

# Shadoo/PrP (*Sprn*<sup>0/0</sup>/*Prnp*<sup>0/0</sup>) double knockout mice

## More than zeroes

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**S**hadoo (Sho) is a brain glycoprotein with similarities to the unstructured region of PrP<sup>C</sup>. Frameshift alleles of the Sho gene, *Sprn*, are reported in variant Creutzfeldt-Jakob disease (vCJD) patients while *Sprn* mRNA knockdown in PrP-null (*Prnp*<sup>0/0</sup>) embryos produces lethality, advancing Sho as the hypothetical PrP-like “pi” protein. Also, Sho levels are reduced as misfolded PrP accumulates during prion infections. To penetrate these issues we created *Sprn* null alleles [Daude et al., Proc. Natl. Acad. Sci USA 2012; 109(23): 9035–40]. Results from the challenge of *Sprn* null and Tg*Sprn* transgenic mice with rodent-adapted prions coalesce to define down-regulation of Sho as a “tracer” for the formation of misfolded PrP. However, classical BSE and rodent-adapted BSE isolates may behave differently, as they do for other facets of the pathogenic process, and this intriguing variation warrants closer scrutiny. With regards to physiological function, double knockout mice (*Sprn*<sup>0/0</sup>/*Prnp*<sup>0/0</sup>) mice survived to over 600 days of age. This suggests that Sho is not pi, or, given the accumulating data for many activities for PrP<sup>C</sup>, that the pi hypothesis invoking a discrete signaling pathway to maintain neuronal viability is no longer tenable.

### Genotype/Phenotype Relationships for PrP and Sho

Genetic analysis can provide profound insights into complex biological systems at the whole organism level. However, few knockouts in systematic studies in *Arabidopsis thaliana*, *C. elegans* and

*S. cerevisiae* produce a demonstrable phenotype—less than 2, 14 and 40–60% respectively.<sup>1–3</sup> For mice, the functional genomic pipeline has generated thousands of null alleles, but—perhaps compounded by under-reporting of “negative results”—this is inferred to have yielded similar low percentages. Broadly speaking, these results can be rationalized by genetic redundancy in the case of gene families, and by genetic robustness for the case where parallel pathways perform similar functions.<sup>3</sup> With regards to the specifics of mammalian prion biology, the cellular prion protein (PrP<sup>C</sup>), a precursor to the infectious prion protein isoform, is encoded by a host locus, the *Prnp* gene. At one stage it was hoped that phenotypes in homozygous null mice would provide a simple penetrating insight into the riddle of PrP<sup>C</sup> function, but unfortunately this was not the case. *Prnp*<sup>0/0</sup> mice are resistant to prion infection but have an ostensibly normal development. Here we review recent results on the knockout of the PrP-like Sho protein, the creation of PrP/Sho double-knockout animals and the implications of these findings for the so-called pi hypothesis.

### PrP<sup>C</sup>

For context, we will first consider properties of these two GPI-linked neuronal glycoproteins (Fig. 1). For PrP<sup>C</sup>, the precursor to misfolded PrP<sup>Sc</sup> protein in prion infection, the situation is quite complex and for the sake of brevity, the reader is directed to recent reviews.<sup>4–6</sup> Nonetheless, it is worth mentioning that PrP has an anti-apoptotic role in vitro<sup>7</sup> and that in vivo it

