# An overlapping reading frame in the *PRNP* gene encodes a novel polypeptide distinct from the prion protein

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ABSTRACT **The prion protein gene *PRNP* directs the synthesis of one of the most intensively studied mammalian proteins, the prion protein (PrP). Yet the physiological function of PrP has remained elusive and has created controversies in the literature. We found a downstream alternative translation initiation AUG codon surrounded by an optimal Kozak sequence in the +3 reading frame of *PRNP*. The corresponding alternative open reading frame encodes a polypeptide termed alternative prion protein (AltPrP) with a completely different amino acid sequence from PrP. We introduced a hemagglutinin (HA) tag in frame with AltPrP in PrP cDNAs from different species to test the expression of this novel polypeptide using anti-HA antibodies. AltPrP is constitutively coexpressed with human, bovine, sheep, and deer PrP. AltPrP is localized at the mitochondria and is up-regulated by endoplasmic reticulum stress and proteasomal inhibition. Generation of anti-AltPrP antibodies allowed us to test for endogenous expression of AltPrP in wild-type human cells expressing PrP. By transfecting cells with siRNA against PrP mRNA, we repressed expression of both PrP and AltPrP, confirming endogenous expression of AltPrP from *PRNP*. AltPrP was also detected in human brain homogenate, primary neurons, and peripheral blood mononuclear cells. These results demonstrate an unexpected function for *PRNP*, which, in addition to plasma membrane-anchored PrP, also encodes a second polypeptide termed AltPrP.—Vanderperre, B., Staskevicius, A. B., Tremblay, G., McCoy, M., O'Neill, M. A., Cashman, N. R., Roucou, X. An overlapping reading frame in the *PRNP* gene encodes a novel polypeptide distinct from the prion protein. *FASEB J.* 25, 2373–2386 (2011). www.fasebj.org**

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The prion protein (PrP) is a glycoprotein anchored to the plasma membrane by virtue of a glycosylphosphatidylinositol (GPI) anchor (1). Transmissible spongiform encephalopathies (TSEs) involve the conversion of $PrP^{C}$, the normally folded conformer of PrP, into $PrP^{Sc}$, an aggregation-prone isoform of PrP that is resistant to proteinase K (2). $PrP^{Sc}$ is known to be the infectious agent in TSEs. However, the presence of normally folded $PrP^{C}$ is absolutely necessary for the onset of disease, as it acts as a continuous supply for the generation of $PrP^{Sc}$. Therefore, *PRNP,* the gene encoding PrP, is essential for the development of TSEs, and not surprisingly, PrP knockout animals are resistant to prion infection (3–5). Many missense mutations within human *PRNP* are also associated with genetic forms of TSEs (6), providing support for a central role of PrP in TSE pathogenesis.

In contrast to its well-established pathogenic role, the quest for the normal physiological function of PrP has proven very difficult. Several functions have been proposed, and controversies on the role of PrP continue in the literature (7). Some of the explanations put forward include the study of different cultured cell models used to investigate the function of PrP, such as neuronal *vs.* non-neuronal cell lines, immortalized cell lines *vs.* primary cells, and mouse neurons *vs.* human neurons. How one gene and the associated protein can result in such a complexity in terms of physiological function is confounding.

It has recently been hypothesized that alternative translation initiation of eukaryotic mRNAs might be used as a method to expand the proteome (8). On the basis of the idea that a single mRNA can produce 3 completely independent amino acid sequences if read in all 3 possible reading frames, this hypothesis suggests that the complexity of the eukaryotic proteome is largely underestimated. Several examples of out-of-frame alternative translation initiation in eukaryotes exist to support this hypothesis. However, almost all of these examples occur at an upstream AUG codon in

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relation to the +1 position of the main open reading frame (ORF) (9, 10). These alternative AUG codons are usually situated within an optimal Kozak context (11). Although extremely rare, a small number of examples of out-of-frame alternative translation initiation at a downstream AUG codon in an optimal Kozak context exist in mammals (12, 13).

On reexamination of the PrP-coding sequence (CDS), we found a potential ORF whose initiator codon is surrounded by an optimal Kozak context in a number of species. In this study, we show that the protein encoded in this overlapping ORF is coexpressed with PrP from the *PRNP* gene. This finding has direct implications regarding the comprehension of the physiological function of *PRNP*.

## MATERIALS AND METHODS

### Cloning of plasmids and transfection

All primer sequences are outlined in **Table 1**. Cloning of human $PrP^{C}$ in pCEP4β vector (Invitrogen, Carlsbad, CA, USA) has been described previously (14). Human $PrP^{(HA)}$ was produced by inserting an HA tag in the +3 frame of human PrP between bases 308 and 309 of the PrP CDS by PCR overlap extension using forward primers 1 and 3 and reverse primers 2 and 4. Human $PrP^{(HA)*}$, in which the alternative AUG at bp 90–92 of $PrP^{(HA)}$ was mutated to CUG, was produced by the Quikchange method (Stratagene, La Jolla, CA, USA) using forward primer 5 and reverse primer 6. Human C-terminally tagged $AltPrP^{HA}$ was produced using $huPrP^{(HA)}$ as a template with forward primer 7 and reverse primer 9. Untagged human AltPrP was amplified from $huPrP^{C}$ with forward primer 7 and reverse primer 8. Human $PrP^{\Delta 1-66(HA)}$, in which the first 66 bp of the $PrP^{(HA)}$ CDS were deleted, was produced using forward primer 10 and reverse primer 2. All constructs were inserted in pCEP4β vector using *Hind*III and *Bam*HI restriction enzymes. Recombinant huAltPrP was produced with forward primer 11 and reverse primer 12. The PCR product was then inserted in pET-21b vector (EMD Chemicals, Gibbstown, NJ, USA) using *Nhe*I and *Bam*HI restriction enzymes. The recombinant protein was then expressed as described previously (15). Bovine PRNP was reverse-transcribed from a total RNA extract using forward primer 13 and reverse primer 14. The bovine PrP CDS was then amplified using forward primer 15 and reverse primer 16. Bovine PrP was then inserted in pCEP4β vector using *Hind*III and *Not*I restriction enzymes. Bovine $PrP^{(HA)}$ and $AltPrP^{HA}$ were produced as described above with primers 15, 16, 17, 18, 19, and 20, and inserted in pCEP4β vector using *Hind*II and *Bam*HI restriction enzymes. White-tailed deer (wtd) PrP in pCEP4β was a kind gift from Debbie McKenzie (Alberta Centre for Prion and Protein Folding Diseases, Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada). $wtdPrP^{(HA)}$ and $AltPrP^{HA}$ were produced as described above with primers 15, 16, 19, 20, 21, and 22. PCR products were then inserted in pCEP4β vector using *Hind*III and *Bam*HI restriction enzymes. Sheep PrP in pCI expression vector was a kind gift from Dr. Michael A. Tranulis (Norwegian School of Veterinary Science, Dept. of Biochemistry and Physiology, Institute of Basic Sciences and Aquatic Medicine, Oslo, Norway). Sheep $PrP^{(HA)}$ and

TABLE 1. *Primer sequences*

| Primer | Sequence, 5′-3′ |
|---|---|
| huPrP F | CCCAAGCTTGTAATGGCGAACCTTGGCTGCTGG |
| huPrP R | CGCGGATCCTCATCCACTATCAGGAAGATG |
| $huPrP^{(HA)}$ F | TATCCGTACGACGTACCAGACTACGCCTAAGCCAAAAACCAAC |
| $huPrP^{(HA)}$ R | GTGGAACAAGCCGAGTATCCGTACGACGTACCAGACTACGCC |
| $huPrP^{(HA)}$* F | CGAAGCCTGGAGGCTGGAACACTGGGG |
| $huPrP^{(HA)}$* R | CCCCAGTGTTCCAGCCTCCAGGCTTCG |
| huAltPrP F | GGGAAGCTTGCCATGGAACTCTGGGGGCA |
| huAltPrP R | CCCGGATCCTTACTCGGCTTGTTC |
| $huAltPrP^{HA}$ R | CGACGTACCAGACTACGCCTAAGGATCCGA |
| huΔ1–66 F | AGTAAGCTTGCCGCCATGGGCCTCTGCAAGAAGCGCCCGAAGC |
| huAltPrP pET F | AGGCTAGCGAACACTGGGGGCAGCCGATA |
| huAltPrP pET R | TCGGATCCTTACTCGGCTTGTTCCACTGACTGT |
| boPrP F | TTCAACCAAGCCGAAGCATCTGTC |
| boPrP R | AGCACGAAATGAGACACCACCACT |
| $boPrP^{(HA)}$ F | TATCCGTACGACGTACCAGACTACGCCTAAGCCAAAAACCAAC |
| $boPrP^{(HA)}$ R | GGCGTAGTCTGGTACGTCGTACGGATACTCCTGGGTTTGTTCC |
| boAltPrP F | ATCAAGCTTAGGAGGATGGAACACTGG |
| $boAltPrP^{HA}$ R | GATGGATCCTTAGGCGTAGTCTGGTAC |
| wtdPrP F | ACACCCTCTTTATTTTGCAG |
| wtdPrP R | AGAAGATAATGAAAACAGGAAGG |
| $wtdPrP^{(HA)}$ F | TATCCGTACGACGTACCAGACTACGCCTAAACCAAAAACCAAC |
| $wtdPrP^{(HA)}$ R | GGCGTAGTCTGGTACGTCGTACGGATACTCCTGGGCTTGTTCC |
| shPrP F | TAGCCTCGAGGTCATCATGGTGAAAAGCCACATAGG |
| shPrP R | CGACTCTAGAGTACTATCCTACTATGAGAAAAATGAGG |
| $shPrP^{(HA)}$ F | TATCCGTACGACGTACCAGACTACGCCTAGCCACAGTCAGTGGAACAAG |
| $shPrP^{(HA)}$ R | GGCGTAGTCTGGTACGTCGTACGGATACTCCCACCTTGACCCCAG |
| $shAltPrP^{HA}$ F | TATCTCGAGGCCACCATGGAACACTGGGGGGAGCC |
| $shAltPrP^{HA}$ R | GGCTGGGGTCAAGGTGGGAGTATCCGTACGACGTACCAGACTACGCCTAGTCTAGATAT |

F, forward; R, reverse.

AltPrP$^{HA}$ were produced as described above with primers 23, 24, 25, 26, 27, and 28. PCR products were then reinserted in the pCI expression vector (Promega, Madison, WI, USA) using *Xho*I and *Xba*I restriction enzymes. All restriction enzymes were acquired from New England Biolabs (Ipswitch, MA, USA). Cells were transfected with ExGen 500 transfection reagent (Fermentas, Burlington, ON, Canada) or GeneCellin transfection reagent (BioCellChallenge, Toulon, France), according to the manufacturer's instructions.

