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Biological properties of the PrP-like Shadoo protein

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1. ABSTRACT

The *SPRN* gene encodes the Shadoo glycoprotein (Sho), a central nervous system-expressed member of the prion protein superfamily. Sho has similarity to two features within PrP^{C}'s natively unstructured N-terminus, a hydrophobic domain and tandem repeats with positively charged residues. Indeed, scrutiny of Sho's biochemical properties in uninfected cells has revealed overlaps with the properties of PrP^{C}, these including shared protein binding partners. *SPRN* is conserved in mammals, as is the prion gene *PRNP*, but in sheep *SPRN* and *PRNP* are both marked by polymorphic variation, suggestive of a shared selection pressure within these scrapie disease-prone livestock animals. In rodent models of prion disease there are reduced levels of Sho in infected tissues, defining a form of cross-regulation between full-length Sho holoprotein and PrP^{Sc}. In human prion disease an *SPRN* signal peptide polymorphism is associated with risk for sporadic Creutzfeldt-Jakob Disease (CJD), while two patients with early-onset variant CJD carried putatively inactive *SPRN* alleles. Further investigation of Sho as a novel tracer or modifier for the accumulation of pathologic forms of PrP may prove advantageous.

2. INTRODUCTION

The world was introduced to the cellular prion protein PrP^{C} and its transformation to the disease associated prion protein PrP^{Sc} over twenty-five years ago. While thousands of publications have since accrued on the properties of these proteins, deciphering physiological function and how this process or processes are nudged towards pathophysiological states have proven challenging. This situation may reflect, in greater part, the observation that PrP^{C} did not belong to a pre-existing protein family of defined activity. The Doppel protein (Dpl) was identified in 1999 (1) but, since serving a crucial role in cells of the male reproductive tract, did not point to functions that relate to the predominant sites of expression of PrP^{C} such as the central nervous system (CNS), heart and lung. It is with this backdrop that a new gene denoted *SPRN* has emerged onto the scene. *SPRN* was identified in 2003 and codes for the protein Shadoo (Sho). Sho has been identified as a new CNS-expressed member of the prion protein superfamily. This protein, the focus of this review, comprises the third PrP^{C}-like protein identified thus far in higher mammals. While PrP and the testis-expressed Dpl protein lie immediately adjacent on chromosome 2, Sho

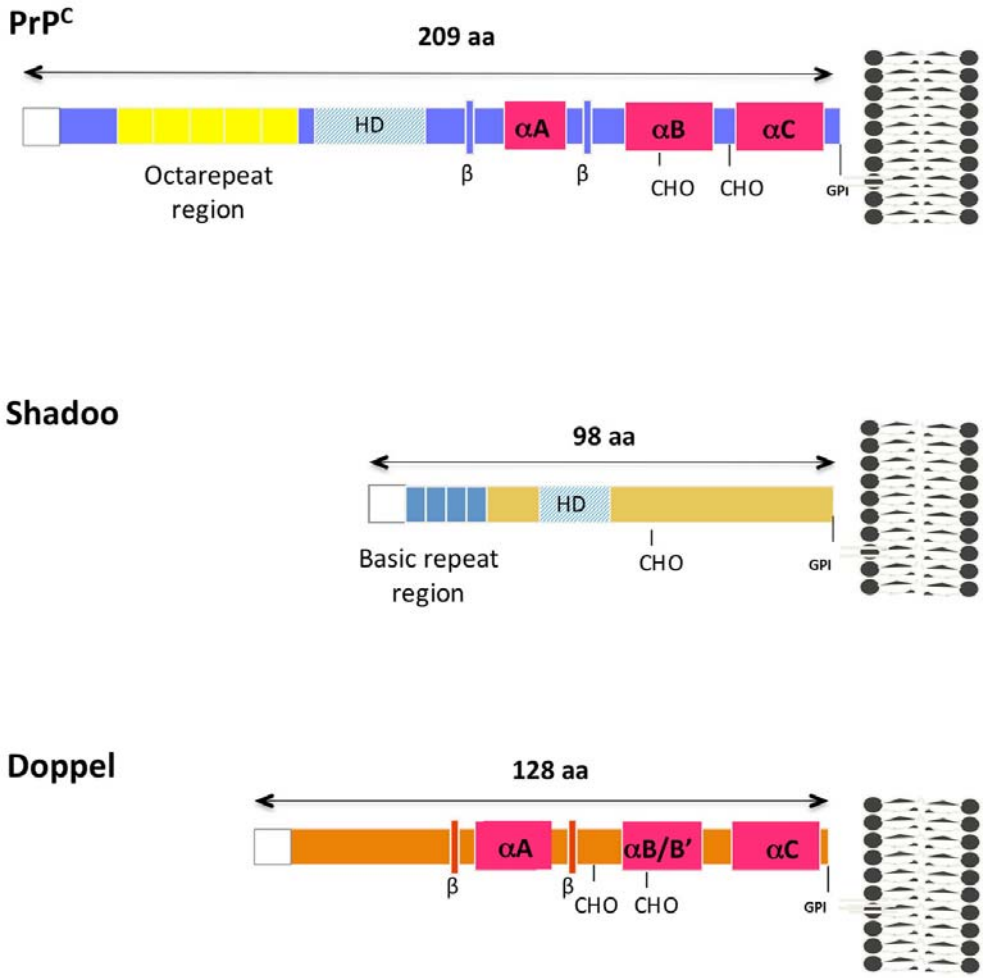

Figure 1. Domain structures of PrP, Doppel and Shadoo proteins. The schematic in PrP includes octarepeats, hydrophobic region ("HD" in diagonal stripes; α, alpha helices; ß-strands ("ß") and *N*-glycosylation sites (CHO). In doppel helix B is not contiguous and has a kink in the middle hence the two parts are labeled B and B'. For Sho the arginine-rich repeats (blue rectangles), HD and the single *N*-glycosylation site are shown. Regions and attachment positions are approximately to scale. Dashed arrow represent the size of the full-length proteins in amino acids (aa). Open boxes represent a charged region present at the mature amino terminus of each protein.