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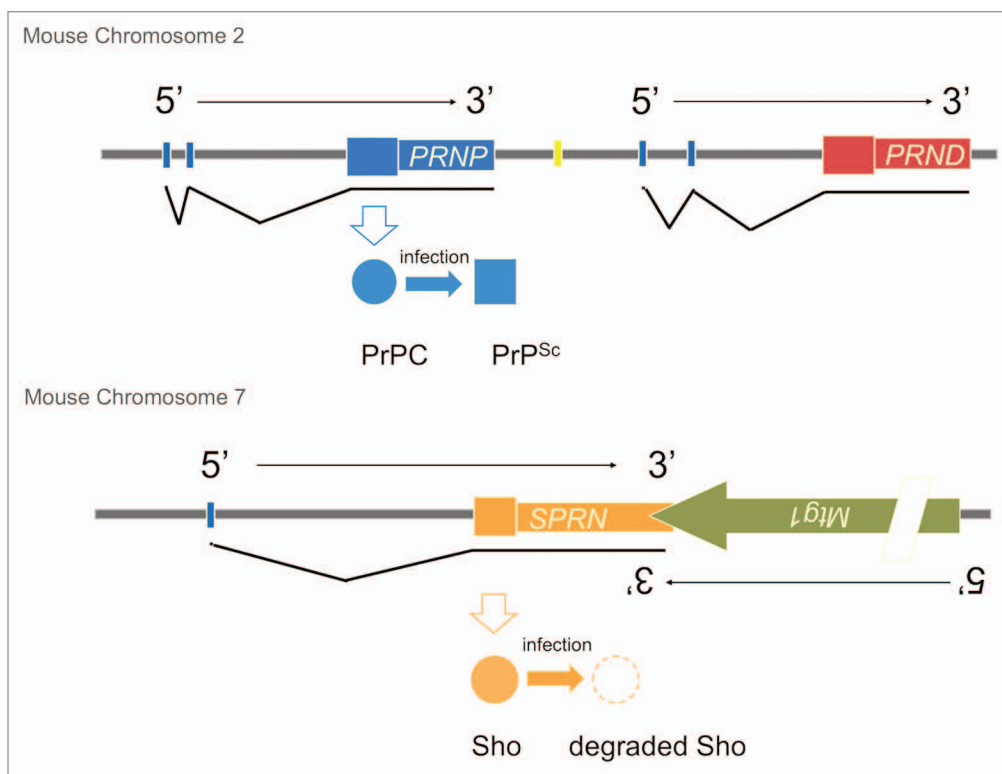
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**Figure 1.** CNS-expressed prion proteins in mice. PrP<sup>C</sup> encoded by *Prnp* and Sho encoded by *Sprn* are located on different chromosomes. Their adjacent flanking genes (*Prnd* and *Mtg1*) are shown and these encode the doppel protein and mitochondrial GTPase 1, respectively. Protein products of *Prnp* and *Sprn* are shown but are omitted for *Prnd* and *Mtg1* for the sake of simplicity. Sizes of the transcription units are not to scale and a complex intron/exon structure for *Mtg1* has been simplified for the purpose of this figure (prime).

is involved in neuroprotection, as *Prnp*<sup>0/0</sup> mice are more sensitive to ischemic insults and to seizure, whereas overexpression of PrP in a rat stroke model is protective. In terms of signaling, PrP<sup>C</sup> is coupled to Fyn kinase in IC11 cells. PrP<sup>C</sup> is also involved in copper binding and modified response to oxidative stress, in cell adhesion and in the glutamatergic system by binding to NMDA receptors. Another way to discern function follows “guilt-by-association,” by identifying protein partners with (hopefully) clear-cut functions. However, this hasn’t proven particularly useful due to the wealth of interactors and the failure of different labs to routinely identify the same proteins.<sup>4,8,9</sup> Two routes to resolution of the PrP<sup>C</sup> puzzle invoke theory. One posits PrP<sup>C</sup> as a generalized scaffold and signaling node for many membrane proteins,<sup>6</sup> and a second posits two hypothetical “missing” partners. Thus, since PrP<sup>C</sup> deficiency is not lethal and since *Prnp*<sup>0/0</sup> mice lack a strong phenotype, it has been inferred from these data, and from transgenic studies with internally deleted

forms of PrP<sup>C</sup>, that another protein takes over PrP<sup>C</sup> function in knockout mice, a hypothetical functional analog termed “pi” ( $\pi$ ),<sup>10</sup> and that both PrP<sup>C</sup> and pi dock an entity termed “L<sub>PrP</sub>” to transmit signals needed for neuronal viability.

### Sho

Could Sho be pi? The Sho glycoprotein encoded by *Sprn* has partial homology to the PrP<sup>C</sup> hydrophobic domain, a series of N-terminal repeats, a C-terminal N-linked glycosylation and a GPI anchor.<sup>11,12</sup> In terms of physiological action, Sho, like PrP<sup>C</sup>, can exhibit neuroprotective properties and also shares a number of protein binding partners with PrP<sup>C</sup>.<sup>12,13</sup> Unlike PrP<sup>C</sup>, this lab failed to define expression of Sho outside the central nervous system (CNS) by immunoblotting, although expression in reproductive tissues has been inferred from the analysis of GFP reporter mice driven by *Sprn* promoter elements ([www.gensat.org](http://www.gensat.org)). Also akin to PrP<sup>C</sup>, we have found that Sho is naturally

