoma cells (subclone N2aPK1) were exposed to 300 μl brain homogenates in 96-well plates for 3 d. Cells were subsequently split three times 1:3 every 2 days, and three times 1:10 every 3 days. After they reached confluence, we filtered 25,000 cells from each well onto the membrane of an ELISPOT plate, treated them with PK (0.5 μg/ml for 90 min at 37°C), denatured, and detected individual infected (PrPSc-positive) cells by immunocytochemistry using alkaline phosphatase-conjugated POM1 mouse anti-PrP and an alkaline phosphatase-conjugated substrate kit (BioRad). We performed serial tenfold dilutions in cell culture medium containing healthy mouse brain homogenate. Scrapie-susceptible PK1 cells were then exposed to dilutions of experimental samples ranging from 10–4 to 10–9, the same for RML, or to a 10–2 dilution of healthy mouse brain homogenate. Samples were quantified in endpoint format, by counting positive wells according to established methods.

Immunoprecipitations

Brains were homogenized in 0.5% CHAPS and protease inhibitors (Complete; Roche, Switzerland) as described above. Mouse monoclonal anti-ncy 4A6 antibody was cross linked to Dynabeads M-280 Sheep anti-Mouse IgG (Dynal, Norway) as recommended by the manufacturer. Four mg of total protein from 5% brain homogenates were diluted to a volume of 1.5 ml of 0.5% CHAPS/1% NP-40. To precipitate the PrPmyc complex, 40 μl of resuspended beads were added and incubated with rotational mixing for 2 hours at 4°C and for 15 min at room temperature. Beads were washed twice in PBS/0.5%CHAPS/1%NP-40 and twice in PBS/1% CHAPS/1%NP-40 at 4°C. To elute the complex, beads were incubated for 2 h at 4°C and another 10 min at room temperature with the synthetic specific peptide (c-myc: H-EQLKISEEDL-NH2, Roche Diagnostics, Basel, Switzerland) and the scrambled nonspeciﬁc peptide (cym: H-IELQKELDES-NH2, jct, Berlin, Germany) respectively. Peptides were added in 10-fold molar excess compared to the 4A6 antibody, in a ﬁnal volume of 380 μl of 1% CHAPS, 1% NP-40.

Tryptic in-gel digestion

Silver stained bands from 12% SDS PAGE were destained and incubated for 1–3 h in 100 mM ammonium bicarbonate (NH4HCO3, pH 8.0, Sigma) in 50% MeOH at 37°C. The proteins were reduced in 2 mM tris(carboxyethyl)phosphine (TCEP•HCl, Pierce, USA) in 100 mM ammonium bicarbonate at 37°C for 40 min and alkylated with 20 mM iodoacetamide (Fluka, Switzerland) for 30 min at room temperature in the dark. Gel pieces were cut into 100 mM ammonium bicarbonate, dehydrated in acetonitrile for 10 min, dried under vacuum for 10 min and reswell in 200–400 ng of sequence-grade modiﬁed trypsin solution (Promega, Madison, WI, USA) for 15 min at RT. Gel pieces were covered with sufﬁcient amount of 100 mM ammonium bicarbonate buffer containing 2 mM CaCl2 and incubated overnight at 37°C. Samples were sonicated for 5 min and supernatant was pooled with an additional peptide extraction round with 50% acetonitrile/1% formic acid for 20 min at RT. Samples were dried under vacuum and kept at −20°C whenever they were not used immediately.

ICAT labeling and sample processing

The IP eluate was precipitated by ethanol precipitation and the pellet was dissolved in 100 μl of cICAT labeling buﬀer (50 mM Tris, pH 8.3; 8 M Urea; 5 mM EDTA; 0.125% SDS and 0.05% RapiGest). The cICAT labeling procedures was performed as described previously [54,55,56]. The control sample was labeled with the light, the specific elution sample with heavy cICAT label (Applied Biosystems, Foster City, CA, USA). Digestion with trypsin (Promega, Madison, WI, USA) was performed at 37°C over night and ICAT-labeled peptides were subsequently puriﬁed according to the manufacturer’s instructions. ZipTip columns
Capillary chromatography and mass spectrometric analysis

