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The Prion-like protein Shadoo is involved in mouse embryonic and mammary development and differentiation

Bruno Passet, Johan Castille, Samira Makhzami, Sandrine Truchet, Anne Vaiman, Sandrine Floriot, Katayoun Moazami-Goudarzi, Marthe Villette, Anne-Laure Gaillard, Louise Helary, Maud Bertaud, Olivier Andréoletti, Daniel Vaiman, Pierre Calvel, Nathalie Daniel-Carlier, Mohammed Moudjou, Christian Beauvallet, Mohamed Benharouga, Denis Laloë, Sophie Mouillet-Richard, Amandine Duchesne, Vincent Béringue, Olivier Andréoletti, Sophie Mouillet-Richard, Jean-Luc Vilotte

Shadoo belongs to the prion protein family, an evolutionary conserved and extensively studied family due to the implication of PrP in Transmissible Spongiform Encephalopathies. However, the biological function of these genes remains poorly understood. While Sprn-knockdown experiments suggested an involvement of Shadoo during mouse embryonic development, Sprn-knockout experiments in 129Pas/C57BL/6J or 129Pas/FVB/NCr mice did not confirm it. In the present study, we analyzed the impact of Sprn gene invalidation in a pure FVB/NJ genetic background, using a zinc finger nuclease approach. The in-depth analysis of the derived knockout transgenic mice revealed a significant increase in embryonic lethality at early post-implantation stages, a growth retardation of young Sprn-knockout pups fed by wild type mice and a lactation defect of Sprn-knockout females. Histological and transcriptional analyses of knockout E7.5 embryos, E14.5 placentas and G7.5 mammary glands revealed specific roles of the Shadoo protein in mouse early embryogenesis, tissue development and differentiation with a potential antagonist action between prp and Shadoo. This study thus highlights the entanglement between the proteins of the prion family.

The prion protein, encoded by Prnp, is extensively studied due to its implication in Transmissible Spongiform Encephalopathies (TSE). Indeed, the misfolded, partially protease-resistant, PrP conformer is the main, if not the sole, component of prions, the infectious agent responsible for TSE in animals and humans. The evolutionary conservation of PrP suggested its involvement in essential cellular processes. Prnp is ubiquitously expressed throughout development and adult life, with a higher expression level in the central nervous system. Surprisingly, naturally occurring or generated mammals or fish devoid of PrP develop normally, suggesting that (an)other host-encoded protein(s) induces() compensatory mechanism(s).

Two other members of the mammalian prion family were described, Shadoo, encoded by Sprn and homologous to the N-terminal part of PrP, and Doppel, encoded by Prnd and homologous to the C-terminal part of PrP. In the adult, Doppel is mainly expressed in male gonads and Prnd or Prnd/Prnp knockout male mice are sterile.

Shadoo shares with PrP some spatial regulation and properties. The knockdown of Sprn in 129Pas/FVB/NCr Prnp-knockout embryos induces a lethal phenotype at early embryonic stages (~E10), originating from a trophectoderm-derived compartment developmental failure. However, Sprn or Sprn/Prnp co-ablation in 129Pas/C57BL/6J or 129Pas/FVB/NCr mice did not reveal embryonic developmental abnormality. These

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divergent observations, sometimes attributed to genetic compensation in mutated animals\textsuperscript{13}, questioned the involvement of Shadoo during embryogenesis.

In this study, we report the establishment, through injection of a Zinc Finger Nuclease (ZFN), of FVB/NJ Sprn\textsuperscript{0/0} mice. Reproductive performances and transcriptomic analyses of these mice demonstrate that Shadoo is involved in embryonic development and more generally in the development and differentiation of several tissues. Furthermore, our data suggest antagonist actions of PrP and Shadoo, at least during embryonic stages.

**Results**

**Generation of Sprn\textsuperscript{0/0} mice.** The ZFN target site is located at the start of the Sprn ORF (Fig. 1). ZFN mRNA was injected into 1 cell-fertilized FVB/NJ mouse eggs that were transferred into pseudo-pregnant mice. Tail-DNA analysis of the 29 resulting pups by PCR amplification of a \~400 bp genomic region surrounding the ZFN-target site and sequencing analysis allowed identifying 9 transgenic founder mice, carrying small deletions ranging from 1 to 13 bp. All founder mice were heterozygous for the mutated allele.

Offspring from two founders (17 and 05) were used to generate Sprn\textsuperscript{0/0} mice. These founders were chosen as they transmitted a mutated allele (deletion of 1 nucleotide associated with the mutation of an adjacent nucleotide and deletion of 10 nucleotides, respectively), inducing a frameshift leading to an early premature stop codon. Translation of the corresponding mRNA is predicted to produce truncated nonsense proteins (Fig. 2) lacking all Shadoo domains.

Intercrosses of Sprn\textsuperscript{0/+} mice produced Sprn\textsuperscript{0/0} mice (Table 1). Knockout mice appear normal and fertile, as previously observed\textsuperscript{12}. However, the percentage of Sprn\textsuperscript{0/0} mice was significantly lower than expected (Table 1), suggesting embryonic lethality. Intercrosses of Sprn\textsuperscript{0/0} mice led to litters with an average size of 6.44 (174 pups/27 litters; 54/8 for line 05, 120/19 for line 17). This litter size was significantly smaller compared to FVB/NJ mice (8.6 pups/litter; 215/25, t-test p \textless 0.05). To assess the fertility of Sprn\textsuperscript{0/0} males and females, crosses between Sprn\textsuperscript{0/0} and WT mice were studied. The average litter sizes were of 9.7 (88/9) and 9.8 (59/6) for Sprn\textsuperscript{0/0} males and females, respectively. These results were similar to those of FVB/NJ mice, suggesting that Sprn knockout is not associated with sub-fertile sex-related phenotype or inability of Sprn\textsuperscript{0/0} females to sustain normal gestations. Of note, such a decrease in litter size was reported for Prnp-overexpressing mice\textsuperscript{14}. As both Sprn\textsuperscript{0/0} lines behaved similarly (average litter sizes, transmission rate), line 17 was chosen for further detailed analyses. It is referred to as Sprn\textsuperscript{0/0} thereafter.