endoproteolytically cleaved into N- and C-terminal fragments, with abundant N1 and C1 fragments but also including species of 12 and 14 kDa.<sup>12,14</sup> Given increasing interest in the protective bioactivity of PrP N1 fragments,<sup>15</sup> analogous studies of Sho N1 in the brain, a neuropeptide of sufficient abundance to be detected in wildtype (wt) mice would appear in order. An important task will be to define the protease that naturally cleaves Sho, which could be the same as the one that cleaves prior to the hydrophobic domain in PrP<sup>C</sup>—the latter activity has sometimes been equated with ADAM proteases but in response to complications in this story<sup>16,17</sup> has also been accorded the more cautious moniker of “alpha-PrPase.”<sup>18</sup>

In light of a potential Sho/pi connection, we looked at neuroanatomical expression in the brain and compared it to PrP. Interestingly, expression of the two proteins was not fully superimposable. In the hippocampus, Sho is expressed in the molecular layer of the dentate gyrus whereas PrP expression is more prominent

in the molecular layer adjacent to CA1 neurons,<sup>14</sup> and future versions of these experiments will benefit from monoclonal antibodies specific to N- and C-terminal fragments of Sho. Since the hippocampus is involved in modification of behavior, attention, and spatial memory, and since expression of Sho and PrP are ablated, memory-based behavioral tests to study the differences between *Sprn*<sup>0/0</sup> and *Prnp*<sup>0/0</sup> mice might be in order to pinpoint specific contributions of the two proteins.

The concept of biological redundancy forms a strand in this review and an implicit assumption that follows from this is that individual knockout stocks are without notable phenotypes in the resting state. This has been mentioned for *Prnp*<sup>0/0</sup> mice and also proves to be the case for *Sprn*<sup>0/0</sup> mice, as both male and female mice are fertile and do not show overt malformations. However, a subtle alteration in body weight was discerned at the  $p < 0.05$  level in *Sprn*<sup>0/0</sup> mice maintained on two genetic backgrounds, with knockout animals a little lighter than their wt counterparts. Furthermore, using *Sprn*<sup>0/0</sup> mice as a negative control we also defined Sho expression, by immunostaining, in hypothalamic neurons in wt mice.<sup>14</sup> Of note, (1) this neuroanatomical structure contains nuclei that control feeding behavior,<sup>19</sup> (2) weight loss can be observed in prion-infected animals,<sup>20</sup> suggestive to some of an endocrinopathy<sup>21</sup> and (3) Sho is downregulated in prion infections. While these three observations are tantalizing, a mechanistic relationship featuring Sho as a determinant of body mass alteration subsequent to PrP<sup>Sc</sup> accumulation remains speculative at this time. In terms of chemical phenotypes, PrP<sup>C</sup> levels in *Sprn*<sup>0/0</sup> mice are comparable to wt mice of the same genotype, arguing against compensatory cross-regulated expression of the two genes.<sup>14</sup>

### Action of Sho in Prion Infections: Witness or Accessory?

A next phenotypic issue at hand is the outcome of prion challenge in *Sprn*<sup>0/0</sup> mice. From a frameshift mutation observed in two vCJD cases, and a polymorphism in the signal sequence associated with risk of sporadic CJD<sup>22</sup> one can infer a potential

role in human prion disease. In our studies so far we have studied prion infection in *Sprn*<sup>0/0</sup> mice, challenging the animals by intracerebral, intraperitoneal and oral routes with the mouse-adapted RML isolate of scrapie prions. These experiments failed to reveal distinctions—either in the symptoms or the duration of the disease—from parallel infections of wt mouse controls. Future studies will need to encompass vCJD challenge of *Sprn*<sup>0/0</sup> × Tg(HuPrP) “humanized” mice and 301V (mouse-adapted BSE) challenge of *Sprn*<sup>0/0</sup> × *Prnp*<sup>b</sup> mice. However, while an active role of Sho in prion replication remains conjectural at this juncture, it is established beyond cavil that the levels of protein are markedly reduced in prion infections with several prion isolates (“strains”) producing slightly different end-stage pathologies.<sup>23-25</sup> Provocatively, BSE, a prion isolate with unusual hyperglycosylated PrP<sup>Sc</sup><sup>26</sup> and with a vast host-range including human and non-human primates, bovidae and felidae,<sup>27</sup> may be an exception to this emerging rule. With regards to reduction in Sho being a non-specific response to tissue damage, this phenomenon was not observed for four other neurodegenerative syndromes. Our laboratory and other workers have generally observed a correlation between the accumulation of protease-resistant PrP<sup>Sc</sup> in the brain and the disappearance of Sho, both in wt animals and in Tg*Sprn* mice overexpressing wt mouse Sho.<sup>23,24</sup> How might this disappearance of Sho be explained? *Sprn* transcript levels are unperturbed by prion infection, so proteolysis is an obvious candidate and we favor the interpretation that Sho levels are passively monitoring an in vivo clearance mechanism directed against PrP<sup>Sc</sup> (rather than Sho actively perturbing this process or, for that matter, perturbing prion replication). Chronically infected prion cultures do not usually exhibit morphological signs of cellular pathology (for a review, see ref. 28) yet can exhibit robust Sho downregulation (Mays CE, in preparation). Besides underscoring the conclusion that the Sho downregulation effect is not related to cellular damage, these systems should provide opportunities for profound mechanistic insight by use of antibiotics and protease inhibitors. Furthermore, the