resides on chromosome 7 of mice (2, 3). As discussed in greater depth below, while Dpl has secondary and tertiary structure similarity to PrP's alpha-helical domain, Sho bears similarity to two features within PrP's natively unstructured N-terminus: these are the hydrophobic domain (HD, referred to by some researchers as the central region), and a series of tandem repeats with positively charged residues (Figure 1).

3. *SPRN* GENE EXPRESSION

3.1. Expression of *SPRN* mRNA

3.1.1. Mice

As in the case of PrP, the entire open reading frame (ORF) of Sho is encoded within one coding exon. Analysis by RT-PCR has shown that *Sprn* mRNA is mostly expressed in brain (3). *In situ* hybridization in mouse shows that *Sprn* is expressed in the adult CNS, with a strong expression in the cell body of pyramidal cells and Purkinje cells in the hippocampus and cerebellum, respectively (4); findings were extended by immuno-histochemistry with polyclonal antisera directed against Sho peptide sequences (see below). However, Sho expression is also present in other area of the brain including the cerebral cortex, thalamus, and the medulla (4). Bioinformatic analyses of expressed sequence tags and SAGE libraries have lead to a tempering of the original contention that this gene is mainly expressed in neurons (5), suggesting systemic expression in a number of tissues including colon, oesophagus, T-cells, muscle and testis, amongst others. Consolidation of these analyses by analysis of protein expression is an important next step.

3.1.2. Sheep

Within the CNS, interrelationships between *PRNP* and *SPRN* expression have been examined by

quantitative RT-PCR (6), using tissues from both infected and healthy (normal brain homogenate inoculated) animals. *PRNP* and *SPRN* were found to be expressed "in step", with the highest expression in the frontal cortex, followed by the cerebellum, the obex and finally the thalamus. However, another study reported no significant difference in relative *SPRN* mRNA expression between sheep cerebrum and cerebellum in healthy animals, again as assessed by RT-PCR, but did report a positive correlation between relative *PRNP* and *SPRN* expression in these two brain regions (7). While the absolute expression levels were not reported by Lampo *et al.* (7), in the Gossner *et al.* studies *SPRN* was expressed 100 to 1000 fold less than *PRNP*, depending of neuroanatomical region examined (6). These numbers are at apparent variance with the situation in the rodent CNS, where Sho protein is readily detected by the same standard western blotting procedures used for PrP^C (i.e. without recourse to the use of ultrasensitive reagents): (4). While species-specific differences could of course apply, since the RT-PCR data from sheep were derived from the use of only a single primer pair (in both studies), and since the 2.5 kb sheep *SPRN* mRNA has a high G+C content (overall figure for the mRNA is 69% GC, and 78% GC for the ORF region alone) that may confound PCR amplification steps, it may be useful to verify the inference of low *SPRN* mRNA abundance relative to *PRNP* by other types of procedures (e.g., quantitative Northern blot analysis, RNA protection assay). Also these findings are at apparent variance with immunohistochemical studies (below, (8)).

Lampo and co-workers have identified genetic variations in the putative promoter region of the sheep *SPRN* gene (8). Five nucleotide substitutions have been found, as well as one insertion and one deletion. Three bases changes and one deletion were defined within the gene intron and eight nucleotide changes have been found in the 3'UTR. The ultimate significance of these changes remains to be established. Thus far, four natural promoter region polymorphisms have been assessed by linking them to a promoter-deficient firefly luciferase reporter plasmid and performing transfections into human SH-SY5Y neuroblastoma cells. In these experiments reporter output was expressed as a ratio to a second *Renilla* luciferase reporter. While the promoter variants do indeed have varying activities, the wild type (wt) promoter region gave a reporter output only 2-fold higher than the promoter-deficient reporter and reporter levels were about one thousandth of those of a co-transfected *Renilla* luciferase reporter control. These data question whether alternative cellular paradigms may be better suited for the assessment of *SPRN* promoter activity. In other analyses described below, relationships of 5' region polymorphisms to scrapie susceptibility were not noted.

3.1.3. Cattle

Bovine *SPRN* expression was found predominantly in brain but also in testis and possibly lung by northern blot analysis, whereas no expression was detected in muscle, heart, kidney, and liver (9).

3.1.4. Humans

Analysis of public domain EST and SAGE databases (http://www.genecards.org/, http://cgap.nci.nih.gov , http://www.cleanex.isb-sib.ch/, http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly) indicates that human *SPRN* is transcribed in the adult hippocampus, cerebellum, eye and spinal cord. Systemically, representation in EST and SAGE databases is consistent with mRNA expression in kidney, liver, lung, pancreas and prostate.