### Antibodies and reagents

Primary antibodies used were monoclonal anti-Cox IV (ab14744), polyclonal anti-VDAC1 (ab15895), polyclonal anti-GRP78 BiP (ab53068), polyclonal anti-GAPDH (ab9485), polyclonal anti-HA (ab9110), and monoclonal anti-phospho-eIF2α (ab32157; Abcam, Cambridge, UK); monoclonal anti-β-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO, USA); monoclonal anti-Hsp70 (SPA-810, Stressgen, Ann Arbor, MI, USA); polyclonal anti-Bax (sc-493; Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-cytochrome *c* (clone 6H2.B4; BD Pharmingen, Franklin Lakes, NJ, USA); monoclonal anti-HA (clone C29F4; Cell Signaling Technology, Danvers, MA, USA); monoclonal anti-mitochondrial Hsp70 (clone JG1; Affinity BioReagents, Waltham, MA, USA); monoclonal anti-PrP (clone SAF32; Cayman Chemical, Ann Arbor, MI, USA); and monoclonal anti-α-tubulin (clone A11126; Molecular Probes, Eugene, OR, USA). Anti-PrP clone 3F4 was purified from hybridoma cell lines. Rabbit polyclonal antibodies against human AltPrP were raised against residues 59–73 and affinity purified by GenScript (Piscataway, NJ, USA). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (NA931V) and HRP-conjugated donkey anti-rabbit IgG (NA934V; GE Healthcare, Little Chalfont, UK) and Alexa Fluor 488-conjugated goat anti-mouse IgG (A-11701) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (A-21069; Invitrogen). All other reagents were obtained from Sigma-Aldrich, unless otherwise stated.

### Cell culture and drug treatments

Human epithelial kidney (HEK293), murine neuroblastoma (N2a), and human astrocytoma (U-118 and U-87) cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC, Canada). Human neuroblastoma [BE(2)-M17] cells were grown in a 1:1 mixture of Eagle's minimum essential medium and F-12 medium supplemented with 5% FBS. All culture media were supplemented with amphotericin B, as well as penicillin/streptomycin. Human primary neurons (1520-5; ScienCell, Carlsbad, CA, USA) were grown in neuronal medium (1521; ScienCell), according to the manufacturer's protocol. Peripheral blood mononuclear cells were purified from human peripheral blood using Ficoll-Paque PLUS (GE Healthcare), according to the manufacturer's protocol. Drug treatments were conducted on cells transfected for 24 h as follows, unless otherwise stated: MG132 and epoxomicin were used for 8 h, each at a concentration of 10 μM. Thapsigargin, tunicamycin, and A23187 were used for 24 h at concentrations of 3.5, 1.5, and 1.3 μM, respectively. Cycloheximide was used at a concentration of 107 μM.

### siRNA treatments

U-118 cells were plated in a 6-well plate at $2 \times 10^5$ cells/well in fresh medium containing no antibiotics. After 24 h, *PRNP* siRNA (SI03019324) or AllStars Negative Control siRNA (1027281; Qiagen, Mississauga, ON, Canada) were transfected into the cells at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. After 72 h, cells were harvested, and lysates were processed for SDS-PAGE and Western blot analysis to assess knockdown efficiency.

### Sample preparation and immunoblotting

Cells were grown in 6-well plates for 24 h and were then transfected as described above. Cells were rinsed and harvested in PBS and centrifuged for 60 s at 5000 rpm. Cells were then lysed in RIPA buffer, and samples were quantified using BCA protein assay reagent (Pierce, Waltham, MA, USA). Preparation of 10% human brain homogenate in PBS supplemented with EDTA Complete protease inhibitor cocktail (Roche Applied Science, Laval, QC, Canada) was prepared using an Omni TH115 tissue homogenizer (Omni International, Kennesaw, GA, USA), according to the manufacturer's protocol. Protein (100 μg) from each sample was precipitated using the chloroform/methanol technique described by Wessel and Flugge (16), and the resulting pellets were resuspended in 4× SDS-PAGE loading dye. After electrophoresis, proteins were transferred to PVDF membranes, according to the manufacturer's protocol. Membranes were then exposed using Luminata Forte Western HRP Substrate (Millipore Corp., Billerica, MA, USA) or Western Lightning ECL reagent (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions. Films used were Amersham Hyperfilm ECL films (GE Healthcare). Membranes were stripped by washing twice in 0.2 N NaOH for 20 min, rinsed in PBS, blocked, and reprobed, as described above. Densitometric analysis was conducted using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Densitometric values were corrected for loading (anti-tubulin or anti-actin signal obtained by Western blot analysis).

### Immunofluorescence

Immunofluorescence was carried out as described previously (17). Confocal analysis was carried out as described previously (18). Briefly, cells were examined with a scanning confocal microscope (FV1000; Olympus, Tokyo, Japan) coupled to an inverted microscope with a ×63 oil-immersion objective (Olympus). Specimens were laser excited at 488 nm (40-mW argon laser) and 543 nm (diode laser). In order to avoid crosstalk, the emitted Alexa Fluor 488 and Alexa Fluor 568 fluorescences were collected sequentially at wavelengths 500–530 and >560 nm, respectively. Serial horizontal optical sections of 512 × 512 pixels with 2 times line averaging were taken at 0.11-μm intervals through the entire thickness of the cell (optical resolution: lateral −0.18 μm; axial −0.25 μm). Images were acquired during the same day, using identical settings of the instrument. For illustration purposes, images were pseudocolored according to their original fluorochromes, merged (FluoView software; Olympus), then cropped and assembled (Adobe Photoshop software, Adobe Systems, Mountain View, CA, USA).

### Mitochondrial fractionation

Mitochondria were isolated and treated with sodium carbonate or digitonin, as described previously (19). Briefly,

mitochondria were subjected to centrifugation at 13,000 *g* at 4°C for 10 min in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany). Mitochondria extracted with sodium carbonate were pelleted by centrifugation at 600,000 *g* for 20 min at 4°C with a Beckman Coulter Optimax LX ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).

**A**

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Human          AT Ggc ga- --- --a cct tgg ctg ctg gat gct ggt tct ctt tgt ggc cac atg gag tga cct ggg cct ctg caa
Chimpanzee     AT Ggc aa- --- --a cct tgg ctg ctg gat gct agt tct ctt tgt ggc cac atg gag tga cct ggg cct ctg caa
Macaque        AT Ggc ga- --- --a cct tgg ctg ctg gat gct ggt tct ctt tgt ggc cac atg gag tga cct ggg cct ctg caa
Cattle         AT Ggt gaa aag cca cat agg cag ttg gat cct ggt tct ctt tgt ggc cat gtg gag tga cgt ggg cct ctg caa
Bison          AT Ggt gaa aag cca cat agg cag ttg gat cct ggt tct ctt tgt ggc cat gtg gag tga cgt ggg cct ctg caa
Deer           AT Ggt gaa aag cca cat agg cag ctg gat cct agt tct ctt tgt ggc cat gtg gag tga cgt ggg cct ctg caa
Elk            AT Ggt gaa aag cca cat agg cag ctg gat cct agt tct ctt tgt ggc cat gtg gag tga cgt cgg cct ctg caa
Moose          AT Ggt gaa aag cca cat agg cag ctg gat cct agt tct ctt tgt ggc cat gtg gag tga cgt ggg cct ctg caa
Sheep          AT Ggt gaa aag cca cat agg cag ttg gat cct ggt tct ctt tgt ggc cat gtg gag tga cgt ggg cct ctg caa
Goat           AT Ggt gaa aag cca cat agg cag ttg gat cct ggt tct ctt tgt ggc cat gtg gag tga cgt ggg cct ctg caa
Cat            AT Ggt gaa agg cca cat agg cgg ctg gat cct ggt tct gtt tgt ggc cac atg gag tga cgt ggg cct ctg caa
Rabbit         AT Ggc g-- --- -ca cct cgg cta ctg gat gct tct ctt tgt ggc cac gtg gag tga cgt ggg cct ctg caa
Mink           AT Ggt gaa aag cca cat agg cag ctg gct cct ggt tct ctt tgt ggc cac atg gag tga cat tgg ctt ctg caa
Rat            AT Ggc ga- --- --a cct tgg cta ctg gct gct ggc cct ctt tgt gac tac atg tac tga tgt tgg cct ctg caa
Hamster        AT Ggc ga- --- --a cct tag ctt ctg gct gct ggc act ctt tgt ggc tat gtg gac tga tgt tgg cct ctg caa
Mouse          AT Ggc ga- --- --a cct tgg cta ctg gct gct ggc cct ctt tgt gac tat gtg gac tga tgt cgg cct ctg caa
Chicken        AT Ggc tag gct cct cac cac ctg ctg cct gct ggc cct gct gct cgc cgc ctg cac cga cgt cgc cct ctc caa
Dog            AT Ggt gaa aag cca cat agg cgg ctg gat cct gct tct ctt tgt ggc cac atg gag tga cgt ggg cct ctg caa
Pig            AT Ggt gaa aag cca cat agg tgg ctg gat cct cgt tct ctt tgt ggc cgc atg gag tga cat agg gct ctg caa