Sprn expression analysis was investigated in Sprn\textsuperscript{0/0} mouse brains. Sprn-transcript levels seemed unaffected, as previously observed\textsuperscript{12}. However, this observation was expected since the ORF is located within the last exon of the gene and the mutated transcript should thus escape nonsense mediated decay\textsuperscript{15}. Microarray transcriptomic analysis of other tissues led to similar observations as Sprn was not detected as differentially expressed between Sprn\textsuperscript{0/0} and FVB/NJ mice (see below). The entire ORF was reverse-transcribed from brain RNAs of WT and Sprn\textsuperscript{0/0} mice. No alternative splicing was detected and Sprn ORF from Sprn\textsuperscript{0/0} mouse

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**Figure 1.** Schematic representation of the Sprn gene and location of the ZFN target site. (A) Schematic representation of the Sprn gene. Bar: flanking and intronic sequences. Grey boxes: exonic untranslated regions. Black box: Open Reading Frame (ORF) sequence. Location of the ZFN target site is indicated on the top. The scheme is not at scale. (B) Representation of the Shadoo protein. (C) Location of the ZFN target site. Blue sequence: partial Sprn exon 2. Red sequence: ZFN target site. Green sequences: oligonucleotides used for genotyping analysis. Bold, underlined nucleotides: start of Sprn ORF (reverse orientation).
mRNAs carried the expected nonsense mutation (Fig. S1A). Of note is the lack of downstream in frame ATG codon close to the nonsense mutation, avoiding translation re-initiation (Fig. 2). Brain western blots analyses did not allow detecting Shadoo in WT or Sprn0/0 brains, despite using several commercially (Abgent # AP4754b, PA5 24688, ab175070) or laboratory homemade (1909 S0, S4 et S5, J74 et J740 from CEA, France and 06sh1 from University Alberta, Canada) antibodies and detection techniques (ECL, IF, data not shown). Nevertheless, RNA investigations clearly demonstrate the knockout genetic status of these mice.

Increased lethality of Sprn0/0 embryos. We next investigated whether the reduced litter size in Sprn0/0 mice was associated with increased embryonic lethality. Pregnant females from Sprn0/0 × Sprn0/0 or WT × WT crossings were analyzed at G7.5 and G14.5. At G7.5, no resorption was observed in FVB/NJ mice (0/76 implantation sites (IS)). In contrast, a statistically significant 12.7% resorption rate was detected in Sprn0/0 females (7/55 IS, $p < 0.05$). Empty decidua suggested that resorptions occurred early after implantation. At G14.5, this significant difference remained with a resorption rate of 19.7% in Sprn 0/0 mice (14/71 IS) compared to 6.2% in WT mice (10/162 IS). Thus, the reduced litter size observed in Sprn0/0 × Sprn0/0 crossings results from an increased fragility of the embryos at early post-implantation, a key period of mammalian fetal development.

Transcriptomic analysis of E7.5 Sprn0/0 embryos. To get deeper insight into the biological consequences of Sprn knockout in embryonic development, comparative microarray transcriptomic analysis of WT and Sprn0/0 embryos was performed at E7.5. Following raw data normalization, differentially expressed genes (DEG) with a p-value $< 0.05$ and a fold change (fc) $< -2$ or $> 2$ were analyzed. Overall, 147 DEG were identified, 86 upregulated and 61 downregulated, of which 29 were small nucleolar RNAs (Table S1). These small RNA genes regulate cell proliferation, migration and invasion by inducing Epithelial-Mesenchymal transition (EMT)\(^1\).\(^2\). Notably, a large set of small nucleolar RNAs with a conserved H/ACA box (snoRA) were downregulated. Such downregulation was found to be associated with cellular apoptosis\(^1\).\(^7\). Further, gene set enrichment analysis (GSEA) identified downregulation of genes involved in dorsal spinal cord development (enrichment score (ESc) 3.37, false discovery rate (FDR) 0.006), mitochondrial translational termination and elongation (ESc 2.7, FDR 3.08 × 10\(^{-5}\)), constituent of ribosomes (ESc 3.47, FDR 4.5 × 10\(^{-17}\)) and proteasome core complex (ESc 2.6, FDR 4.2 × 10\(^{-8}\)). Identification of these biological pathways revealed that Sprn0/0 E7.5 embryos suffer from developmental defects.
Intra-uterine growth retardation. We thus analyzed whether normal or vascularization modification (data not shown). The relative proportions of the labyrinth layer, spongio-
significance (Fig. S2).

SerpinA1 and Fibrinogen. The results confirmed the tendency observed at the transcriptomic levels, with on

Igsf11 (Table S1). Importantly, Prnp altered cell fate determination and inducing embryonic lethality soon after the implantation.