3.2. Synthesis, maturation and structure of the Shadoo protein

3.2.1. Studies *in silico*

Based upon bioinformatic analysis, Sho was predicted to be synthesized in the secretory pathway with biochemical features of the protein including an N-terminal signal peptide, a series of arginine containing tetrarepeats, a central HD with strong homology to PrP, a less conserved C-terminal domain with one consensus N-linked glycosylation site, and lastly, a signal peptide for attachment of a GPI anchor (3). The last of these features were first demonstrated *in vitro* for one isoform of zebrafish Shadoo (SHO2) by transfection of a FLAG-tagged allele into N2a cells followed by PIPLC digestion (10). Most of the other predictions have now been validated with regards to mammalian Sho, as discussed below.

In terms of structural features deduced from primary structure, application of contemporary structural prediction algorithms (some capable of accurately predicting the three helices in the NMR structure of the globular domain of PrP, in contradistinction to early studies which arrived at a 4-helix model (11)) has revealed little of interest, other than the puzzling observation that the HD region is predicted as a helix (12). The sequence of PrP analyzed by the same means yields the same result, with the added corollary that transmembrane (TM) forms of PrP can be identified in cell-free systems and in cell lysates in the instance of the A117V Gerstmann–Straussler–Scheinker (GSS) mutation (13, 14). The significance of this finding for Sho remains to be explored but in this context it is noteworthy that the structure of the wt HD region (AAAG-AAAG-AAAG-VAAG-LAAG) is arranged in tandem repeats R1-R5 that can be seen to encompass GxxxG motifs. In other proteins GxxxG motifs are associated with lateral association of TM helices (15-17) and may also play a role in packing of amyloid fibrils derived from the TM helices of a model substrate glycophorin A (18). Closer to the realm of *in vivo* pathogenesis, the GxxxG motif is found in the coronavirus spike protein of Severe Acute Respiratory Syndrome and the precursor membrane protein of Japanese encephalitis virus (JEV) flavivirus (19, 20). Furthermore, GxxxG motifs have been investigated in the context of CNS protein misfolding disorders, in the amyloid precursor protein TM region giving rise to amyloid beta (Abeta) peptide, as well as in the C-terminal part of PrP^C's HD at residues 119-130 (21-23).

Since the Cu or Zn-binding octarepeats found in PrP have been subject to intense scrutiny, the function of the most closely analogous structure in Sho – the arginine repeats – rises to the fore. In this regard, bioinformatic

analysis has shown that these repeats include an "RGG-box" motif. This is defined as a sequence of closely spaced Arg-Gly-Gly (RGG) repeats interspersed with other, often aromatic, amino acids (24). Previous RGG box proteins are proposed to be RNA-binding proteins involved in various aspects of RNA processing and mediating interactions with other proteins. What this might mean *in vivo* is moot as, by all measures, Sho is made as a cell-surface protein, that, even if released and endocytosed, would have to cross the lipid bilayer of a vesicle perimeter before it could gain access to the cytoplasm and RNA binding partners. This would simply suggest that the important feature of the tetrarepeats is their net positive charge, rather than any cryptic ability related to nucleic acids. This viewpoint is simple and economical but, before discounting any participation of Sho in nucleic acid metabolism, it is worth noting that there is an extensive strand in the literature that PrP^C can bind to nucleic acids (25, 26) and has interactor proteins defined genetically that do *not* occur in vesicular compartments (27). However, in terms of protein architecture PrP has two positively charged patches sometimes referred NLS1 and NLS2 that occur at the N-terminus and just prior to the HD (28) and are therefore not analogous in this sense to the Sho tetrarepeats.

The issue of sub-cellular localization also colours the validity of other Sho motifs identified *in silico*. Thus one arginine (Arg) methylation site and 3 potential protein kinase C phosphorylation sites have also been identified (24). The putative Arg methylation site in Sho is completely conserved in all species from fish to humans. Arg methylation is a common post-translational modification in RGG-box domains (29) carried out by a family of enzymes called protein-arginine N-methyltransferases (PRMTs). This modification may affect protein-protein interactions (30) and modulate nucleic acid binding (31, 32). As a corollary of the second observation, PRMTs have protein substrates that are more typically found in nucleocytoplasmic compartments.

3.2.2. Shadoo *in vitro*

Recombinant mouse Sho appears to be natively unstructured, returning a random-coil signature by circular dichroism spectroscopy (4, 12) and attempts to discern secondary structure features within NMR spectra have as yet been unsuccessful (33). These data suggest that Sho may acquire structure upon meeting binding partners, as it has been reported for natively disordered (unfolded) proteins. Provocatively, native disorder is a frequent feature of RNA and protein chaperones, with the former echoing the theme of RGG repeats and the latter suggesting a link to prion misfolding issues (34). Other studies into the issue of Sho's secondary structure have taken a pathological, rather than a physiological point of departure. Thus, based upon the similarity in domain structure between wt Sho and pathogenic stop codon variants of PrP that lack a complete globular domain and generate parenchymal amyloid deposits, Daude *et al.* tested the hypothesis that wt Sho can convert to an amyloid-like form. Indeed, merely by extended incubation at neutral pH, recombinant mouse and sheep Sho were able to form amyloid-like assemblies, as ascertained by electron microscopy, thioflavin and Congo Red binding (12). The extent to which this type of event might occur *in vivo*, for example to make a putative "physiological amyloid" form of wt Sho, remains to be established as does the alternative possibility that these assemblies would have toxic or infectious properties.

Going beyond recombinant proteins made in *E. coli*, our own studies have demonstrated that the murine *Sprn* locus encodes an authentic glycoprotein (4) and have confirmed CNS expression by *in situ* hybridization, western blot analysis and immunohistochemistry in the adult mouse (4). Shadoo protein is differentially expressed during embryogenesis, with mRNA expression starting at day 7-8 and increasing (35), and with protein detectable at day 15 (4). Mature Sho is N-glycosylated, and antennary carbohydrate structures glycosylation can be removed by PNGase F digestion or by mutation of Asn107 to Gln ((4); unpublished data of Joel Watts). Shadoo can be removed from the surface of cells by treatment with a GPI-specific phospholipase PIPLC digestion (4, 12).