Human          gaa gcg ccc gaa gcc t-- -gg Agg ATG Gaa cac tgg ggg --- cag ccg ata ccc ggg gca ggg cag ccc tgg agg
Chimpanzee     gaa gcg ccc gaa gcc t-- -gg Agg ATG Gaa cac tgg ggg --- cag ccg ata ccc ggg gca ggg cag ccc tgg agg
Macaque        gaa gcg ccc aaa gcc t-- -gg Agg ATG Gaa cac tgg agg --- cag ccg ata ccc ggg gca ggg cag ccc tgg agg
Cattle         gaa gcg acc aaa acc tgg agg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc agg aca ggg cag tcc tgg agg
Bison          gaa gcg acc aaa acc tgg agg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc agg aca ggg cag tcc tgg agg
Deer           gaa gcg acc aaa acc tgg agg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc ggg aca ggg aag tcc tgg agg
Elk            gaa gcg acc aaa acc tgg agg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc ggg aca ggg aag tcc tgg agg
Moose          gaa gcg acc aaa acc tgg agg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc ggg aca ggg aag tcc tgg agg
Sheep          gaa gcg acc aaa acc tgg cgg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc ggg aca ggg cag tcc tgg agg
Goat           gaa gcg acc aaa acc tgg cgg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc agg aca ggg cag tcc tgg agg
Cat            gaa gcg gcc gaa gcc tgg tgg Agg ATG Gaa cac tgg ggg --- gag ccg gta ccc ggg gca ggg cag tcc tgg agg
Rabbit         gaa gcg gcc gaa gcc tgg agg Agg ATG Gaa cac agg ggg --- gag ccg gta ccc ggg tca gag cag ccc tgg agg
Mink           gaa gtg gcc aaa gcc tgg agg Agg GTG Gaa cac tgg ggg --- gag ccg ata ccc agg gca ggg cag tcc tgg agg
Rat            aaa gcg gcc aaa gcc t-- -gg Agg GTG Gaa cac tgg tgg --- aag ccg gta ccc tgg gca ggg aag ccc tgg agg
Hamster        gaa gcg gcc aaa gcc t-- -gg Agg GTG Gaa cac tgg cgg --- aag ccg ata ccc tgg gca ggg cag ccc tgg agg
Mouse          aaa gcg gcc aaa gcc t-- -gg Agg GTG Gaa cac cgg tgg --- aag ccg gta tcc cgg gca ggg aag ccc tgg agg
Chicken        gaa ggg c-- --- --- --- -aa Agg CAT Acc cag tgg tgg --- ggg ttg ggg cgc cgg gag cca tcg cca gcc cag
Dog            gaa gcg gcc gaa gcc t-- -gg Agg ATG Gaa cac agg ggg tgg gag ccg ata ccc agg cca ggg TAG
Pig            gaa gcg acc aaa gcc tgg cgg Agg ATG Gaa cac tgg ggg --- gag ccg ata ccc agg gca ggg TAG

Human          caa ccg cta ccc acc tca ggg cgg tgg tgg ctg ggg gca gcc tca t-- -gg tgg tgg ctg --- --- --- --- ---
Chimpanzee     caa ccg cta ccc acc tca ggg cgg tgg tgg ctg ggg gca gcc tca t-- -gg tgg tgg ctg --- --- --- --- ---
Macaque        caa ccg cta ccc acc cca ggg tgg tgg tgg ctg ggg gca gcc tca t-- -gg tgg tgg ctg --- --- --- --- ---
Cattle         caa ccg tta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg ggg cca gcc tca tgg
Bison          caa ccg tta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg ggg cca gcc tca tgg
Deer           caa ccg cta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg --- --- --- --- ---
Elk            caa ccg cta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg --- --- --- --- ---
Moose          caa ccg cta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg --- --- --- --- ---
Sheep          caa ccg cta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg --- --- --- --- ---
Goat           caa ccg cta tcc acc tca ggg agg ggg tgg ctg ggg tca gcc cca t-- -gg agg tgg ctg --- --- --- --- ---
Cat            caa ccg tta ccc acc cca ggg cgg tgg cgg ctg ggg tca gcc cca cgc cgg cgg ctg --- --- --- --- ---
Rabbit         caa ccg cta ccc acc cca ggg cgg tgg cgg ctg ggg aca gcc cca t-- -gg cgg cgg ctg --- --- --- --- ---
Mink           caa ccg cta ccc acc cca ggg tgg tgg cgg ctg ggg cca gcc cca c-- -gg ggg tgg ctg --- --- --- --- ---
Rat            caa ccg tta ccc acc tca gag tgg tgg tac ctg ggg gca gcc cca t-- -gg tgg tgg ctg --- --- --- --- ---
Hamster        caa ccg tta ccc acc tca ggg --- --- --- --- --- --- --- --- t-- -gg cgg cac ctg --- --- --- --- ---
Mouse          caa ccg tta ccc acc tca ggg --- --- --- --- --- --- --- --- --- --- tgg cac ctg --- --- --- --- ---
Chicken        cta ccc ccg cca gcc --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Dog
Pig

Human          --- --- --- ggg gca gcc tca tg- --g tgg tgg ctg ggg gca gcc cca tg- --g tgg tgg ctg ggg aca gcc tca
Chimpanzee     --- --- --- ggg gca gcc tca tg- --g tgg tgg ctg ggg gca gcc cca tg- --g tgg tgg ctg ggg aca gcc tca
Macaque        --- --- --- ggg gca acc cca tg- --g tgg cgg ctg ggg aca gcc tca tg- --g tgg cgg ctg ggg aca gcc tca
Cattle         agg tgg ctg ggg cca gcc tca tg- --g agg tgg ctg ggg tca gcc cca tg- --g tgg tgg ctg ggg aca gcc aca
Bison          agg tgg ctg ggg cca acc tca tg- --g agg tgg ctg ggg tca gcc cca tg- --g tgg tgg ctg ggg aca gcc aca
Deer           --- --- --- ggg cca acc tca tg- --g agg tgg ctg ggg tca gcc cca tg- --g tgg tgg ctg ggg gca gcc aca
Elk            --- --- --- ggg cca acc tca tg- --g agg tgg ctg ggg tca gcc cca tg- --g tgg tgg ctg ggg aca gcc aca
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**Figure 1.** An alternative ORF overlapping with the PrP octarepeat (OR) region exists in the +3 frame of several species. *A*) DNA sequence alignment of *Prnp* in several species. *Prnp* DNA sequences of several species were aligned starting from the PrP start codon (capitalized letters) until the AltPrP stop codon (boxed and capitalized). All larger mammals analyzed contain a start codon (boxed and capitalized) surrounded by a Kozak consensus sequence (underscored). Most smaller species analyzed do not possess a conventional ATG start codon (shown in gray), though they all possess a Kozak consensus sequence, except chicken. Sequences were obtained from GenBank and aligned using ClustalW software. GenBank accession number of each sequence

(*continued on next page*)

After digitonin treatment, mitoplasts were pelleted by centrifugation at 13,000 *g* at 4°C for 10 min in an Eppendorf centrifuge.

### PNGase F treatment and solubility assay

PNGase F treatment of transfected cell lysates (New England Biolabs) was carried out according to the manufacturer's instructions. Briefly, 100 μg of proteins extracted in RIPA buffer was adjusted to 0.5% SDS, boiled 10 min, and digested with 2 U of PNGase F for 18 h at 37°C. Solubility of PrP was assessed as described previously (20).

### Ethics statement

The human brain sample was a kind gift from Dr. Cheryl Wellington (Department of Pathology, University of British Columbia, Vancouver, BC, Canada). All experiments were performed according to University of British Columbia ethics protocol UBC C04-0595.

### Bioinformatics

DNA sequences of *Prnp* in several species were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and aligned using ClustalW software (http://www.clustal.org).

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Moose         --- --- --- ggg cca acc tca tg- --g agg tgg ctg ggg gca gcc cca tg- --g tgg tgg ctg ggg gca gcc aca
Sheep         --- --- --- ggg cca acc tca tg- --g agg tgg ctg ggg tca gcc cca tg- --g tgg tgg ctg ggg aca gcc aca
Goat          --- --- --- ggg cca acc tca tg- --g agg tgg ctg ggg tca gcc cca tg- --g tgg tgg ctg ggg aca gcc aca
Cat           --- --- --- ggg tca gcc cca cgc cgg cgg agg ctg ggg tca gcc cca cgc cgg cgg tgg ctg ggg tca gcc cca
Rabbit        --- --- --- ggg gca gcc tca cg- --g tgg tgg ctg ggg aca gcc cca cg- --g cgg tgg ctg ggg gca gcc cca
Mink          --- --- --- ggg aca gcc cca cg- --g ggg tgg ctg ggg tca gcc cca cg- --g ggg tgg ctg ggg aca gcc gca
Rat           --- --- --- ggg aca acc tca tg- --g tgg tgg ctg ggg aca acc tca tg- --g tgg tgg ctg ggg tca gcc cca
Hamster       --- --- --- ggg gca acc cca tg- --g tgg tgg ctg ggg aca gcc cca tg- --g tgg tgg ctg ggg tca gcc cca
Mouse         --- --- --- ggg gca gcc cca cg- --g tgg tgg ctg ggg aca acc cca tg- --g ggg cag ctg ggg aca acc tca
Chicken       --- --- --- ggg cta ccc tca TAA
Dog
Pig

Human         t-- -gg tgg tgg ctg ggg tca agg agg tgg cac cca cag tca gtg gaa caa gcc gag TAA          M13899
Chimpanzee    t-- -gg tgg tgg ctg ggg tca agg agg tgg cac cca cag tca gtg gaa caa gcc gag TAA          AY665268
Macaque       t-- -gg tgg tgg ctg ggg tca agg agg tgg cac cca caa tca gtg gca caa gcc cag TAA          U08307
Cattle        tgg tgg tgg agg ctg ggg tca agg tgg t-- -ac cca cgg tca atg gaa caa acc cag TAA          AB001468
Bison         tgg tgg tgg agg ctg ggg tca agg tgg t-- -ac cca cgg tca atg gaa caa acc cag TAA          AY769958
Deer          tgg tgg tgg agg ctg ggg tca agg tgg t-- -ac cca cag tca gtg gaa caa gcc cag TAA          AY286008
Elk           tgg tgg tgg agg ctg ggg tca agg tgg t-- -ac cca cag tca gtg gaa caa gcc cag TAA          AF016228
Moose         tgg tgg tgg agg ctg ggg tca agg tgg t-- -ac cca cag tca gtg gaa caa gcc cag TAA          AY225484
Sheep         tgg tgg tgg agg ctg ggg tca agg tgg TAG                                                 U67922
Goat          tgg tgg tgg agg ctg ggg tca agg tgg TAG                                                 S82626
Cat           cgc cgg cgg cgg ctg ggg tca agg tgg tgg cac cca cgg tca gtg ggg caa acc cag TAA          AF003087
Rabbit        c-- -gg tgg tgg ctg ggg tca agg agg tgg tac cca caa cca gtg ggg caa gcc cag TAA          AF015603
Mink          tgg tgg cgg tgg ctg ggg tca agg tgg tgg gag cca cgg tca gtg ggg caa gcc cag TAA          EF508270
Rat           t-- -gg cgg ggg ctg gag tca agg agg ggg tac cca TAA                                      D50093
Hamster       t-- -gg tgg tgg ctg ggg tca agg agg tgg cac cca caa tca gtg gaa caa gcc car TAA          M14054
Mouse         t-- -gg tgg TAG                                                                         M18071
Chicken                                                                                               GU991271
Dog                                                                                                   AF042843
Pig                                                                                                   AY835633
```

**B**