Some pathways identified above are reminiscent of those described for PrP modulation of EMT18,19. PrP regulates embryonic stem cell pluripotency and differentiation, and Shadoo and PrP share biological properties9 although recent experiments suggest that above could depend of the model system used20,21. As we previously performed transcriptomic analysis of E7.5 Sprno/0 embryos11, we searched for potential shared DEG between Sprno/0 and Sprno/0 embryos. Two master regulators of pluripotency were differentially expressed in E7.5 Sprno/0, Oct4 and TGF/ir2. Oct4 was overexpressed and TGF/ir2 downregulated in Sprno/0 embryos11, i.e. oppositely to what is observed in Sprno/0 (note S1). Furthermore, out of the 147 DEG observed in Sprno/0 embryos, 12 were also differentially expressed in Sprno/0 embryos11 but in an opposite sense, those indicated in Table 2 alongside Hspa1b and lgsf11 (Table S1). Importantly, Prnp was not differentially expressed in Sprno/0 embryo, excluding this observation as an indirect consequence of Prnp overexpression.

Collectively, these transcriptomic analyses suggest that Sprn knockout induces alteration of EMT pathways, impacting cell fate determination and inducing embryonic lethality soon after the implantation.

### Table 2. Identified networks with differentially expressed genes in E7.5 embryos. Bold-faced genes: positively differentially expressed genes in Sprno/0 embryos. Underlined genes: negatively differentially expressed genes in Sprno/0 embryos. Yellow colored genes: genes also found differentially expressed in Prnp0/0 E7.5 embryos11. Of note, these genes were all differentially expressed in an opposite way between Sprno/0 and Prnp0/0 embryos.

| Network | Differentially expressed genes in E7.5 embryos |
|---------|-----------------------------------------------|
| Developmental Disorder | A0CL1, C3, C5AR2, CASP8, CAT, CAV1, CD68, Cyb5r3, CYBB, DGN, DE3, EHD2, FABP4, GLP1R, GZMH, HSPA1A/HSPA1B, ITGA2, LY96, mir-15, Met4a, NFYA, PGE, SLCO2A12, SLCO6A4 |
| Cell-to-cell signaling and interaction | ANXA1, C15, DAG1, FADS3, IRAK3, LAPTMS, 5Y96, MFS2DA, mir-302, OSMR, RNASE4, SLCO3A3, Srgn, TN54, ZFAND2A |
| Immunological and/or hematological Disease | AGPAT2, AMPD3, AT1R, AT1R1, C3, Gstm3, IFI44, IFI44, IFI44, IFI44, LAMAT1, mir-17, mir-19, MUCF3, Nan1, Prl2c2, RPL14, Rpph1, SKE1 |
| Organismal survival | ALG12, COQ5, EHD3, Erv3, FERTM1, FSTL3, GPX3, GREM2, Hpya1b, mir-467, MPEG1, PAP52, Serpinh1, SLCO2A1 |
| Cellular Growth and Proliferation | BIN2, DHR9, Ear2, mir-17, NFYA, PEG10, RBM3, SCUBE2, SDC3, SNORA74A, SnoD1B, Speer4a, TC2N, Tpsab1 |

Ingenuity® Pathway Analysis (IPA®) (http://www.ingenuity.com/) of the 147 DEG identified 4 significant networks (score > 22); developmental disorder, cell-to-cell signaling and interaction, immunological and/or hematological disease, organism survival and cell growth and proliferation (Table 2). It suggested that Shadoo deficiency alters cell proliferation and differentiation resulting in abnormal early development. Potential related master regulator genes were identified in further analyses of the DEG, but at reduced fc (note S1).

Comprehensive analyses of Sprn0/0 pups' growth curves and of Sprn0/0 placentas. To assess whether pups body weight is affected in Sprno/0 mice, as previously reported10, Sprn0/0 pups fed by WT lactating females. The body weight gain was lower for Sprn0/0 versus WT pups (statistically significance between L3-L10, Fig. 3A). At variance with the previous data10, there was no difference according to the pup sex. Abnormal growth could have various origins, one being placental dysfunction as in Prnp0/0 mice14. Placental dysfunction would explain that at birth, the weight of the Sprn0/0 pups were significantly lighter (1.34 ±/− 0.14 g for Sprno/0 vs. 1.4 ±/− 0.16 g for WT pups (n = 97), t-test p < 0.05), suggesting intra-uterine growth retardation. We thus analyzed whether Sprn knockout affects placental efficiency. Fetuses and placental tissues from E14.5 WT and Sprno/0 were weighted. No significant difference was observed between the two genotypes (Table 3). Histological examination of E14.5 and E17.5 placentas did not reveal structural or vascularization modification (data not shown). The relative proportions of the labyrinth layer, spongio-trophoblast and decidua zones were unaffected.

We next performed comparative transcriptomic analysis on E14.5 WT and Sprno/0 placentas. Following raw data normalization, DEG with a p-value < 0.05 and a fc < −2 or > 2 were selected. 133 genes were identified, of which 17 were downregulated (Table S2), including 12 small nuclear RNAs. Seven DEG were common between placentas and embryos, all of them behaved similarly between the two tissues: 4 small nuclear RNAs alongside Glp1r, Ly96 and SLCO4C1 genes. This small number of shared DEG was expected due to transcriptomic diver-
generates between organs besides early stages of development22. Validation of the transcriptional regulation of five genes, TBPA, Gli3, GC, PSG23, SerpinA1 (a−antitrypsin), was tested by RT-qPCR. All results were concordant (Table S3). Furthermore, immunoblot analyses were performed to examine the expression levels of two proteins, SerpinA1 and Fibrinogen. The results confirmed the tendency observed at the transcriptomic levels, with an average a higher expression level of the two proteins in the knockout animals, which did not reach statistical significance (Fig. S2).

IPA® of the DEG identified two major associated networks (score > 35); lipid metabolism, molecular transport, small molecule biochemistry and tissue development, cellular movement (Table 4). Global gene activation
within these networks led to the overexpression of various protein families, including apo-lipoproteins and fibrinogens. Both gene families exert protective effects against stresses, supporting placentation and embryonic development\(^{23,24}\). Potential related master regulator genes were identified in further analyses of DEG, but at reduced fc (note S2).