early preclinical disappearance of Sho—or perhaps the appearance of telltale proteolytic fragments—could provide a new diagnostic angle on prion infections.

### Sho, PrP<sup>C</sup> and Embryonic Development

Returning to physiology there is an implicit question as to the viability of “double” knockout mouse embryos. The first attempt at addressing this issue was taken in *Prnp*<sup>0/0</sup> embryos, where *Sprn* knockdown using lentiviral vectors was reported to result in embryonic lethality,<sup>29</sup> potentially validating the pi hypothesis with pi equated to Sho. Curiously, in contrast to this robust effect, using penetrant null alleles of *Sprn* and *Prnp* to generate double knockouts (*Sprn*<sup>0/0</sup>/*Prnp*<sup>0/0</sup>) we found the adult mice to be viable and fertile.<sup>14</sup> Moreover, we performed inbreeding crosses over several generations to ensure that this phenomenon is not due to maternal effects (e.g., carryover of maternally encoded Sho mRNAs into *Sprn* homozygous null embryos). So, it seems that although PrP and Sho have overlaps in their chemical properties, neither alone nor in combination are they required for the completion of embryogenesis when using fully penetrant, constitutive null alleles. How then did the lentiviral studies in *Prnp*<sup>0/0</sup> embryos yield a seemingly contradictory result?

Besides a theoretical compensation phenomenon by other genes (discussed below), technical explanations also need consideration. The possibility that *Sprn* phenotypic effects are extremely sensitive to conditions of husbandry cannot be excluded. In terms of genetic effects, inbred strain background, a standard argument invoked in the case of divergent allelic phenotypes, is not so different between the two experiments (FVB/N for the knockdown studies vs. FVB/NCr × 129Pas for our study). Perhaps more pivotal is the adopted strategy, genetic deletion of all Sho ORF codons in the germline vs. virally-vectored shRNAs that target the 3' untranslated region (UTR) of *Sprn* mRNA within cells of infected embryos. It is known that the *Sprn* transcription unit overlaps with an adjacent gene encoding a mitochondrial protein, *Mtgi*, and it is

possible that shRNAs against the *Sprn* 3' UTR might affect *Mtg1* expression if there is transcriptional interference between the two loci. It is of interest to recall that an early interpretation of the impact of PrP knockout on cerebellar degeneration<sup>30</sup> had to be revisited subsequent to a demonstration of an artifactual effect of certain *Prnp* null alleles on the adjacent *Prnd* transcription unit.<sup>31,32</sup> However, *Mtg1* transcript levels are reported as being unaltered by shRNA vectors in a follow-up study,<sup>33</sup> tipping the balance to a consideration of off-axis effects of shRNAs against irrelevant genes. Further validation of shRNA knockdown strategies could involve (1) confirming successful knockdown of the Sho protein and (2) genetic rescue of the knockdown effect on protein levels and embryonic development by co-administration of a second vector with the shRNA vector: this second vector would encode a wt Sho protein open reading frame upstream of a heterologous 3'UTR (i.e., a 3' UTR not targeted by the current anti-*Sprn* shRNAs). Data to ascertain and distinguish (3) the phenotypic impact of lentiviral *Mtg1* knockdown would also comprise a useful, additional point of reference.