```
Human         MEHWG-QPIPGAGQPWRQPLPTSGRWWLGAAS-WWWLGA--------ASWWWLGAAP-WWWLGTASWW-W-LGSRRWHPQSVEQAE  73
Chimpanzee    MEHWG-QPIPGAGQPWRQPLPTSGRWWLGAAS-WWWLGA--------ASWWWLGAAP-WWWLGTASWW-W-LGSRRWHPQSVEQAE  73
Macaque       MEHWR-QPIPGAGQPWRQPLPTPGWWWLGAAS-WWWLGA--------TPWWRLGTAS-WWRLGTASWW-W-LGSRRWHPQSVAQAQ  73
Cattle        MEHWG-EPIPRTGQSWRQPLSTSGRGWLGSAP-WRWLGPASWRWLGPASWRWLGSAP-WWWLGTATWW-WRLGSR-WYPRSMEQTQ  81
Bison         MEHWG-EPIPRTGQSWRQPLSTSGRGWLGSAP-WRWLGPASWRWLGPTSWRWLGSAP-WWWLGTATWW-WRLGSR-WYPRSMEQTQ  81
Deer          MEHWG-EPIPGTGKSWRQPLSTSGRGWLGSAP-WRWLGP--------TSWRWLGSAP-WWWLGAATWW-WRLGSR-WYPQSVEQAQ  73
Elk           MEHWG-EPIPGTGKSWRQPLSTSGRGWLGSAP-WRWLGP--------TSWRWLGSAP-WWWLGTATWW-WRLGSR-WYPQSVEQAQ  73
Moose         MEHWG-EPIPGTGKSWRQPLSTSGRGWLGSAP-WRWLGP--------TSWRWLGAAP-WWWLGAATWW-WRLGSR-WYPQSVEQAQ  73
Sheep         MEHWG-EPIPGTGQSWRQPLSTSGRGWLGSAP-WRWLGP--------TSWRWLGSAP-WWWLGTATWW-WRLGSR-W            64
Goat          MEHWG-EPIPRTGQSWRQPLSTSGRGWLGSAP-WRWLGP--------TSWRWLGSAP-WWWLGTATWW-WRLGSR-W            64
Cat           MEHWG-EPVPGAGQSWRQPLPTPGRWRLGSAPRRRRLGS-A------PRRRRLGSAPRRRWLGSAPRR-RRLGSRWWHPRSVGQTQ  77
Rabbit        MEHRG-EPVPGSEQPWRQPLPTPGRWRLGTAP-WRRLGA--------ASRWWLGTAP-RRWLGAAPRW-W-LGSRRWYPQPVGQAQ  73
Mink          LEHWG-EPIPRAGQSWRQPLPTPGWWRLGPAP-RGWLGT--------APRGWLGSAP-RGWLGTAAWWRW-LGSRWWEPRSVGQAQ  74
Rat           VEHWW-KPVPWAGKPWRQPLPTSEWWYLGAAP-WWWLGT--------TSWWWLGTTS-WWWLGSAPWR-G-LESRRGYP        66
Hamster       VEHWR-KPIPWAGQPWRQPLPTSG---------WRHLGA--------TPWWWLGTAP-WWWLGSAPWW-W-LGSRRWHPQSVEQAX  65
Mouse         VEHRW-KPVSRAGKPWRQPLPTSG----------WHLGA--------APRWWLGTTP-WGQLGTTSWW                   48
Chicken       ------------RLHRRRPLQEGQRQ--------------------TQWWGLGRRE-----PSPAQL----------PPPAGLPS  38
Dog           MEHRGWEPIPRPG                                                                           13
Pig           MEHWG-EPIPRAG                                                                           12
```

**Figure 1.** (*continued*) used is included at the end of the DNA sequence. *B*) Amino acid sequence alignment of AltPrP in several species. AltPrP is present in several large mammals and has conserved tryptophan-rich repeats. Some smaller mammals contain the AltPrP ORF, yet have interrupted tryptophan-rich repeats replaced by arginine (such as cat and rabbit). Other species do not possess a conventional initiator methionine residue (mink, rat, hamster, and mouse) or contain a stop codon early on in the peptide sequence (dog and pig). Length of the ORF in each species is indicated at the end of the peptide sequence. Sequences were translated from DNA sequences obtained from GenBank using the ExPaSy Translate tool and then aligned using ClustalW software. Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under accession numbers TPA: BK007887–BK007890.

TABLE 2. *Amino acid sequences of constructs used to detect AltPrP*

| Construct | Reading frame | Sequence |
|---|---|---|
| huPrP | 1 | MANLGCWMLVLFVATWSDLGLCKKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGGG-WGQPHGGGWGQPHGGGWGQPHGGGWGQGGGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGY-MLGSAMSRPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHTVTTT-TKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSSPPVILLISFLIFLIVG |
| | 3 | MEHWGQPIPGAGQPWRQPLPTSGRWWLGTASWWWLGAASWWWLGAAPWWWLGTASWWWLGSRRW-HPQSVEQAE |
| huPrP$^{(HA)}$ | 1 | MANLGCWMLVLFVATWSDLGLCKKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGG-GWGQPHGGGWGQPHGGGWGQPHGGGWGQGGGTHSQWNKPS*IRTTYQTTP*KPKTNMKHMAGAAA-AGAVVGGLGGYMLGSAMSRPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCV-NITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSSPPVILL-ISFLIFLIVG |
| | 3 | MEHWGQPIPGAGQPWRQPLPTSGRWWLGTASWWWLGAASWWWLGAAPWWWLGTASWWWLGSRR-WHPQSVEQAEYPYDVPDYA |
| huPrP$^{(HA)*}$ | 1 | MANLGCWMLVLFVATWSDLGLCKKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGG-GWGQPHGGGWGQPHGGGWGQPHGGGWGQGGGTHSQWNKPS*IRTTYQTTP*KPKTNMKHMAGAAA-AGAVVGGLGGYMLGSAMSRPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCV-NITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSSPPVILL-ISFLIFLIVG |
| | 3 | LEHWGQPIPGAGQPWRQPLPTSGRWWLGTASWWWLGAASWWWLGAAPWWWLGTASWWWLGSRRW-HPQSVEQAE |
| huAltPrP | 1 | MEHWGQPIPGAGQPWRQPLPTSGRWWLGTASWWWLGAASWWWLGAAPWWWLGTASWWWLGSRRW-HPQSVEQAE |
| huAltPrP$^{HA}$ | 1 | MEHWGQPIPGAGQPWRQPLPTSGRWWLGTASWWWLGAASWWWLGAAPWWWLGTASWWWLGSRRW-HPQSVEQAEYPYDVPDYA |
| PrP$^{\Delta 1-66(HA)}$ | 1 | MGLCKKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGW-GQPHGGGWGQGGGTHSQWNKPS*IRTTYQTTP*KPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMS-RPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHTVTTTTKGENF-TETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSSPPVILLISFLIFLIVG |
| | 3 | MEHWGQPIPGAGQPWRQPLPTSGRWWLGTASWWWLGAASWWWLGAAPWWWLGTASWWWLGSRRW-HPQSVEQAEYPYDVPDYA |
| boPrP | 1 | MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPH-GGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGTHGQWNKPSKPKTNMKHVAGAAA-AGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYYRENMHRYPNQVYYRPVDQYSNQNNFVHDCVN-ITVKEHTVTTTTKGENFTETDIKMMKRVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLIS-FLIFLIVG |
| | 3 | MEHWGEPIPRTGQSWRQPLSTSGRGWLGSAPWRWLGPASWRWLGPASWRWLGSAPWWWLGTATW-WWRLGSRWYPRSMEQTQ |
| boPrP$^{(HA)}$ | 1 | MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPH-GGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGTHGQWNKPS*IRTTYQTTL*KPKTN-MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYYRENMHRYPNQVYYRPVDQYSNQ-NNFVHDCVNITVKEHTVTTTTKGENFTETDIKMMKRVVEQMCITQYQRESQAYYQRGASVILFS-SPPVILLISFLIFLIVG |
| | 3 | MEHWGEPIPRTGQSWRQPLSTSGRGWLGSAPWRWLGPASWRWLGPASWRWLGSAPWWWLGTATW-WWRLGSRWYPRSMEQTQYPYDVPDYA |
| boAltPrP$^{HA}$ | 1 | MEHWGEPIPRTGQSWRQPLSTSGRGWLGSAPWRWLGPASWRWLGPASWRWLGSAPWWWLGTATW-WWRLGSRWYPRSMEQTQYPYDVPDYA |
| wtdPrP | 1 | MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPH-GGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGTHSQWNKPSKPKTNMKHVAGAAAAGAVVGGL-GGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQYNNQNTFVHDCVNITVKQHTV-TTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLISFLIFLIVG |
| | 3 | MEHWGEPIPGTGKSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAPWWWLGAATWWWRLGSRW-YPQSVEQAQ |
| wtdPrP$^{(HA)}$ | 1 | MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPH-GGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGTHSQWNKPS*IRTTYQTTL*KPKTNMKHVAGAA-AAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQYNNQNTFVHDCV-NITVKQHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLI-SFLIFLIVG |
| | 3 | MEHWGEPIPGTGKSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAPWWWLGAATWWWRLGSRW-YPQSVEQAQYPYDVPDYA |
| wtdAltPrP$^{HA}$ | 1 | MEHWGEPIPGTGKSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAPWWWLGAATWWWRLGSRW-YPQSVEQAQYPYDVPDYA |

(*continued on next page*)

TABLE 2. *(continued)*

| Construct | Reading frame | Sequence |
|---|---|---|
| | | MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPH-GGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGSHSQWNKPSKPKTNMKHVAGAAAAGAVVGGL-GGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITVKQHTV- |
| shPrP | 1 | TTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLISFLIFLIVG |
| | 3 | MEHWGEPIPGTGQSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAPWWWLGTATWWWRLGSRW |
| shPrP$^{(HA)}$ | 1 | MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPH-GGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG*IRTTYQTTP*SHSQWNKPSKPKTNMKHVAGAA-AAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCV-NITVKQHTVTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLI-SFLIFLIVG |
| | 3 | MEHWGEPIPGTGQSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAPWWWLGTATWWWRLGSR-WYPYDVPDYA |