Altogether, these data suggest that at E14.5, Sprn\(^{0/0}\) placentas underwent an adaptive biological process, involving LXR/RXR and FXR/RXR activation highlighted by the apo-lipoproteins and serpinA1 upregulations, to compensate earlier developmental dysfunctions.

Sprn-invalidation affects mouse lactation. Since Sprn appears to be involved in embryonic EMT and in cellular proliferation and tissue development and since PrP\(^{c}\) is involved in the self-renewal of mammary adult stem cells\(^{25}\), we assessed whether Sprn knockout affects lactation. We compared the growth curves of WT pups fed by WT or Sprn\(^{0/0}\) lactating females. Their weight gain was significantly lower when fed by Sprn\(^{0/0}\) females between L2 and L10 (Fig. 3B). No significant difference was observed between WT and Sprn\(^{0/0}\) milk protein concentrations at L7.5 (Table S4). Consistently, SDS-PAGE and western analyses did not reveal modification of the milk protein profiles (Fig. S3). Iron, zinc and copper concentrations were also similar between Sprn\(^{0/0}\) and WT milks (data not shown). These data thus suggest that Sprn invalidation affects mouse lactation.

Figure 3. Analysis of pup's growth curves. (A) Analysis of potential growth differences according to the genotype and sex of the pups. Male or females pups from FVB/NJ (blue) or Sprn\(^{0/0}\) (orange) genotype were fed by FVB/NJ females. Statistically different mean weights between pups according to their genotypes (t test \(p < 0.05\)) are indicated by *. At least 6 pups of each genotypes for both sexes were weighted per stage (see Material and Method section) (B) Analysis of potential lactating differences according to the genotype of the females. FVB/NJ male or female pups were fed by either FVB/NJ (blue) or Sprn\(^{0/0}\) (orange) females. Statistically different mean weights between pups fed by the two types of lactating female (t test \(p < 0.05\)) are indicated by *. At least 6 pups of each genotypes for both sexes were weighted per stage (see Material and Methods).

| Mouse genotype | FVB/NJ | Sprn\(^{0/0}\) |
|----------------|--------|---------------|
| Placenta Weight E14.5 (average +/- SEM) | 0.064 ± 0.008 | 0.065 ± 0.012 |
| Fetal Weight E14.5 (average +/- SEM) | 0.236 ± 0.029 | 0.230 ± 0.034 |
| Placental efficiency | 3.754 ± 0.526 | 3.585 ± 0.580 |
| Number of analyzed Pups | 66 | 63 |
| Number of analyzed Litters | 7 | 11 |

Table 3. Analysis of placenta and fetal weights.
Sprn-invalidation affects milk fat globules volumes and morphology. Analysis of milk fat globules (MFGs) revealed a significant reduction of the medians volume of Sprn<sup>0/0</sup> MFGs (n = 3166) compared to that of FVB/NJ MFGs (n = 5643) (Fig. 4A, kruskal-Wallis chi-squared = 22.114, p-value = 2.57 × 10<sup>-6</sup>). Analysis of the size distribution of the MFGs showed that the majority of MFGs were smaller in Sprn<sup>0/0</sup> milk with a significant increase (up to 30% of total MFGs) of very small MFGs (size between 0 and 100 µm<sup>3</sup>) (Fig. 4Ab). Intriguingly, the detailed analysis of this size range (Fig. 4A,b,b) showed that more than 30% of the very small MFGs in Sprn<sup>0/0</sup> milk were <10 µm<sup>3</sup> (Fig. 4Ab). Moreover, while size distribution of MFGs from FVB/NJ mice between 0 and 100 µm<sup>3</sup> was in favor of the largest ones, those from Sprn<sup>0/0</sup> mice were evenly distributed (Fig. 4AB). To what extent this difference in MGF size distribution affects milk digestibility and pups’ growth remain unknown. Nevertheless, these observations suggest that the growth of intracellular lipid droplets (CLDs), potentially linked to their apical polarity and organization of the myoepithelial cells were generally smaller in Sprn<sup>0/0</sup> mice, which may impact their size distribution.<ref>26,27</ref>

Sprn inactivation affects mouse mammary epithelial tissue development. As Sprn<sup>0/0</sup> MGFs morphological properties were altered, we investigated whether differences could be observed at the MG level. Confocal imaging on tissue sections were performed at three MG developmental stages: mid-gestation (G10) and full lactation (L10) in the presence or absence of pups. Key features such as lipid droplets morphology, apical apoptosis of mammary epithelial cells. MG sections of each genotype were labeled for Ki67, active caspase3 at mid-gestation and full lactation. At these stages, the lipid droplets, while correctly associated with the raft specific marker GM1 ganglioside, were generally smaller in Sprn<sup>0/0</sup> mammary epithelial cells with a more disperse distribution. The myoepithelial cells morphology and general organization around forming or active alveoli was markedly disturbed in Sprn<sup>0/0</sup> mice, as assessed by glycoproteins (WGA) and actin (PHA) staining. These data thus suggest that the lack of shadoo disturbs the establishment of the apico-basal polarity of mammary epithelial cells and alveoli formation.

Sprn-invalidation affects mouse mammary proliferation. We next investigated the potential incidence of Sprn<sup>0/0</sup> inactivation on mammary gland development by whole mount analyses. The selected pictures showed in Fig. 6 are representative of the mammary morphology of at least 3 animals of both genotypes. In virgin, G7.5 and lactating MGs, some mammary primary canals were enlarged in Sprn<sup>0/0</sup> tissues. In gestating and lactating tissues, Sprn<sup>0/0</sup> MGs had less and only primary side branching compared to their wild-type counterparts and were overall less dense. This suggests that Sprn-knockout is associated with developmental defects.