PrP is endoproteolysed close to the centre of the molecule to create a metabolically stable carboxy-terminal fragment denoted "C1" and there has been growing interest in C1 (and a cognate N-terminal fragment "N1") as the PrP fragments generated by endoproteolysis may have important biological activities (36-38). Consequently, it bears mention that Sho resembles PrP^C in that a C-terminal fragment of Sho is readily detected in brain samples and in the cell-associated fraction from lysed cultured cells (4, 12). The protease responsible for generating PrP C1 is debated (38, 39) precluding - at least for the time being - an assessment of whether the apparent similarity between PrP and Sho metabolic fragments extends to processing by the same endoprotease. Nonetheless, it will be of interest to determine if Sho N1/C1 metabolites share biological properties with those emerging for PrP N1/C1.

Besides endoproteolysis in the central region, PrP^C (and PrP^{Sc}) is endoproteolysed adjacent to the GPI attachment site to create a virtually full-length molecule, this species being found both in brain homogenates and in conditioned medium from cultured cells (40, 41). Recent work has implicated ADAM proteases in this "shedding" event (42). A similar situation pertains to Sho, with a glycosylated form of the protein being easily detectable within the conditioned medium of Sho expressing cells (12), again raising the issue of protease processing partners shared between PrP^C and Sho.

4. SHADOO AND CELLULAR PHYSIOLOGY

4.1. Pathways and binding partners

These are early days in discerning the physiology of the wt Sho protein and at this stage our questions can be reduced to a number of simple preliminary issues that need to be established beyond cavil. One issue has already been mentioned, namely the sub-cellular localization, while the second issue is the nature of Sho's interacting partners.

PrP^C is an instructive point of reference when considering Sho and, as noted above, the issue of PrP^C as a

classical secretory pathway protein equipped with N- and C-terminal signal peptides for translocon insertion and GPI-attachment has been enlightened (or muddied, depending upon perspective) by the notion of cytoplasmic PrP. While this topic has been reviewed extensively (43-49), the notion that some PrP can "escape" into the cytoplasm and thence to the nucleus cannot be excluded. This debate may equally apply to ascertaining the life history of a typical Sho protein molecule. Based purely upon consideration of steady-state levels in tissue lysates and extracellular fluid, most of Sho bears the hallmarks of synthesis and release in the secretory pathway (i.e., N-glycosylation, GPI-attachment, shedding into conditioned medium) and the protein is prominently labelled at the cell-surface in cytological analyses. However, as per PrP (46, 50, 51), immunoelectron microscopy with monoclonal antibodies may be necessary to ascertain whether a subset of Sho embarks on a more complicated journey to the other side of a lipid bilayer, i.e., to a cytoplasmic environment.

'Guilt-by-association' can be used to infer protein activity and in the studies of Watts *et al*, parallel interactomes were assembled to discern the protein partners of prion proteins expressed in the same context (52). Besides mouse Sho, the analyses extended to a FLAG-tagged version of PrP^{C}. Dpl protein, although not normally expressed in the CNS or neural-like cells, was included as a third "bait" protein as the biological interactions of PrP^{C} and ectopically expressed Dpl have been of considerable interest (see below). Interactors were retrieved by a process involving crosslinking of live N2a cells with formaldehyde, detergent lysis, affinity chromatography with alpha-FLAG antibodies, denaturation, trypsin digestion and labelling of peptide fragments with isobaric tags. Following MS/MS analysis some major Sho interactors identified in this paradigm are listed in Table 1. This list is noteworthy for the number of partner proteins also appearing in the PrP and Dpl interactomes. Candidates of note are lactate dehydrogenase (Ldha), protein disulfide isomerases Pdia3, Pdia6 and P4hb, galectin-1, NCAM, neuromodulin (Gap43), laminin receptor (40S ribosomal protein SA) and its ribosomal protein partner Rps21, heat shock proteins Hspa5 and Hsp90ab1, histocompatibility antigens H2-K1 and H2-D1, the secreted differentiation factor Fam3c, peptidyl prolyl isomerase B, calnexin, calreticulin, 14-3-3 zeta/delta and basigin, as these proteins were identified with the highest sequence coverage (25–75%) and/or enrichment versus the 114 Da internal control. As can be appreciated, several of these proteins are associated with a cytoplasmic localization, an observation that harkens back to issue of the predominant subcellular locale for Sho. Others proteins are in the secretory pathway or organelles associated with recycling, in accord with demonstrated properties of Sho.

A finding also arising out of these studies addresses the extent to which PrP, Sho and Dpl might have physical interactions between themselves, an issue pertinent to the genetic and biological studies of synergism to be described below. In the formaldehyde crosslinking studies of Watts *et al.* it was observed that endogenous PrP was enriched in FLAG-Sho pull-downs from Sho-transfected cells, and that endogenous Sho was apparently enriched in FLAG-PrP and FLAG-Dpl pull-downs. Other experimental settings have been used to gain a purchase on the exact same issue; it has been shown by a yeast two-hybrid system, co-immunoprecipitation and surface plasmon resonance that PrP^{C} and Sho interact together. By deletion analysis of yeast bait and prey constructs the interaction site of PrP^{C} is in the interval of residues 108-126 and for Sho in the interval of residues 61-77 (53). Although not mentioned by the authors it is difficult to ignore that these deletion intervals span the HD's of the respective proteins. For PrP the HD serves as putative function as a binding site for other proteins such as Stress-inducible protein 1 (54), modulates the formation of transmembrane PrP topologic isoforms (13) and contributes to basolateral sorting in polarized cells (55). Furthermore, the AGAAAAGA palindrome in the N-terminal part of the PrP HD may play a part in the disease specific PrP^{Sc}-PrP^{C} interaction (56) as peptides containing this sequence are neurotoxic (57) and the A117V variant of the palindrome AGAAVAGA is linked to GSS syndrome, a genetic form of human prion disease (58).