| shAltPrP$^{HA}$ | 1 | MEHWGEPIPGTGQSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAPWWWLGTATWWWRLGSR-WYPYDVPDYA |

Amino acid sequences of all constructs used are included. Note that wherever possible, the amino acid sequence of both the PrP reading frame (+1) and the AltPrP reading frame (+3) are included for each construct. Underscored letters represent the hemagglutinin (HA) tag. When shown in roman type, the HA sequence is in its proper reading frame in order to be detected by an anti-HA antibody. When shown in italics, the HA sequence is in another reading frame, which cannot be detected by an anti-HA antibody. Note that for huPrP$^{(HA)*}$, the initiator methionine codon (M) is replaced by leucine (L, double underscored).

DNA sequences were translated using the ExPaSy Translate tool (http://expasy.org).

## RESULTS

### An alternative ORF exists in the coding sequence of PrP

In an attempt to uncover the reason for the numerous functions attributed to PrP, we have reexamined the sequence of *PRNP* from several species. We noticed an ORF in the +3 reading frame, in which the AUG initiation codon (bp 90–92 in the human PrP CDS) is positioned within an optimal Kozak context (**Fig. 1*A***). We termed the putative polypeptide encoded by this ORF alternative PrP (AltPrP). The AltPrP ORF covers the entire octarepeat (OR) region of PrP. AltPrP ranges in length between 64 and 81 aa, and contains several tryptophan-rich repeats resulting from translation of the OR region of PrP in the +3 reading frame (Fig. 1*B*). In other species, such as mouse and hamster, the alternative initiation AUG codon is absent and is replaced by a GUG codon still located within an optimal Kozak sequence (Fig. 1*A*). Although there is at least one example in which GUG is an efficient initiation codon to translate a protein (21), we focused on the expression of AltPrP in humans, cattle, sheep, and white-tailed deer in this study.

### AltPrP is expressed from PrP cDNA of several species

A detailed list of all constructs used, along with amino acid sequences of each construct, is outlined in **Table 2**. To test whether AltPrP is expressed, we introduced a hemagglutinin (HA) tag in frame with AltPrP to produce carboxy-tagged AltPrP (AltPrP$^{HA}$), within the human PrP cDNA (**Fig. 2*A***). For clarity reasons, this construct is termed PrP$^{(HA)}$, where (HA) indicates that the HA tag is silent within the reading frame of PrP. As a positive control, we created a construct encoding solely AltPrP$^{HA}$. We also engineered a PrP$^{(HA)}$ construct with an inactivated alternative initiation codon. In this construct, termed PrP$^{(HA)*}$, the AUG at bp 90 was changed to CUG. Lysates from mammalian cells transfected with PrP, PrP$^{(HA)}$, or PrP$^{(HA)*}$ were probed with both anti-PrP and anti-HA antibodies to test for the expression of PrP, PrP$^{(HA)}$, PrP$^{(HA)*}$, and AltPrP$^{HA}$. As expected, the introduction of the HA tag resulted in a slight increase in the molecular weight of PrP (Fig. 2*B*). On a SDS-PAGE gel, PrP$^{(HA)}$ migrated as several bands, indicating the presence of glycosylations similar to native PrP. Remarkably, a band corresponding to the expected molecular weight for AltPrP$^{HA}$ was detected with an anti-HA antibody in cells transfected with PrP$^{(HA)}$. The identity of this band was confirmed by probing lysates from cells directly transfected with cDNA encoding AltPrP$^{HA}$. AltPrP$^{HA}$ was not detected in cells transfected with PrP$^{(HA)*}$ (Fig. 2*B*), clearly showing that translation of AltPrP is indeed initiated at the identified alternative AUG codon (Fig. 1*A*), most likely by alternative translation initiation. Analogous results were obtained in neuronal, as well as in non-neuronal cell lines (Fig. 2*B*). We then confirmed these results by immunofluorescence on N2a and HEK293 cells expressing each of these constructs (Fig. 2*C*). Detection of PrP, PrP$^{(HA)}$, PrP$^{(HA)*}$, and AltPrP$^{HA}$ was done using anti-PrP and anti-HA antibodies. In both cell types, expression of PrP$^{(HA)}$ and AltPrP$^{HA}$ resulted in an HA signal with a granular cytoplasmic distribution. Expression of PrP or PrP$^{(HA)*}$ did not provide an HA signal, confirming the results described in Fig. 2*B*. The addition of the out-of-frame HA tag in PrP$^{(HA)}$ and PrP$^{(HA)*}$ did not affect the subcellular localization of PrP, which

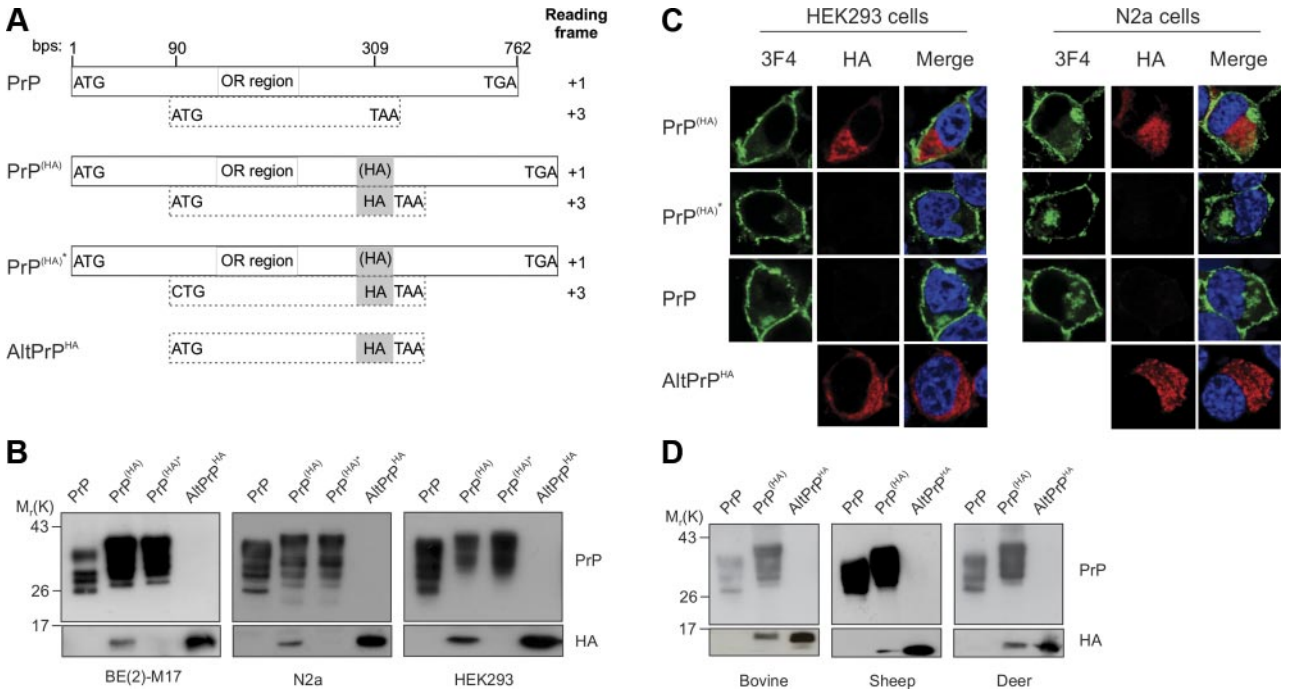


**Figure 2.** Detection of AltPrP. *A*) Strategy used to detect AltPrP by introducing an HA tag at the C-terminus of AltPrP. AltPrP is in the +3 reading frame in relation to PrP and spans the entire OR region in the N-terminal domain of PrP. Top box in each construct represents the PrP reading frame (+1); dashed bottom box represents the AltPrP reading frame. In $PrP^{(HA)}$, $PrP^{(HA)*}$, and $AltPrP^{HA}$, an HA tag (shaded box) was inserted at the C terminus of AltPrP. Parentheses surrounding the HA in the PrP reading frame represent the fact that the HA epitope sequence is encoded in the AltPrP reading frame, and is, therefore, undetected if expressed from the ATG codon at bp 1 of the PrP CDS. $PrP^{(HA)*}$ is identical to $PrP^{(HA)}$ except that the ATG codon at bp 90 has been mutated to CUG. *B*) Western blot against PrP (3F4 epitope) and AltPrP (HA epitope) in BE(2)-M17, N2a, and HEK293 cells transfected with PrP, $PrP^{(HA)}$, $PrP^{(HA)*}$, and $AltPrP^{HA}$ constructs. *C*) Cells transfected with PrP, $PrP^{(HA)}$, $PrP^{(HA)*}$, or $AltPrP^{HA}$ were immunostained with anti-PrP (3F4; green) or anti-HA (red) antibodies. Merged images also show nuclei stained with Hoechst (blue). *D*) Expression of $AltPrP^{HA}$ from bovine, sheep, and deer cDNA PrP constructs. Note the slight change in molecular weight between species.

is typically localized at the plasma membrane and the Golgi apparatus.

The ORF encoding AltPrP is also present in *PRNP* from cattle, sheep, and white-tailed deer (Fig. 1*A*). An HA tag was inserted at the C terminus of AltPrP in PrP cDNA of each of these species using the same strategy as above for human PrP. Western blot analysis of HEK293 cells transfected with cattle, sheep, and deer $PrP^{(HA)}$ cDNA demonstrated the presence of AltPrP in these 3 species, in addition to humans (Fig. 2*D*). These data clearly demonstrate that under normal conditions, the prion protein cDNA of numerous species directs the synthesis of 2 coexpressed polypeptides: PrP and the newly identified AltPrP.

### AltPrP is a mitochondrial protein

We then addressed the subcellular localization of AltPrP by immunofluorescence. The precise cytoplasmic localization of AltPrP was determined in both N2a and HEK293 cells expressing human $AltPrP^{HA}$ using antibodies against different cytoplasmic organelles. We observed a clear colocalization of $AltPrP^{HA}$ with mitochondrial Hsp70 (**Fig. 3*A***) and cytochrome *c* (data not shown). The presence of AltPrP at the mitochondria was confirmed by subcellular fractionation and differential centrifugation of HEK293 cells stably transfected with $AltPrP^{HA}$. $AltPrP^{HA}$ was detected in the crude cellular fraction, as well as in the mitochondrial fraction, but not in the cytosol (Fig. 3*B*). Bioinformatic analysis predicts a transmembrane domain within AltPrP (Fig. 3*C*). To test this hypothesis, mitochondria isolated from cells expressing $AltPrP^{HA}$ were treated with sodium carbonate. Similar to other mitochondrial membrane-integrated proteins, including VDAC and Cox IV, AltPrP was not extracted after alkali treatment. In contrast, Bax, a peripheral mitochondrial membrane protein in nonapoptotic cells (19) was completely extracted by treatment with sodium carbonate (Fig. 3*D*). This result demonstrates that AltPrP is a mitochondrial membrane-integrated protein. We next tested whether AltPrP is inserted in the mitochondrial inner or outer membrane with digitonin to selectively solubilize the outer membrane (19). In the presence of digitonin, most of the VDAC (an outer membrane component) was extracted from mitochondria, while Cox IV (localized at the inner membrane) remained associated with the mitochondria. In these conditions that allow selective extraction of the outer membrane, AltPrP did not remain attached to the mitochondria (Fig. 3*E*), indicating that AltPrP is inserted in the outer mitochondrial membrane.