To consolidate these observations, we explored whether Shadoo invalidation impacts proliferation and/or apoptosis of mammary epithelial cells. MG sections of each genotype were labeled for Ki67, active caspase3 at mid-gestation and full lactation (Fig. S4). At mid-gestation both proliferating and apoptotic cells were observed at the periphery of the alveoli in the mammary tissue around forming or active alveoli was markedly disturbed in Sprn<sup>0/0</sup> mice. These results suggest that the absence of Shadoo generates an imbalance in favor of apoptosis in the growing mammary epithelial tissue, associated with a defect/delay in lumen formation.

Sprn-invalidation affects mouse mammary transcriptomic. Comparative transcriptomic analysis was investigated on G7.5 WT and Sprn<sup>0/0</sup> MGs. Fifty-three DEG were identified with a p-value < 0.05 and fc < −2 or >2, 21 downregulated and 32 upregulated (Table S5), including 8 downregulated small nuclear RNAs. Five differentially expressed genes were common between the placentas and the MGs (SLC27A2, Sndr13, Sndr45b, SLC3A1, SLC40A1, NDK1, NDUFA3, NDUG, LRP2, SERPINA10, SERPIND1, SLC22A2, SLC5A1, SLC6A9, SLC2A2, SLC3A1, STRA13, Tchb2, VDR, GIPC2, HABP2, HGD, HSD3B2, KLK3, LY96, NDUF2, PEGK1, PEG, PGLKRT, Pigl8 (includes others), SERPINA1, SLC27A2, SLC2A2, SLC3A1, STRA13, Tchb2, VDR.
Figure 4. Effects of Sprn invalidation on Milk fat globules volume and morphology. (A) (a,a') Comparative representations of the milk fat globule volumes distribution between FVB/NJ and Sprn0/0 milks. At least 3 individual milks were analyzed per genotype, as described in the Material and Methods section. (A) (b,b') Comparative representations of 0 to 100 µm³ milk fat globule distribution between FVB/NJ and Sprn0/0 milks. At least 3 individual milks were analyzed per genotype, as described in the Material and Methods section. (B) Immunofluorescence imaging of MFGs from FVB/NJ or Sprn0/0 mice at day 10 of lactation. MFGs were co-stained for for neutral lipids (green) with Bodipy 493/503 and (red) for either cytoplasmic crescents (arrowheads) with acridine orange, membranes with FM4–64, glycoproteins with wheat germ agglutinin (WGA) or free cholesterol with filipin. Bar, 10 µm.

Figure 5. Mammary gland morphology and mammary epithelial cell architecture. Confocal microscopy imaging of mammary gland sections from FVB/NJ and Sprn0/0 mice at mid-gestation (G10) and full lactation (L10, in the presence or absence (+pups) co-stained for neutral lipids (Bodipy 493/503 (bod), green), rafts microdomains (cholera toxin B subunit (GM1), red), glycoproteins (wheat germ agglutinin (WGA), green), and/or actin (Phalloidin (Pha), red). Nuclei were counterstained with nuclear marker 4′,6-diamidino-2-phenylindole (DAPI, blue). The Asterisks indicate the lumen. Bar, 10 µm.

Rnu3a, Snora73a) and 9 between the embryos and the MGs (AF357399, Hist2H2BF, MRP536, Ms4a4b, Snora23, Snora74a, Snora81, Rnu3a and Snora73a), all of them behaving similarly between the different tissues, suggestive of shared but not yet identified pathways.

Sprn-knockout induces in the MG an immune response with the upregulation of 14 immunoglobulin genes, of CD209b and the downregulation of 2 MHC genes and 1 interferon inducible gene. IPA identified 3 networks
(score > 17); Connective Tissue Development and Function, Humoral Immune Response, Infectious Disease (Table 5). It suggests that in Sprn0/0, gestating mammary epithelial cells undergo, associated with an immune reaction, a growth inhibition correlating with the whole mount observations. Search for deregulation of known

Figure 6. Mammary gland development analysis. Representative whole mount analysis of mammary gland structure from WT (FVB/NJ; a,c,e,g,i) or mutant (Sprn0/0; b,d,f,h,j) mice. It highlights defects in duct tree formation in 6-week-old virgin, in duct side-branching during early gestation (G7,5) and consequently in the density and organization of the mammary epithelium during lactation (L2: day 2 of lactation, 10: day 10 of lactation; +pups: in the presence of pups, −pups: in the absence of pups). Bar = 0.5 mm. Enlarged views of each developmental stage show the differences in mammary epithelial tissue organization between WT and Sprn0/0 mice. Bar = 200 nm.
regulators of mammary growth and branching morphogenesis revealed miR-145 overexpression in Sprn0/0 MGs. MiR-145 acts as an antitumor miRNA that targets Wnt5B whose overexpression is associated with mammary tumors. Wnt5B was indeed downregulated in Sprn0/0 MGs but at a reduced fold-change (fc −1.4, p < 0.01). CIDEA, another potential regulator contributing to the control of mammary epithelial growth, is also upregulated (Table 5).

Furthermore, upregulation of both CIDEA and PPARGC1A (Table S5) may contribute to the observed difference in milk fat globules’ size distribution. GSEA analysis confirmed an abnormal proliferation of Sprn0/0 mouse mammary tissue, highlighting a downregulation of genes related to protein localization to kinetochore (ESc4.593, FDR 2.75 × 10^−6) and to DNA replication (ESc3.4, FDR 4.01 × 10^−6).