Binding partners other than proteins have already come up in the context of PrP in the form of transition metals. Sho itself has no histidine residues that can play a crucial role in binding Cu and Zn, at least as in the case of PrP and Dpl (59) so this particular avenue is closed. However, the more general consideration that other macromolecular species or metabolites - perhaps negatively charged species such as sulphated glycosaminoglycans - might interact productively with Sho remains to be explored (26).

4.2. Neuroanatomical expression and function

In terms of neuroanatomy, mouse *Sprn* transcripts detected by *in situ* hybridization are quite widely expressed, equalling *Prnp* in the number of positive anatomical regions, and on occasion yielding similar levels of signal intensity (albeit assessed here in the context of a semi-quantitative technique). Thus, as ascertained in the Allen Brain Atlas data consortium, the areas with patterns of expression lower than PrP include cerebellum, hypothalamus, midbrain, medulla, olfactory bulb, pallidum and the pons. Some of these findings have been cross-correlated with western blot and immunohistochemical studies performed with polyclonal antisera such as a pattern of low expression in the cerebellum, perhaps restricted to Purkinje cells (4). These immunohistochemical studies of mice have recently been augmented by analyses performed with a polyclonal alpha-Sho antibody on sheep tissues, again defining Purkinje cell expression as well as signals in the hippocampus and pituitary (60). At the level of subcellular localization some but not all neurons have a pattern of reciprocal staining with anti-PrP and anti-Sho antibodies. This effect is apparent in hippocampal CA1 neurons where Sho signal is more somatodendritic and PrP signal is more in axonal projections, and in cerebellar Purkinje cells where somatodendritic staining is seen for Sho but is absent for PrP. Neurons of the cerebral cortex comprise an instance where Sho and PrP expression may overlap (4).

Table 1. Prion protein family interactome in mouse neuroblastoma cells

IPI accession #	Symbol	Identified proteins [1]	Pept [2]	Unique [3]	% Cov.[4]	114 Control[5]	115 Dpl	116 PrP	117 Sho
IPI:IPI00131622.1	Prnd	prion protein dublet (doppel, Dpl)	8	13	39.1	2.0	92.5	3.0	2.5
IPI:IPI00120793.1	Prnp	prion protein (PrP)	5	5	26.8	4.0	20.5	65.1	10.5
IPI:IPI00226455.1	Sprn	shadow of prion protein (shadoo, Sho)	3	6	69.4	2.9	11.4	20.5	65.2
IPI:IPI00751369.1	Ldha	lactate dehydrogenase A	15	19	67.3	6.0	26.3	44.0	23.7
IPI:IPI00230108.6	Pdia3	protein disulfide isomerase associated 3	14	15	61.2	6.0	46.7	18.8	28.4
IPI:IPI00229517.5	Lgals1	galectin-1 (lectin, galactose binding, soluble 1)	5	7	57.8	5.3	32.8	37.4	24.4
IPI:IPI00122971.1	Ncam1	neural cell adhesion molecule 1 (NCAM)	23	34	54.3	3.4	58.6	16.6	21.3
IPI:IPI00128973.1	Gap43	growth associated protein 43 (neuromodulin)	2	2	46.7	7.0	30.5	24.5	38.0
IPI:IPI00850840.1	Rpsa	ribosomal protein SA (laminin receptor precursor, LRP)	7	8	43.4	11.8	33.8	26.9	27.5
IPI:IPI00319992.1	Hspa5	heat shock protein 5	15	20	42.7	7.4	32.9	22.5	37.3
IPI:IPI00515173.1	H2-K1	histocompatibility 2, K1, K region	6	6	40.6	3.6	60.9	17.0	18.5
IPI:IPI00762203.2	Ftl1	ferritin light chain 1	3	4	39.9	10.4	36.9	29.4	23.3
IPI:IPI00454042.2	Fam3c	family with sequence similarity 3, member C	4	4	35.7	4.7	64.4	12.5	18.5
IPI:IPI00133522.1	P4hb	protein disulfide-isomerase (prolyl 4-hydroxylase beta)	8	9	35.4	5.7	22.5	28.4	43.4
IPI:IPI00135686.2	Ppib	peptidylprolyl isomerase B	4	4	34.3	10.3	30.3	24.2	35.1
IPI:IPI00132950.1	Rps21	ribosomal protein S21	2	2	31.3	8.6	18.7	37.9	34.9
IPI:IPI00110805.1	H2-D1	histocompatibility 2, D region locus 1	5	5	29.3	3.4	56.7	19.9	20.0
IPI:IPI00119618.1	Canx	calnexin precursor	8	8	28.8	7.1	40.7	18.8	33.4
IPI:IPI00554929.2	Hsp90ab1	heat shock protein 90 alpha (cytosolic), class B member 1	10	11	28.6	11.5	15.6	33.4	39.6
IPI:IPI00123639.1	Calr	calreticulin	3	3	27.9	7.2	32.5	24.7	35.6
IPI:IPI00116498.1	Ywhaz	14-3-3 protein zeta	4	5	27.8	10.0	21.6	35.4	33.0
IPI:IPI00462199.1	Bsg	basigin	5	5	27.3	4.1	32.0	23.2	40.7
IPI:IPI00230682.6	Ywhab	14-3-3 protein beta	2	2	27.0	10.5	26.3	23.4	39.8
IPI:IPI00857709.1	Tmed2	transmembrane emp24 domain trafficking protein 2	2	2	26.9	6.0	33.6	26.1	34.4
IPI:IPI00854971.1	Pdia6	protein disulfide isomerase associated 6	2	2	25.6	7.8	29.5	27.9	34.9