### Activities of Sho and PrP<sup>C</sup> in the Adult CNS

The viability of *Sprn*<sup>0/0</sup> and *Prnp*<sup>0/0</sup> knockout mice leads next to a consideration of action and synergy beyond embryos and, certainly, within the CNS of adult mice that has the most fulminant expression of these two proteins. While aging *Prnp*<sup>0/0</sup> mice have a polyneuropathy<sup>34,35</sup> that is not apparently enhanced by the absence of Sho,<sup>14</sup> it will be important to pursue other parameters in *Sprn*<sup>0/0</sup> mice (e.g., susceptibility to stroke and seizure) and to compare the results with *Prnp*<sup>0/0</sup> animals.

A theoretical concern with the constitutive null alleles is that phenotypic impacts might be masked by a counterbalancing expression of a functional homolog

(or homologs) induced as a result of the gene deficiency. For PrP<sup>C</sup>, the possibility of other CNS proteins with functional homology is not inconceivable.<sup>36</sup> One way to address this is to perform “-omic” analyses (for example, but not limited to, microarray analyses), to compare *Sprn*<sup>0/0</sup>, *Prnp*<sup>0/0</sup> and *Sprn*<sup>0/0</sup>/*Prnp*<sup>0/0</sup> mice. Another tack is to use conditional null alleles. This is based on the assumption that compensatory proteins, induced transiently in embryogenesis, might not be available once adulthood is attained: hence a single conditional knockout in adults or activation of conditional null PrP alleles in adult *Sprn*<sup>0/0</sup> mice might reveal the “true” phenotype of protein deficiency. This could lie in the behavioral domain and be revealed by forced swim and open field tests, for example, or it might comprise a neurodegenerative syndrome. If a neurologic phenotype was still absent under standard housing conditions, then focus would turn to conditions of CNS duress or adaptation. Since both Sho and PrP are putatively neuroprotective proteins, lesioning or neurotoxic challenge of double knockout mice might yield impairments that transcend the individual knockouts.

### Pi, on the Face of It

While the equation “Sho = pi” might have been a foregone conclusion a few years back<sup>29</sup> this is not so clear now. One viewpoint is that Sho is as plausible a candidate for pi as can be mustered from biochemical data, and that the pi hypothesis may be erroneous. Observations that underpin this position are (1) largely overlapping patterns of Sho/PrP gene expression, (2) failure to pass a critical genetic test and (3) a vacant candidacy for a transmembrane protein (L<sub>PrP</sub>) with two distinct PrP binding sites. The fact that much of PrP is endoproteolyzed such that the N- and C-termini are separated might comprise a further caveat for the hypothesis (which posits simultaneous docking by

N- and C-terminal PrP sites to the L<sub>PrP</sub> partner). One may also note that another hypothesis that seeks to explain the properties of internally-deleted PrP does not concern signaling for neuronal survival under basal conditions, but instead as a response to stress events.<sup>37</sup> Alternatively, if one believes in the pi hypothesis (which includes an assumption of genetic redundancy), then Sho cannot be pi because of the health of the *Prnp*<sup>0/0</sup>/*Sprn*<sup>0/0</sup> mice.

In reaching a final position, there are other questions and subtleties that apply to the failure to discern a phenotype in a knockout strain. First, there is always a question as to whether the “right” phenotype is being tested, and, beyond redundancy from members of a gene family, there is another concept to explain a lack of a phenotype in knockouts, this being “genetic robustness.” Even if two proteins function in the same pathway, a parallel pathway might exist such that functionality and/or viability is maintained.<sup>3</sup> For PrP<sup>C</sup> there is already evidence against high affinity interaction with just single protein, evidence for interactions with diverse proteins and evidence for a diversity of actions including functional modulation of distinct ion channels and protection against different neurotoxic insults. Since these complex activities must inevitably involve a variety of accessory proteins (e.g., ligand and voltage gated channels are typically multi-subunit complexes), and, since parallel systems from neuroprotection go beyond just biochemical pathways in neurons to include contributions from glia and microglia,<sup>38</sup> then the complex cell-surface landscape of PrP<sup>C</sup> and Sho seems far more reconcilable with the concept of genetic robustness than genetic redundancy. Thus, rather than double knockouts excluding that Sho is pi, we are more inclined to conclude that the pi hypothesis is closed as an avenue in prion biology research. Hopefully, however, the new knockout mouse lines will contribute to a better understanding of these small, enigmatic glycoproteins.



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