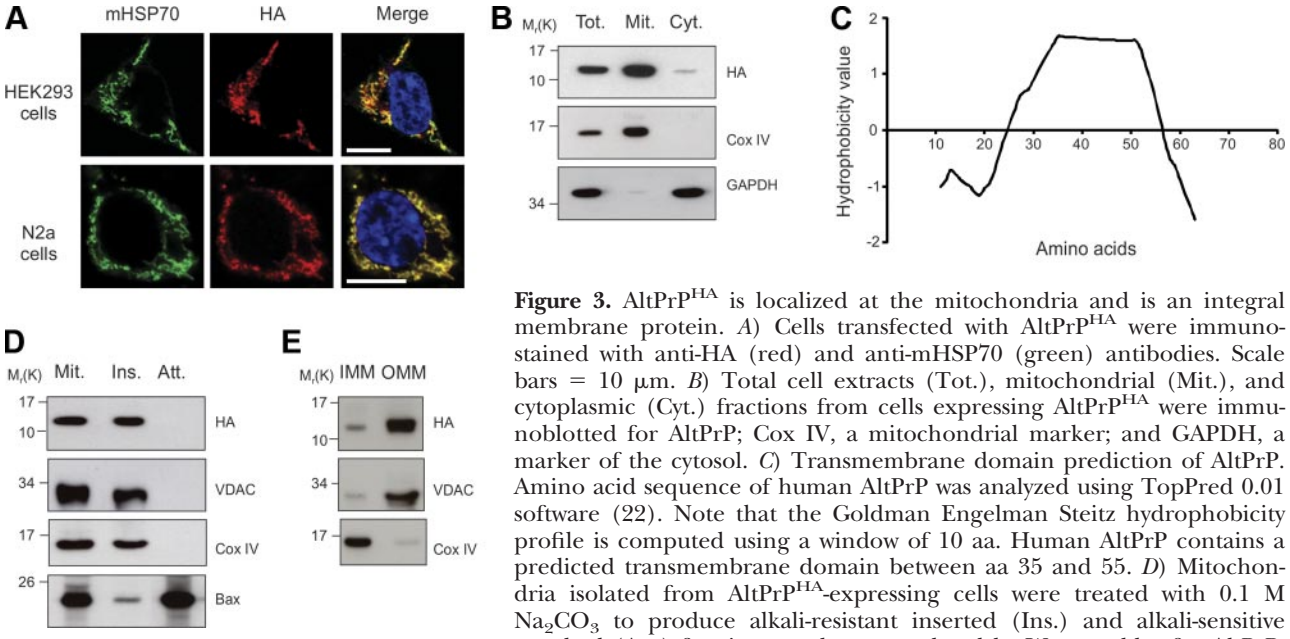


**Figure 3.** AltPrP$^{HA}$ is localized at the mitochondria and is an integral membrane protein. *A*) Cells transfected with AltPrP$^{HA}$ were immunostained with anti-HA (red) and anti-mHSP70 (green) antibodies. Scale bars = 10 μm. *B*) Total cell extracts (Tot.), mitochondrial (Mit.), and cytoplasmic (Cyt.) fractions from cells expressing AltPrP$^{HA}$ were immunoblotted for AltPrP; Cox IV, a mitochondrial marker; and GAPDH, a marker of the cytosol. *C*) Transmembrane domain prediction of AltPrP. Amino acid sequence of human AltPrP was analyzed using TopPred 0.01 software (22). Note that the Goldman Engelman Steitz hydrophobicity profile is computed using a window of 10 aa. Human AltPrP contains a predicted transmembrane domain between aa 35 and 55. *D*) Mitochondria isolated from AltPrP$^{HA}$-expressing cells were treated with 0.1 M $Na_2CO_3$ to produce alkali-resistant inserted (Ins.) and alkali-sensitive attached (Att.) fractions, and were analyzed by Western blot for AltPrP, VDAC, Cox IV, and Bax. *E*) After treatment with 0.2 mg/ml digitonin, the digitonin-sensitive outer mitochondrial membrane (OMM) and digitonin-resistant inner mitochondrial membrane (IMM) fractions were analyzed by Western blot for the presence of AltPrP$^{HA}$, VDAC, and Cox IV.

### AltPrP expression is increased by proteasome inhibition and endoplasmic reticulum (ER) stress

The observation that expression levels of AltPrP$^{HA}$ in cells transfected with PrP$^{(HA)}$ are lower than levels in cells directly transfected with AltPrP$^{HA}$ suggests that expression of AltPrP from PrP cDNA is constitutively negatively regulated (Fig. 2). Certain cellular stresses modulate the expression level of a great number of proteins (23, 24). Because AltPrP originates from *PRNP*, a locus linked to neurodegeneration, we decided to test whether AltPrP expression levels were affected by stresses implicated in neurodegenerative diseases. One such example is proteasomal dysfunction, which is often associated with neurodegeneration (25, 26). Treatment of HEK293 cells expressing PrP$^{(HA)}$ or AltPrP$^{HA}$ with the proteasome inhibitors MG132 or epoxomicin for 8 h resulted in a 2- to 3-fold increase of AltPrP$^{HA}$ levels (**Fig. 4*A*, *B***). Neither drug modified the levels of PrP$^{(HA)}$ or tubulin in the same experimental conditions, showing that this increase is specific to AltPrP. The effect of MG132 was rapid, since a significant increase of AltPrP$^{HA}$ levels was observed as soon as 2 h after the addition of the drug (Fig. 4*C*), an indication that AltPrP is a naturally labile protein with a short half-life, probably degraded in a proteasome-dependent manner. The half-life of AltPrP was determined in the presence of cycloheximide, an inhibitor of protein synthesis. In the absence of MG132, the half-life of AltPrP was estimated to be $<0.5$ h (Fig. 4*D*). The half-life of AltPrP in the presence of MG132 was $>2$ h, confirming its stabilization by proteasome inhibition.

Another stress associated with neurodegenerative disorders is ER stress (27, 28). It is known that the rate of alternative translation initiation in several mRNAs can be significantly increased during ER stress (29, 30). Since AltPrP is most likely produced by alternative translation initiation (Fig. 2*B*), we tested whether ER stress could modulate its expression. HEK293 cells expressing PrP$^{(HA)}$ or AltPrP$^{HA}$ were treated with drugs known to perturb ER homeostasis: thapsigargin (an ER-calcium ATPase inhibitor), tunicamycin (an *N*-glycosylation inhibitor), and A23187 (a calcium ionophore). The 3 drugs induced an ER stress, as monitored by interfered glycosylation of PrP (demonstrated by the disappearance of higher molecular-weight bands detected by the anti-PrP antibody), as well as increased levels of BiP/GRP78 chaperone (**Fig. 5*A***; refs 31, 32). All 3 drugs also induced a $\geq$2-fold increase in AltPrP$^{HA}$ levels in cells expressing PrP$^{(HA)}$. However, this effect was not observed in cells directly transfected with AltPrP$^{HA}$, in which AltPrP$^{HA}$ is no longer produced by alternative translation initiation at a downstream AUG codon (Fig. 5*A*, *E*). We concluded that ER stress specifically increases the synthesis of AltPrP from PrP cDNA.

We hypothesized that the target of this regulation is located upstream of the AltPrP start codon (bp 1–90 of the human PrP CDS). If so, this region is expected to exert an inhibitory effect on the translation of AltPrP from PrP cDNA that might be abolished during ER stress. To test this hypothesis, we engineered a mutant cDNA construct, termed PrP$^{\Delta 1-66(HA)}$, with a deletion of the first 66 bp of the PrP CDS. Since bp 1–66 encode the N-terminal signal peptide, PrP$^{\Delta 1-66(HA)}$ is expressed as a cytoplasmic protein and does not undergo post-translational modifications, migrating as a single band (Fig. 5*B*). AltPrP$^{HA}$ was constitutively expressed in cells transfected with PrP$^{\Delta 1-66(HA)}$ similarly to cells trans-

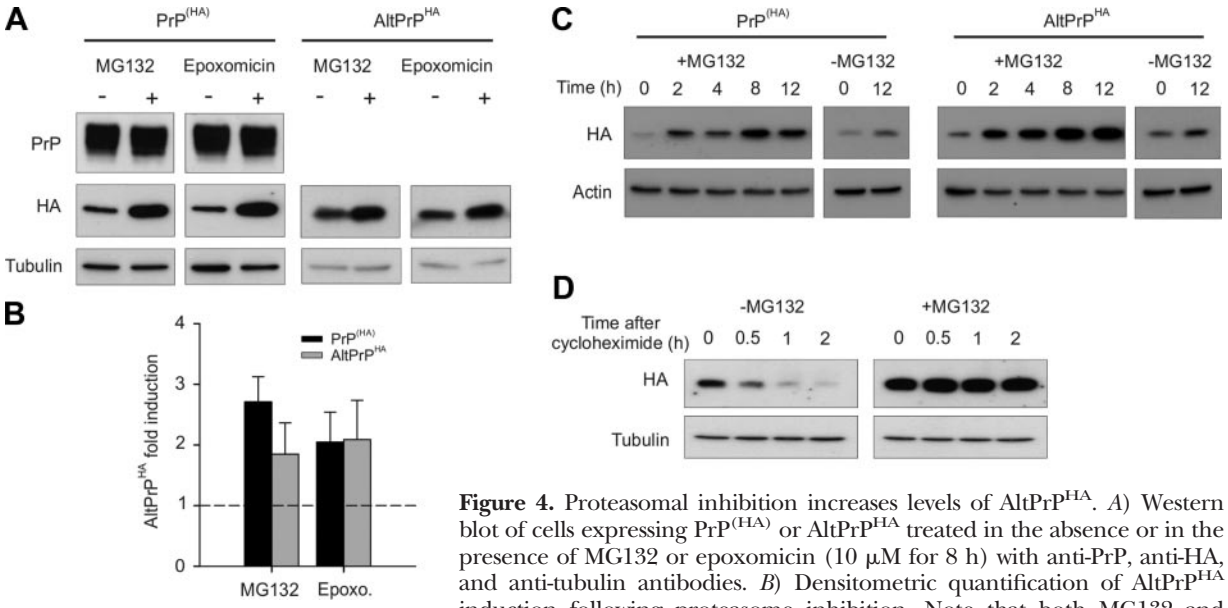


**Figure 4.** Proteasomal inhibition increases levels of AltPrP$^{HA}$. *A*) Western blot of cells expressing PrP$^{(HA)}$ or AltPrP$^{HA}$ treated in the absence or in the presence of MG132 or epoxomicin (10 μM for 8 h) with anti-PrP, anti-HA, and anti-tubulin antibodies. *B*) Densitometric quantification of AltPrP$^{HA}$ induction following proteasome inhibition. Note that both MG132 and epoxomicin (Epoxo.) induce 2- to 3-fold increase of AltPrP$^{HA}$ whether expressed from PrP$^{(HA)}$ or AltPrP$^{HA}$. No significant differences (*t* test) in induction of AltPrP$^{HA}$ could be observed between the 2 transfection conditions, regardless of the proteasome inhibitor used. Dotted line represents AltPrP$^{HA}$ levels in untreated transfected cells normalized to 1. Values are expressed as means ± SD from ≥3 independent experiments. *C*) Time course analysis of MG132-mediated proteasome inhibition on the accumulation of AltPrP in cells expressing PrP$^{(HA)}$ or AltPrP$^{HA}$. Cell extracts were immunoblotted with anti-HA and anti-actin antibodies. *D*) AltPrP$^{HA}$-expressing cells preincubated with MG132 (10 μM for 6 h) were treated with cycloheximide for various times and immunoblotted for HA and tubulin.

fected with PrP$^{(HA)}$. More important, induction of AltPrP$^{HA}$ by thapsigargin was abolished, confirming that bp 1–66 comprise at least part of the regulation domain for AltPrP expression from PrP cDNA (Fig. 5*B*, *E*).

Other stresses, including heat shock and oxidative stress, did not induce any change in the levels of AltPrP. In these experiments, cellular stress was monitored by increased levels of phospho-eIF2α (Fig. 5*C–E*). Therefore, the synthesis of AltPrP from PrP cDNA is specifically regulated by ER stress.