Adapate with permission from 52. [1]Candidate interactors are listed in order, with the position of a given protein in the table reflecting the percentage of primary structure corresponding to the combined unique CID spectra. In instances where a subset of CID spectra were matched to more than one isoform or member of a protein family, only the highest scoring entry was selected unless an independent identification was supported by at least two unique CID spectra. Proteins were sorted into specific versus unspecific binder categories based on their iTRAQ distribution, i.e. proteins were considered unspecific interactors if their derived CID spectra revealed iTRAQ114 signature mass peak signal intensities which exceeded 15% of combined and normalized (100%) intensities for iTRAQ114-117 mass peaks. [2]Only CID spectra underlying different peptides were considered, i.e. if the same peptide was identified with different charge states or modifications it counted as one hit. [3]Total number of unique CID spectra. Please note that the same peptide was only counted more than once if it was identified with different charge states or modifications. [4]Percent sequence coverage based on the presence of peptides for which no higher ranked assignment to other proteins could be made. [5]For the calculation of iTRAQ values the intensity of individual peptide associated iTRAQ signature peaks was normalized to combine to 100% per peptide and subsequently averaged.

In terms of normal function and appropriate assays, settling the situation for Sho's forbear PrP has proven a lengthy and contentious issue, with the conclusion that the ultimate phenotypic outputs from PrP may be subtle, at least in laboratory rodents assessed at the beginning of adulthood. This area has been reviewed extensively (61, 62), with putative activities falling in the areas of neuroprotection and reduction of oxidative damage, long-term maintenance of white matter, transition metal sensing and scavenging, toxic mediator of Alzheimer disease Abeta oligomers, modulation of apoptotic signalling and progression to neoplasia, modulation of synaptic activity and a role in cell adhesion. The diversity in this list of activities rather begs the question is there anything this small GPI-anchored protein does *not* do, and injects a bracing note of caution for future studies asking the same question of Sho. The biochemical signatures of the Sho protein are limited at this stage, perhaps not surprisingly given its recent arrival on the scene, suggesting an approach through other disciplines. Genetics inevitably comes to mind and in this context Sho can be seen to align with a theory concerning the lack of overt phenotypes in PrP-deficient mice, the neurotoxic attributes of Dpl and internally deleted forms of PrP (*delta-PrP*), and the action of a notional PrP-like molecule called pi. In short, PrP is suggested to interact with a partner protein with two docking sites and failure to engage this partner at both sites results in aberrant signalling and neuronal death. In PrP-deficient mice correct signalling is taken over by the PrP-like pi molecule such that phenotypic deficits are not scored. In mice expressing *delta-PrP* the neurotoxic consequences are registered most prominently in the cerebellum, and can be offset by co-expression of wt PrP. Since Sho a) has PrP-like biochemical attributes and is indeed the only CNS-expressed paralog, b) shares a number of binding partners with PrP^C and Dpl (52), c) abrogates toxic properties of *delta-PrP* and Dpl in transfected granule cell neurons (4) and d) is normally expressed at low levels in cerebellar granule cells (4) (and hence might be unable to offset the toxic property of *delta-PrP* expressed in these cells) this protein is a plausible candidate for pi. Indeed, it may be the only candidate as there are no other CNS-expressed PrP-like proteins in mammalian databases and since ZIPs 5, 6 and 10 metal ion transporters discussed as putative progenitors of PrP and with a PrP-like domain are not characterized by a GPI anchor (and thus may not inhabit the same "raft" membrane domains populated by PrP^C) (63). Hence putting Sho through its paces in this neurodegenerative paradigm defined by a gain-of-pathological function form of PrP^C might be an informative first step.

Other approaches to Sho's function may turn upon loss-of-function paradigms. For example, in a context that is not "challenged" by a specialized toxic form of PrP such as *delta-PrP*, there are divergent theories as the phenotype of mice lacking the signalling engendered by normal PrP^C and 'backed-up' by pi (i.e. in mice lacking

both PrP and pi). One viewpoint suggests a deleterious phenotype at the level of the organism, and, if one assumes that Sho is pi, a phenotype that could surface in the behaviour of $Prnp^{00}$ + $Sprn^{00}$ mice held under normal caging conditions. An experiment that lies close to this perspective is the finding that $Prnp^{00}$ embryos 'knocked-down' for *Sprn* expression by administration of virally encoded anti-*Sprn* shRNAs do not all develop normally, with some embryos examined prior to E11 exhibiting an incomplete closure of the neural tube (35). Another viewpoint pegs PrP^{C} in a protective system that only comes into play under conditions of neurologic stress or challenge (e.g. exposure to hypoxic episodes) (64). Since PrP^{C}'s protective functions map to N-terminal sequences and since Sho has homology to the N-terminus of PrP^{C}, it is not an enormous stretch to attribute analogous properties to Sho. On a practical level defining the CNS expression profile of Sho with monoclonal antibodies and determining the starting and "stressed" phenotypes of $Sprn^{00}$, or even $Prnp^{00}$ + $Sprn^{00}$ adult mice should be of great utility. Furthermore, given that a) Sho is implicated in an orally-transmitted prion disease with a systemic phase (dietary exposure to BSE prions to give variant Creutzfeldt-Jakob Disease (vCJD): see below) and b) that recent EST and SAGE indicate *SPRN* mRNA expression in peripheral tissues, documenting systemic expression of Sho and its N1 and C1 fragments could provide a framework for new hypotheses of physiological action.