Overall, transcriptomic analysis of Sprn0/0 gestating MG revealed the potential deregulation of pathways corroborating phenotypic and histologic analyses of this tissue. These results highlight a yet undescribed role of Shadoo in lactation.

Discussion

The biological role of the members of the prion family remains mostly unknown, despite intensive researches focusing on PrP due to its major implication in TSE and potential involvement in other more prevalent neurodegenerative diseases such as Alzheimer’s disease. Less attention focused on the related Shadoo protein, probably resulting from its recent discovery and potentially limited implication in TSE. The previously reported knockout of Sprn did not induce overt phenotypes beside a moderate, transient and sex-related, growth deficiency that could originate from a role of Shadoo in the control of feeding behavior, in association with its expression in hypothalamic neurons.

A role of PrP in mouse embryonic development, around the implantation stage and the transition from anaerobic to aerobic metabolism, was suggested as well as in the development of the nervous system and of extra-embryonic tissues. Shadoo shares with PrP some biological properties, although other biological roles are specific to this protein and/or even an antagonist to PrP. A biological redundancy between PrP and Shadoo was evocated following the observation that the knockdown of Sprn induces early embryonic lethality in Prnp−/− mouse transgenic lines. This hypothesis was weakened by the absence of phenotype of double Prnp/Sprn knockout mice. The present report reinvestigates the role of Shadoo in FVB/NJ embryonic development through the generation of knockout mice using a ZFN approach, allowing its assessment in a non-mixed genetic background. This study highlights that Sprn0/0 mice suffer from subtle, sometimes transient, but significant alterations of developmental and differentiation processes that affect both embryonic and adult tissues.

Sprn0/0 mice were viable and fertile, but analysis of the transmission rate in heterozygous intercrosses and of the average litter size in knockout homozygous crossings revealed a non-Mendelian distribution of the genotypes and a reduced number of pups, respectively. Further investigations highlighted an abnormal lethality rate of Sprn0/0 embryos at an early post-implantation developmental stage, between E4.5 and E6.5. This suggested that the absence of Shadoo is not detrimental for embryonic implantation but weakens the survival potential of the embryos early post-implantation.

Absence of Shadoo transiently also affects the growth rate of the pups in this genetic background but independently from their sexes. This slightly differs from previous observations. The difference could originate from the genetic backgrounds used in relation to the invalidation strategies. Although not formally exclusive with an abnormal feeding behavior, this transient phenotype could correlate with an abnormal biology of the placenta, as judged by our transcriptomic studies (Table 6). Invalidation of Sprn led to placental defects, recalling observations made following PrnP-knockout. However, in Sprn0/0 mice, no histological placental defect were evidenced and the few genes described as DEG in Prnp0/0 placentas were not transcriptionally affected in Sprn0/0. Our transcriptomic analysis rather suggests that Sprn0/0 extra-embryonic tissues might undergo early developmental defects, highlighted by transcriptomic alteration in E7.5 embryos of genes affecting the formation of mouse extra-embryonic tissues such as Smad1. These early potential defects seem at least partially corrected during development, as exemplified by the activation of pathways at G14.5 supporting placenta development and embryonic development.

Studies of earlier developmental stages to assess placenta development should help validating this hypothesis.

We also evidenced an impact of the absence of Shadoo on MG physiology, objectivized by (i) growth curve analyses of FVB/NJ pups fed by Sprn0/0 females, (ii) transcriptomic alterations of G7.5 mammary tissue, (iii) morphological development defects and alteration of milk fat globules synthesis that correlate with transcriptomic alterations, (iv) mammary cell/membrane alterations. Implication of the Prion protein family in the physiology of the MG per se has received little attention. Implication of PrP in mammosphere formation and repopulating activity was documented, but its relationship with lactation performances remains controversial. Of note is the

**Table 5.** Identified networks with differentially expressed genes in G7.5 mammary glands. Bold-faced genes: positively differentially expressed genes in Sprn0/0 mammary glands. Italized genes: negatively differentially expressed genes in Sprn0/0 mammary glands.
heterozygous mice were used to derive transgenic knockout lines. Mutations.

amplified 400 bp long DNA genomic region surrounding the ZFN target site was sequenced to search for putative

elevation should occur. Absence of such a scenario in that of 

Sprn0/0 NJ pups fed either by FVB/NJ or females as foster mothers. Lactation performance was indirectly assessed by analysis of growth curves of FVB/

Sprn0/0 -knockdown/Prnp0/0 females. Six pups were placed under each lactating females and 3 females

Summary of observed phenotypes and transcriptomic alterations in Sprn0/0 mice.

| Offspring | Phenotype | Transcriptomic |
|-----------|-----------|----------------|
| Foetus (E7.5) | Early post-implantation increased lethality rate, growth retardation | Developmental disorder, cell-to-cell signaling and interaction, cell growth and proliferation, immunological and/or hematological disease, organismal survival |
| Placenta (G14.5) | None detected | Lipid metabolism, molecular transport, small molecule biochemistry, tissue development and cellular movement |

Table 6. Summary of observed phenotypes and transcriptomic alterations in Sprn0/0 mice.

absence of any mammary phenotype described so far for Prnp0/0 mammals. In the present study, invalidation of Shadoo appears to induce various gestational/lactation mammary tissue and cellular defects that results in transient lactation deficit.