### Absence of AltPrP has no obvious effect on PrP biology

Because PrP and AltPrP are produced from the same gene, it was important to test for a direct functional relation between these proteins. We verified whether the absence of AltPrP would alter the biology of PrP, namely its post-translational modifications, localization, and solubility. Since AltPrP expression is abolished when the AUG codon at bp 90 is mutated (Fig. 2*B*), we compared cells expressing either PrP$^{(HA)*}$ with cells expressing PrP$^{(HA)}$ to answer these questions. As a control, we also included cells transfected with wild-type PrP to ensure that each treatment was performed properly. PNGase F is a specific *N*-glycosidase commonly used to deglycosylate PrP in order to characterize its post-translational modifications. After PNGase F treatment of lysates of HEK293 cells transfected with human PrP, PrP$^{(HA)}$, and PrP$^{(HA)*}$, only the unglycosylated band was detected by Western blot (data not shown). Thus, neither the HA tag nor the coexpression of AltPrP interfered with post-translational modifications of PrP. Similarly, AltPrP did not perturb the trafficking of PrP, since both PrP$^{(HA)}$ and PrP$^{(HA)*}$ were mainly localized at the plasma membrane and in the Golgi apparatus (Fig. 2*C*). Finally, an essential characteristic of PrP is its ability to switch from a soluble into a disease-associated insoluble isoform (20). To test whether the solubility of PrP was affected by coexpression of AltPrP, we monitored PrP, PrP$^{(HA)}$, and PrP$^{(HA)*}$ solubility in HEK 293 cells by ultracentrifugation. Each construct displayed the same solubility (data not shown). We concluded that the presence of AltPrP has no apparent effect on the biology of PrP. This result was not unexpected, since under normal conditions, PrP and AltPrP are not localized in the same cellular compartments and are therefore unlikely to interact.

### AltPrP is endogenously expressed from *PRNP*

Next, we raised and affinity-purified a polyclonal antibody against the C terminus of human AltPrP to detect wild-type AltPrP encoded by the endogenous *PRNP* gene. The anti-AltPrP antibody was validated in Western blot experiments using lysates from untransfected (mock) HEK293 cells and cells transfected with human PrP or AltPrP. A band was detected with the expected molecular weight in lysates from PrP- and AltPrP-expressing cells (**Fig. 6*A***). The addition of the immunogenic peptide to neutralize the antibodies completely prevented the detection of AltPrP. AltPrP also was not detected with preimmunized serum. To determine the expression of AltPrP from cells expressing endogenous PrP, we used human astrocytoma U-118 cells, which express high levels of PrP. A band was

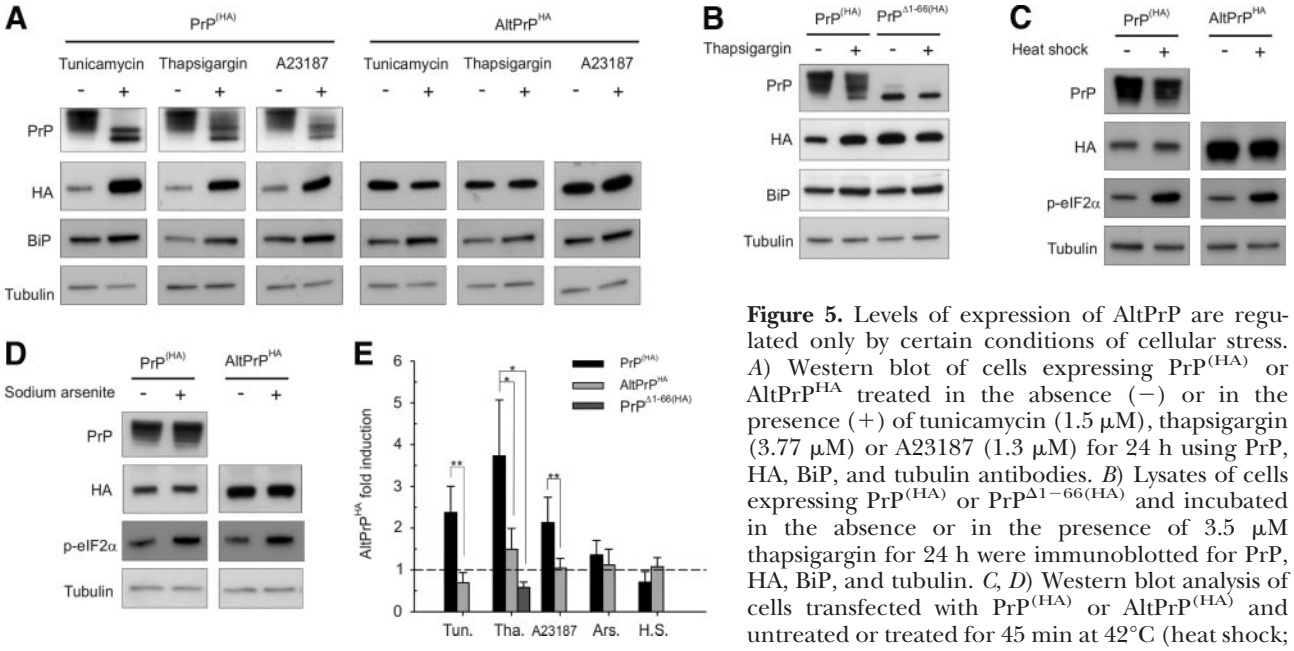


**Figure 5.** Levels of expression of AltPrP are regulated only by certain conditions of cellular stress. *A*) Western blot of cells expressing $PrP^{(HA)}$ or $AltPrP^{HA}$ treated in the absence (−) or in the presence (+) of tunicamycin (1.5 μM), thapsigargin (3.77 μM) or A23187 (1.3 μM) for 24 h using PrP, HA, BiP, and tubulin antibodies. *B*) Lysates of cells expressing $PrP^{(HA)}$ or $PrP^{\Delta 1-66(HA)}$ and incubated in the absence or in the presence of 3.5 μM thapsigargin for 24 h were immunoblotted for PrP, HA, BiP, and tubulin. *C*, *D*) Western blot analysis of cells transfected with $PrP^{(HA)}$ or $AltPrP^{(HA)}$ and untreated or treated for 45 min at 42°C (heat shock; *C*), or for 30 min with 0.5 mM sodium arsenite (*D*) with anti-PrP, anti-HA, anti-phospho-eIF2α (p-eIF2α), and anti-tubulin antibodies. Cells were allowed to recover for 8 h before harvesting. *E*) Densitometric quantification of $AltPrP^{HA}$ induction under several cellular stress conditions. Tunicamycin (Tun.), thapsigargin (Tha.), and A23187 each significantly induced $AltPrP^{HA}$ expression from $PrP^{(HA)}$ as compared to the $AltPrP^{HA}$ construct. Stimulation of AltPrP expression in the presence of thapsigargin was abolished by the deletion of bp 1–66 relative to translation initiation site of PrP ($PrP^{\Delta 1-66(HA)}$). Control experiments using cells transfected with $PrP^{(HA)}$ and treated with heat shock (H.S.) or sodium arsenite (Ars.) did not produce significant changes in $AltPrP^{HA}$ expression levels. Dotted line represents $AltPrP^{HA}$ levels in untreated transfected cells normalized to 1. Values are expressed as means ± SD from ≥3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$; *t* test.

detected at the expected size in U-118 whole-cell lysates, suggesting that endogenous AltPrP is, indeed, detected by the antibody (Fig. 6*B*). The identity of AltPrP was confirmed by treating the cells with siRNA against *PRNP*. Western blot analysis proves that AltPrP is endogenously expressed in these cells, since *PRNP* knockdown resulted in a 60% decrease of the intensity of the bands corresponding to both PrP and AltPrP (Fig. 6*B*, *C*). Furthermore, mitochondrial fractionation shows that, like $AltPrP^{HA}$, the band corresponding to AltPrP is enriched in mitochondria, and is absent in the cytosolic fraction (Fig. 6*D*). Notably, expression of endogenous AltPrP was upregulated following treatment with thapsigargin, further indicating that the level of AltPrP expression might be increased during neurodegeneration (Fig. 6*E*, *F*). Following this experiment, primary human cells that express PrP were tested for AltPrP expression using the same antibody. In addition to U-118 cells, human primary neurons, as well as human peripheral blood mononuclear cells, express AltPrP, judging by the prominent band at the same molecular weight as the band present in a control cell lysate transfected with AltPrP (Fig. 6*G*). Furthermore, a healthy human brain homogenate was tested for AltPrP expression using the same technique. As expected, the homogenate expressed a band at the same molecular weight (Fig. 6*H*).

Overall, these results clearly establish that, in different tissues, primary cells, and cell lines, two protein products, PrP and AltPrP, are endogenously expressed from two distinct overlapping reading frames present in the *PRNP* gene.

## DISCUSSION

In this study, we provide evidence that out-of-frame alternative translation initiation in the human, sheep, bovine, and deer *PRNP* gene, which encodes the cellular prion protein, results in the synthesis of a novel polypeptide that we termed AltPrP. In-frame alternative translation in PrP mRNA has been reported to produce N-terminally truncated cytoplasmic or nucleocytoplasmic forms of hamster, human, and sheep PrP, which might have a different function from that of GPI-anchored PrP (33, 34). Here, we propose that coexpression of AltPrP along with PrP represents an additional level of complexity regarding the functions attributed to the *PRNP* locus. The production of several polypeptides from this single gene, with modulation depending on cellular conditions, represents a very plausible explanation for the difficulty in assessing the physiological function of PrP, and more generally of the *PRNP* locus. Knockdown or knockout of PrP expression by targeted inactivation of PrP mRNA or the *PRNP* gene most likely results in knockdown of all PrP isoforms as well as AltPrP (Fig. 6*C*). To get insights into the molecular mechanisms of PrP function, these considerations should be taken into account in the interpretation of experimental results in the future.

The investigation of AltPrP functions is likely to shed some light on the physiological role of PrP, as controversies have emerged in the literature (35–37). We propose that part of the difficulty in assessing the physiological relevance of the *PRNP* gene could be

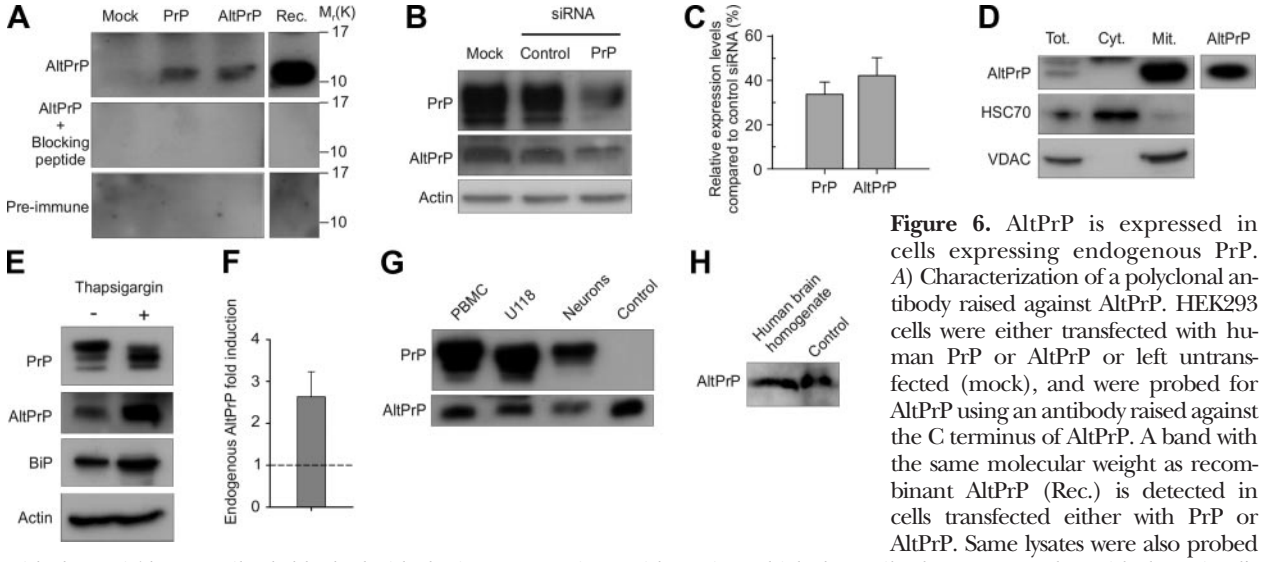