5. SHADOO AND THE PATHOGENESIS OF PRION DISEASE

5.1. Down-regulation of Shadoo protein in prion-infected mice

We have reported previously that expression of mature Sho protein is diminished after infection by the RML isolate of scrapie prions, as observed in two independent cohorts of animals (4). Similar changes were not apparent in a partial model of Alzheimer's disease (AD) based upon expression of a double mutant form of human amyloid precursor protein (65). In both prion and AD animal models of neurodegenerative disease the analyses were performed on brain homogenates from animals in the clinical phase; in terms of effect size, an ~90% diminution was seen for Sho versus a ~40% diminution for another GPI-anchored protein, Thy-1. Subsequent analyses have now demonstrated that this down-regulation phenomenon can be observed prior to disease onset (unpublished data of Joel Watts, George Carlson and DW). Aspects of the phenomenon have been replicated in a second series of studies. CD1 mice infected with rodent-adapted sporadic CJD (sCJD), GSS, vCJD, kuru, and sheep scrapie, and *tga*20 mice overexpressing $Prnp^{a}$ infected with kuru were employed for this purpose (66). Incubation times to disease onset of these different infectious agents ranged from 154-163 to 403-494 days with infectious samples from clinical phase of disease being processed for Sho immunoblot, and with baseline levels of Sho defined by parallel analysis of brain homogenates from uninfected mice. While age-related changes in uninfected mice were not described, the degree of reduction of Sho protein varied with the agent. In general, samples with high levels of protease-resistant PrP had low levels of Sho, although two CJD isolates with similar (low) levels of protease-resistant PrP differed 2-fold in their reduction in Sho protein. In the same analyses *tga*20 mice infected with prions originating from a P102L GSS case (somewhat confusingly referred to as "FU-CJD") produce a high level of Sho compared to CD-1 non-transgenic mice, but had lower levels of protease-resistant PrP than CD-1 mice infected with the same inoculum. In sum, these data hint at intriguing relationships between the levels of PrP^{C}, PrP^{Sc} and Sho, relationships almost suggestive of a titration of the respective protein levels.

The mechanistic basis of the Sho down-regulation effect is poorly understood. In prion disease the key pathogenic events are at the level of protein misfolding and altered protein trafficking so a perturbation at the level of *SPRN* mRNA abundance seems unlikely. Such a change, if it occurred, would be expected to involve a reduction in *SPRN* mRNA abundance however Lloyd *et al.* reported increased levels of mRNA level in RML-infected mice versus normal controls in three inbred strains (C57, NZW and RIIIS) (67). Whether the age of the control mice is an important variable for these analyses is unknown. Based upon the importance of proteostasis in neurodegenerative disease, examination of protein translation, turnover and subcellular trafficking are likely to yield insights into this process.

5.2. Shadoo and scrapie disease of sheep

Given that ovine *PRNP* variants have been associated with differential susceptibility to scrapie, it is not surprising that attempts have been made to link *SPRN* polymorphisms to aspects of the same disease process. In a study of sheep populations in Belgium comprising 25 different breeds, the authors reported that a deletion of two alanine residues in the HD of Sho was associated with increased susceptibility to natural scrapie (i.e., the classical form of the disease, this standing in distinction to "atypical" scrapie). This sequence variation is referred to as "2263_2268del". Heterozygotes were underrepresented in the scrapie samples by a factor of ~2, with a commensurate increase in homozygotes for the reference allele. Using a Cochran-Mantel-Haensel test, the data returned a *p* value of 0.022. These data warrant comment because of the nature of the reference allele. Alignments of the HD domain from different species reveal that the consensus wt structure in mammals is of the form AAAG-AAAG-AAAG-VAAG-LAAG, and analysis of the sheep *SPRN* gene structure and allelic frequencies vindicate this point of view (12). Thus the "2263_2268del" allele described by Lampo *et al.* is in fact the predominant allele. This interpretation is supported by their own data where their "wt" (i.e +6 nucleotide insertion) allele has a frequency of less than 50% (38% and 46% in the scrapie-negative and scrapie-positive samples, respectively). While the observation that animals homozygous for wt *SPRN* alleles can succumb to natural scrapie may not be an important advance (and is in any event prefigured by experimental studies of prion-infected mice with wt *SPRN* alleles), the effect of *SPRN* heterozygosity is reminiscent of *PRNP* variation and prion strain susceptibility and may bear further scrutiny in

datasets encompassing a single sheep breed. Lastly, in the same dataset comprising 25 breeds Lampo *et al.* found a mutation His-Pro at position 126 only within the scrapie-positive samples, but insofar as it was present in only 5 animals the significance of this finding is unclear; we also note that this polymorphism has been found in the normal sheep population in other studies (68).