Cleaned samples were resuspended in equilibration buffer (3% acetonitrile/0.1% formic acid in MilliQ-water) and loaded onto a microcapillary column constructed by slurry packing 8 cm of reversed-phase (RP) material (Magic C18, 5 μm, 200 A) Micromich BioResources, Auburn, CA, USA) into a 75 μm fused-silica capillary (BGB Analytik AG, Böckten, Switzerland). Mass spectrometric analyses were performed on an LTQ-FITM (Thermo Scientific, Bremen, Germany) systems directly coupled to a nanoLC™ HPLC system (eksigent, Dublin, CA, USA) at a flow rate of 200 nl/min. Peptides were eluted with an acetonitrile gradient from 3 to 45% in approximately 55 min and data-dependent acquisition of tandem mass spectra was continuously repeated during the course of the analysis. Each high accuracy MS full scan was followed by four MS/MS scans of the four most intense peaks. High mass accuracy data was search with Mascot Integra (Matrix Science, UK) using the UniProt mouse protein database (ftp.ebi.ac.uk/pub/databases/SPproteomes/fasta/Mus_musculus.fasta.gz), allowing for two missed trypsin cleavage sites and precursor- and fragment ion tolerances of 5 ppm and 0.8 Da, respectively. Peptides from ICAT samples were identified by searching MS/MS spectra against the same mouse protein database using Sequest [24]. PeptidePhophet was used to assess the validity of peptide assignments. Proteins were filtered using ProteinPhopht with a computed overall probability of ≥0.95 for a protein being present in the sample. Only peptide pairs that had a mass difference of 9.0301 Da were included. Both peptide contained cysteins and belonged to a protein that was identified with an Xcorr value ≥1.5. Averages and standard deviations were calculated for each protein expression value when multiple peptide measurements were available. We only considered peptides with double and multiple charges, and manually evaluated the expression values by inspecting the areas of integration that the software had chosen and by adjusting them as needed. To calculate protein ratio between different pull down samples, XPRESS [56] was used.

Prion inoculations

8–12 weeks old mice were inoculated intracerebrally (ic) or intraperitoneally (ip) with 3x10^6 infectious units (IU) or 10x10^6 IU, respectively, of Rocky Mountain Laboratory strain (RML, passage 5.0) brain homogenate, prepared as described [57]. Beginning 50 days after inoculation, mice were examined daily for neurological dysfunction and sacrificed on the day of onset of terminal clinical signs of scrapie. For transmission experiments, mice were inoculated ic with up to 30 μl of 10% sonicated brain homogenate. Mice were monitored clinically every other day in order to ascertain the onset of clinical signs and the course of the disease. Clinical signs exacerbated over time and included progressive akinesia, priapiism (males), hunchback, and stiff tail. Mice were sacrificed on the day of onset of terminal clinical signs of scrapie, defined as the time point at which they became unable to drink and/or eat.

Supporting Information

Table S1 Inoculation of Prnp+/− and PrPmyc+/−

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| at | doi:10.1371/journal.pone.0004446.s001 |

Table S2 Inoculation of PrPmyc−/−

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| at | doi:10.1371/journal.pone.0004446.s002 |

Table S3 Transmission to PrPmyc+/− and PrPmyc−/−

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Table S4 Proteins identified by GeLC-MS/MS after epitope elution

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Table S5 Proteins with Xcorr 1.5

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Acknowledgments

We thank Ralph Schlaphach and the Functional Genomics Center Zurich for access to technologies, Christina Sigurdson for help with inoculations, Giuseppe Manco for technical assistance, and Carl Lagenaur for providing M6-7 antibody.

Author Contributions

Conceived and designed the experiments: DR KDM RM EB AMC AA. Performed the experiments: DR KDM RM TR AMC. Analyzed the data: DR KDM AMC. Wrote the paper: DR KDM AA.

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Author Contributions

Conceived and designed the experiments: DR KDM RM EB AMC AA. Performed the experiments: DR KDM RM TR AMC. Analyzed the data: DR KDM AMC. Wrote the paper: DR KDM AA.

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