**Figure 6.** AltPrP is expressed in cells expressing endogenous PrP. *A*) Characterization of a polyclonal antibody raised against AltPrP. HEK293 cells were either transfected with human PrP or AltPrP or left untransfected (mock), and were probed for AltPrP using an antibody raised against the C terminus of AltPrP. A band with the same molecular weight as recombinant AltPrP (Rec.) is detected in cells transfected either with PrP or AltPrP. Same lysates were also probed with the anti-AltPrP antibody blocked with the immunogenic peptide against which the antibody was targeted or with the animal's preimmunized serum in order to demonstrate the specificity of the antibody. *B*) Endogenous AltPrP was detected in U-118 cells, which express high levels of PrP. An siRNA against the 3′UTR of *PRNP* (PrP) notably reduced the expression of PrP, as well as the band corresponding to AltPrP at comparable levels, whereas a control siRNA did not change PrP and AltPrP expression. *C*) Densitometric analysis of siRNA treatment revealed that U-118 cells transfected with an siRNA against PrP showed a decrease (60–70%) in expression of both PrP and AltPrP following treatment. No statistically significant difference was observed between the knockdown levels of PrP and AltPrP by paired *t* test ($n=2$). *D*) Total cell extracts (Tot.), mitochondrial (Mit.), and cytoplasmic (Cyt.) fractions from U-118 cells were immunoblotted for AltPrP; VDAC, a mitochondrial marker; and HSC70, a marker of the cytosol. Band corresponding to AltPrP was enriched in mitochondria, as expected. AltPrP represents a lysate from cells transfected with AltPrP and is used as a control. *E*) Western blot of human astrocytoma U-87 cells expressing endogenous PrP treated in the absence (−) or in the presence (+) of thapsigargin (3.77 μM) for 24 h using PrP, AltPrP, BiP, and actin antibodies. *F*) Densitometric quantification of endogenous AltPrP induction in the presence of thapsigargin. Dotted line represents AltPrP levels in untreated U-87 cells normalized to 1. Values are expressed as means ± SD from ≥4 independent experiments. *G*) Detection of endogenous AltPrP in several cell types. Human peripheral blood mononuclear cells (PBMCs, U-118 cells, and human primary neurons (neurons) were immunoblotted for both PrP and AltPrP. All cell types tested showed a prominent band at the same molecular weight as a positive control consisting of cells transfected with human AltPrP (control). *H*) Hman brain homogenate was probed for AltPrP using the anti-AltPrP antibody. A band at the same molecular weight as the one seen in cells transfected with human AltPrP was detected (control).

attributed to the coexpression of AltPrP together with PrP from the *PRNP* gene. For instance, the neuroprotective role of PrP has been largely debated and seems to be context dependent (38, 39). Because AltPrP is localized at the mitochondria (Fig. 3), an essential organelle for energy metabolism, stress response, and apoptosis (among other functions), it is tempting to think that some toxic or protective functions have been misattributed to PrP. Though these speculations remain to be proven, we have provided evidence that support this hypothesis. The increased level of AltPrP synthesis under conditions of ER stress (Fig. 5) represents a characteristic feature of various proteins implicated in the unfolded protein response that participate in stress recovery or, if cellular damage is irreversible, induction of apoptosis (30). It is to be noted that AltPrP does not seem to be proapoptotic on its own, since we were able to maintain cell lines stably overexpressing $AltPrP^{HA}$ without any noticeable change in growth rate or cellular morphology (data not shown). However, one can imagine that AltPrP might participate in either a proapoptotic or antiapoptotic response under specific cellular conditions.

An interesting consideration regarding the discovery of AltPrP is its high degree of conservation and homology in a large number of mammals (Fig. 1). Although numerous studies have demonstrated the conservation of the C-terminal portion of PrP among different species, explained by the highly structured nature of this domain, as well as its necessity for forming $PrP^{Sc}$ (40, 41), there still lacks a convincing argument explaining why the unstructured N-terminal domain of PrP is conserved as well (40, 42–44). Despite the fact that the function of AltPrP remains unknown, this newly discovered protein may be the reason for the conservation of the N-terminal portion of PrP. Selective pressure might act on the *Prnp* gene in order to conserve AltPrP rather than the unstructured fragment of PrP. At this point, this is merely a speculation, but it provides another compelling reason to continue studying both the *Prnp* gene and AltPrP.

The role of PrP in the pathogenesis of TSEs is very well established, as self-templated transition of the native isoform of PrP ($PrP^{C}$) to its misfolded, disease-linked isoform ($PrP^{Sc}$), is at the origin of the neurotoxic mechanisms implicated in these neurodegenerative disorders. As a result, the *PRNP* locus is the main genetic risk factor in these diseases (45). Since this gene also directs the synthesis of AltPrP, the possibility exists that this novel polypeptide might be linked to

TSEs. AltPrP could be functionally implicated in TSEs, although our data suggest that basic features of PrP biology, including glycosylation, localization, and solubility, do not seem to depend on coexpression of AltPrP. Our results are based on a cell culture system, a model that does not reproduce the events taking place at the level of a whole organism during disease. Moreover, it is plausible that the currently unknown function of AltPrP will have no direct effect on PrP biology. Nevertheless, the possibility that AltPrP might participate in the pathogenesis of TSEs deserves further investigation, and determining its function will likely answer this question. Regardless of whether AltPrP will be proven to have a functional relationship with the pathogenesis of TSEs, the fact that its expression is greatly enhanced following proteasome inhibition and ER stress (Figs. 4 and 5) makes it a potential biomarker for neurodegenerative disorders. Indeed, these two cellular stresses have been shown to be hallmarks not only of TSEs, but also of other neurodegenerative diseases, such as Huntington's, Parkinson's, and Alzheimer's diseases (27, 28, 46).

Other examples of polypeptides synthesized from out-of-frame downstream alternative translation initiation sites exist in the literature, although this mechanism occurs most often in viruses (47–49). Very few mammalian examples of this phenomenon have so far been described (12, 13). Klemke and colleagues (12) have discovered and characterized ALEX, a protein encoded by an alternative ORF in the XLαs/Gαs gene and that is expressed *in vivo* in both rats and humans. Cryptic T-cell epitopes represent other examples of this phenomenon in humans. However, except for their immunogenic potential, these polypeptides have not been further characterized (50). To our knowledge, AltPrP represents the second extensively characterized product of out-of-frame alternative translation initiation in a human gene. The discovery of AltPrP could not only change our understanding of the *PRNP* locus, but the fact that AltPrP is endogenously expressed supports the idea that out-of-frame alternative translation initiation in mammals is very likely to participate in protein diversity. This reveals an additional level of complexity to the proteome and to gene function, which deserves further investigation in order to determine its physiological importance. FJ

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