This phenotype exemplified Shadoo implication in the control of cellular proliferation and differentiation, also revealed at early post-implantation embryonic stages. Such an involvement in cell proliferation and differentiation was highlighted for PrP31. Implication of these two related proteins in similar biological functions alongside their expression in developing mammalian fetuses could substantiate the hypothesis of their biological redundancy. Surprisingly, our data rather suggest that Sprn and Prnp exert opposite effects on expression of master regulators of pluripotency and/or embryonic development, at least at E7.5. Thus, one could expect their co-invalidation to be neutral, as documented32. However, comparison of the data described here and for Sprn-knockdown/Prnp0/0 embryos10,11 highlights a developmental delay in the embryonic lethality between Sprn0/0 and Sprn-knockdown/Prnp0/0 embryos. Lethality of Sprn0/0 embryos occurs early after implantation while that of Sprn-knockdown/Prnp0/0 embryos was evidenced only at E10.536, with E7.5 decidua carrying abnormal embryos31. It could suggest that Sprn-knockdown induces a fragility of the embryos at implantation that becomes detrimental to the further development of the Prnp0/0 ones, between E8.5 and E9, when upturn of PrP RNA expression should occur32. Absence of such a scenario in Prnp0/0/Sprn0/0 mice might result from genetic adaptation32.

In conclusion, this study provides evidence for a yet controversial role of the Prion-related protein Shadoo during mouse embryogenesis and more generally in the control of cellular proliferation and differentiation of various tissues, including adult ones such as the mammary epithelium. It also highlights a potential embryonic antagonist action of PrP and Shadoo, revisiting alongside recent papers the current view of a potential biological redundancy between these two proteins.

Methods

Ethics statement. All animal experiments were carried out in strict accordance with the recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 2016/63/UE). All efforts were made to minimize suffering. Experiments were approved by the INRAE local animal experiment ethics committee of Jouy-en-Josas (Comethea, Permit Number 02532.01). All animal manipulations were done according to the recommendations of the French Haut Conseil aux Biotechnologies, HCB (Permit Number N°6461 and DUO N°5468).

Generation and establishment of Sprn-knockout mice. A preparation of mRNA encoding a ZFN targeting the start of the Sprn ORF was purchased from Sigma-Aldrich Corp (CompoZrTM ZFN design). The ZFN target site was 5′-cttgacagcttgccgcanwgccgcggcggagccgcggttg-3′. ZFN mRNA was injected at 2 ng/μl into 1 cell-fertilized FVB/NJ mouse eggs. Surviving injected eggs were transferred into pseudo-pregnant recipient mice. Tail-DNA analysis of the 29 resulting live pups was performed by PCR using oligonucleotides 5′-gcctattcccaagccctgatac-3′ and 5′-gccggccttacgcgtactcttga-3′. PCR conditions were made of 40 amplifications cycles; 94 °C-30 s, 60 °C-30 s and 72 °C-30 s. The amplified 400 bp long DNA genomic region surrounding the ZFN target site was sequenced to search for putative mutations.

Transgenic founder mice were crossed with FVB/NJ mice to establish transgenic lines. Intercrosses between heterozygous mice were used to derive transgenic knockout lines.

Reproductive performance analyses. All examined females were on their first pregnancy or lactation. Interccrosses between heterozygous mice and between homozygous mice were performed and the number and genotype of pups recorded. To assess embryonic lethality, pregnant FVB/NJ or Sprn0/0 females, following matings with males of the same genotype, were euthanized at different gestation stages. Implantation points were recorded. Development and vitality of the embryos were observed under binocular microscope.

Comparative analysis of growth curves of FVB/NJ and Sprn0/0 pups was performed, using FVB/NJ lactating females as foster mothers. Lactation performance was indirectly assessed by analysis of growth curves of FVB/NJ pups fed either by FVB/NJ or Sprn0/0 females. Six pups were placed under each lactating females and 3 females.
in their first lactation of each genotype analyzed. Milk samples were collected at L7.5 on other lactating females following pups removal for 3 hours and 300 µl – 10 UI intra-peritoneal injection of oxytocin.

**RNA purifications and RT-(q)PCR.** Total RNA was isolated from (i) E7.5 mouse embryos, (ii) E14.5 placentas and (iii) G7.5 MGs (first pregnancy). Six gestating females of each genotype were used at each analyzed stages. Individual RNA extractions were performed using the RNeasy Lipid Tissue Mini kit (Qiagen cat # 75842). RNA concentration was calculated by electro-spectrophotometry and the RNA integrity checked with the Agilent Bioanalyzer (Waldbroom, Germany). All extracted RNA samples had a RIN value > 8.5.

Reverse transcription was performed on 5 µg of total RNA, using Invitrogen SuperScript™TM reverse transcriptase kit (18080051) or RTVilo (11754050) and random primers, according to the manufacturer's instructions. Amplification of the mouse Sprn cDNA ORF was performed using oligonucleotides 5′-taggtcttgacatcctgctc-3′ and 5′-gagttagctgcttgctagaagc-3′, that are located on the two separate Sprn exons to avoid amplification of potential contaminating genomic DNA. PCR conditions were made of 40 amplifications cycles; 94°C-30 s, 60°C-60 s and 72°C-30 s, using GC-rich adapted PCR amplification kit (KAPA2G Robust PCR kit, SigmaAldrich). Two WT and two Sprn00 mouse brains were analyzed.

For RT-qPCR, quantification was performed on triplicates, using 3 different RNA samples for each genotype, using the ABsolute Blue QPCR SYBR Green ROX Mix (Thermo Scientific) and standard PCR conditions. Primers were designed on separate exons, using Primer 3 software (http://primer3.ut.ee) to produce 100-bp amplicons, with a Tm of 60°C. The TBP and SDHA genes were used for normalization, using primers 5′-ttccggaatcagagaaa-3′ and 5′-gctttgttcctgtctcatacg-3′, GC using primers 5′-gcaagacgctctggttcagc-3′ and 5′-aggtggtcgcagaactttc-3′, Gli3 using primers 5′-tccccgagagatgtatc-3′ and 5′-tgagctgtcatgttgactgc-3′, TPBA using primers 5′-tgaagggctgaacccatgg-3′ and 5′-ccagctgaatagctg-3′ and 5′-ggccagtgcaaggtgctc-3′.