Beyond the promoter-reporter studies mentioned above, in studies to addressing *SPRN* gene expression in ovine tissue, infection with SSBP/1 had no significant effect ($P > 0.05$) on expression of either *PRNP* or *SPRN* in any tissue examined (frontal cortex, cerebellum, obex, thalamus, spleen, and prescapular lymph nodes) (6). Given the likelihood that biological modulation of Sho protein is at a posttranslational level, this finding is not unexpected.

5.3. Germline *SPRN* mutations and human prion disease

A single base-pair insertion at codon 46 causing a frameshift in the Sho protein has been found in two unrelated vCJD patients from the UK (69). vCJD is closely connected to the occurrence of BSE. Since the frameshift a) leaves only about one fifth (~ 20 amino acid residues) of the mature protein coding region intact and results in the addition of ~ "extra" amino acid residues, it is extremely unlikely that the mutant protein would retain any functional attributes of the wt Sho protein. Furthermore, the frameshift has been suggested to even hasten the disappearance of corresponding mRNA by nonsense mediated decay: in sum it is to be regarded to cause a penetrant null allele. While the neurological phenotype of vCJD in the two frameshift patients was not overtly different from other vCJD cases, it is noted that these two patients "were amongst the most susceptible patients based on time of onset" (i.e. youngest: dates at onset unspecified) and were among the first British patients reported to die of the epidemic of BSE (69). The simple inference from these data is that hemizygosity for *SPRN* strongly disposes to an orally-transmitted prion disease, and, in consequence, the action of Sho in peripheral prion infections may need to be considered in addition to any role possible in the CNS. For the most widely studied epidemic of orally transmitted prion disease in humans, ie kuru, a putative protective allele of *PRNP* (G127V) was found in an allelic frequency gradient decreasing from the disease epicentre in the central highlands of New Guinea (70). Selection pressure to enhance the frequency of this allele may have arisen because children were exposed at the ritualistic cannibalism ceremonies. By extrapolation, selection pressure may have existed in early stages of human evolution to retain both copies of the Sho gene *SPRN*. While to the best of our knowledge copy-number variation has not been reported for human *SPRN* or *PRNP*, 11 mammalian species not overtly prone to cannibalistic episodes do lack an *SPRN* locus (71).

A further finding in the studies of Beck *et al* is that two linked single nucleotide polymorphisms, one in intron 1 and the other a missense variant at codon 7 were found to be associated with risk of sCJD (69). Signal peptide polymorphisms in PrP have been associated with differential usage of the optional "Ctm" isoform adopting a transmembrane topology using the HD (72) and analogous studies into the biogenesis of human Sho may be warranted.

6. PERSPECTIVES

PrP's centre-stage role in prion infections has been established by biochemical, immunological, and genetic analyses. Recent experiments describing the generation of prion infectivity from manipulation of recombinant PrP further highlight this position (73). However, the situation for the origins of sporadic disease, the basis of prion strain variability and the biology of normal prion proteins is less than clear, and it is perhaps within these contexts that Sho may expand our boundaries. To date the overlapping chemical features between CNS-expressed PrP^C and Sho include N-terminal repeats, a HD, endoproteolysis to a stable C1 fragment, and a C-terminal glycosylation site prefacing a GPI anchor. While Sho is natively unstructured akin to the PrP N-terminal region, it has a propensity, at least *in vitro*, to adopt an alternate beta-enriched conformation. In one school of thought in evolutionary genetic analyses *SPRN* is placed as the antecedent to *PRNP* (2) and, from this perspective, overlaps in biochemical properties may come as no surprise. Expression of Sho at the cell surface, shedding into extracellular medium and overlapping binding partners all point to further similarities with PrP^C. Shared biological properties are also coming into view in terms of phenotypes in cells and in mice (4, 35), measured in terms of cell death endpoints and in developmental biology. Lastly, we believe two observations in the crucial area of pathobiology bear emphasis as we look to the future.

The first observation is down-regulation of full-length Sho protein in infected animals, a finding that was adventitious in the sense that it was not predicted by an *a priori* hypothesis. From a practical point of view this defines Sho as an indicator or tracer to follow pathogenic event, a potentially useful advance. In the theoretical realm we now realize that as PrP^{Sc} is being created the Sho protein is also disappearing, pointing to a previously cryptic sensing or regulatory mechanism indicative of cross-talk between Sho and PrP^{Sc} pathways. The second finding is the discovery, perhaps the seminal discovery, of putative *SPRN* null alleles in two unrelated vCJD patients. While further scrutiny of the normal population is warranted to exclude the existence of these rare alleles (rare even in the vCJD dataset, found in only 2 cases out of 107 cases versus no occurrences in 861 normal subjects), the simple interpretation is that the frame shift allele facilitates an aspect in the pathogenesis of a medically important, orally transmitted prion disease. Since the patients carry only one copy of the putative null allele, this leads to the conclusion that a drop of only 50% in protein level influences disease outcome: in other words Sho is a *de facto* vCJD suppressor. In a more speculative vein it is tempting to consider a link between this genetically programmed drop in Sho expression with the aforementioned drop in expression seen in the brains of animals in the clinical phase of disease. These possibilities

and other confluences in Sho/PrP biology may create avenues in previously impenetrable thickets of prion biology.

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Abbreviations: AD, Alzheimer's disease; Arg, arginine; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; Dpl, Doppel; GSS, Gerstmann–Straussler–Scheinker syndrome; HD, hydrophobic domain, ORF, open reading frame; PRMT, protein-arginine N-methyltransferase; sCJD, sporadic CJD; Sho, Shadoo; TM, transmembrane; vCJD, variant CJD; wt, wild type

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