**Western blot analysis.** Following mouse cervical dislocation, FVB/NJ and Sprn00 mouse brains were crushed in 0.32 M Sucrose solution containing cocktail of protease inhibitors (Roche) and enriched in subcellular membranes by ultra-centrifugation (150 000 × g) as described in Westaway et al. (2011). Proteins were extracted from membrane pellets by N-PER Neuronal Protein reagent (Thermo). Amounts of 50 µg sample proteins were separated on a home-cast 14% polyacrylamide gel SDS-PAGE and were transferred on PVDF membrane by the Trans-blot Turbo (Bio-Rad). Non-specific reactivity was blocked by incubation for 1 h with a 5% skimmed milk in 0.1% TBS-T for two hours at 4°C. Shadoo, obtained from Covalab (https://www.covalab.com/), at 1/750e dilution in 0.1% TBS-T for two hours at room temperature or with other antibodies directed against Shadoo that are listed in the text at recommended dilutions. Horseradish peroxidase-conjugated anti-IgG from various origins according to the primary antibody were used as secondary antibody with 1 h incubation. Excess first and second antibodies were removed by washing 3 × 10 min in TBS-T buffer. Detection was accomplished with a chemiluminescence system (ECL Prime, GE Healthcare) and exposure in ChemiDoc Touch device (Bio-Rad).

Fibrinogen and α1-antitrypsin protein ratio between wild type and Sprn00 mouse were estimated in placenta samples with Image Lab software 5.2 (BioRad). They were crushed with ultra-turrax in N-PER Neuronal Protein reagent containing cocktail protease inhibitors (Roche). The experimental protocol was followed as described above according recommended factor dilutions, using Abcam ab189490 and Abcam ab231093 antibodies, respectively.

**Milk collect and analysis.** Milk was collected from L7.5 Sprn00 and FVB/NJ females on their first lactation. Milk protein concentration was estimated using Bradford’s procedure and fractionation of its proteins was performed on home-cast SDS-PAGE 12.5% polyacrylamide followed by coomassie blue staining. A western blot was performed with an Axio observer Z1 microscope (Zeiss) equipped with standard filters for FITC, Rhodamine and DAPI emissions, using a 40x objective and a Greyscale CCD camera CoolSnap HQ2 camera (Photometrics) coupled to the Axiosvision imaging system software. About thirty pictures of each condition were randomly acquired for each condition and both the number and the perimeter (µm) of the MFGs were estimated using ImageJ (1.47q software 2012, http://rsb.info.nih.gov/ij/). The volume of the MFGs (µm3) were calculated and a Kruskall-Wallis rank test was performed using the version 3.5.2 of the R Software (R core Team 2018).

**Placenta histological analysis.** Immediately after dams’ sacrifice by cervical dislocation at G14.5 or G17.5, the uterus were removed and the placentas collected. Intact placentas (6 per genotype and stage from 3 different dams) were fixed in formalin (48 hours). After fixation, each placenta was bisected adjacent to the...
umbilical vessels. Both halves were embedded in paraffin. Section (3–5 µM) from each placenta were processed for Hematoxylin-Eosin coloration and examined by a qualified veterinary pathologist.

**MG whole mount and immunohistological analyses.** Experiments involved analyses of at least 3 mice per genotype and condition. Inguinal MGs (4th, left) were excised, spread on glass slides and fixed in Carnoy’s fixative (100% EtOH, chloroform, glacial acetic acid; 6:3:1) for 4h at room temperature, washed in 70% EtOH for 15 min, rehydrated in water, and stained in carmine alum (2% carmine and 5% aluminum potassium sulfate in water) overnight. Tissues were then gradually dehydrated through serial ethanol baths and cleared in xylene overnight. MGs were kept in Permount Mounting Media (Fisher Scientific) until images were captured.

For immunohistological analyses, mouse MGs were collected at different physiological stages and processed as described26. Tissue sections were labeled using Alexa488-conjugated WGA, rhodamine-conjugated phalloidin (Thermo Fisher), or filipin (Sigma-Aldrich) diluted in PBS for 30 min at 4°C. Neutral lipids and nuclei were counterstained with BODIPY 493/503 or Nile Red and DAPI (4’-6-diamidino-2-phenylindole), respectively. Slides were mounted with Vectashield (Vector Laboratories LTD, United Kingdom) and stored at 4°C until observation. For some experiments, Tissue sections or MGs were also labeled for actin with rhodamine-conjugated phalloidin (Molecular Probes), GM1 ganglioside with Alexa 594–conjugated CTxB (Invitrogen), or free CH using filipin (Sigma-Aldrich). Each experiment was performed at least twice and included negative control without primary antibody. At least 20 images were randomly acquired per condition tested and further analyzed. All images were analyzed using ImageJ 1.47 software (http://rsb.info.nih.gov/ij/).

**Transcriptomic analyses.** Transcriptomic analyses were performed using Affymetrix MouseGene 2.0ST microarray on the Institute Cochin Genomic’s platform. Each analyzed embryonic RNA sample derived from the equal mixing of 6 individual total RNA preparations from E7.5 embryos. Different embryonic samples were used for each condition. Similarly, each analyzed placenta RNA sample derived from the equal mixing of 6 individual total RNA preparations from E14.5 placentas. Different individual samples were used for each mixture. Each MG RNA sample derived from the equal mixing of 3 individual total RNA preparations from G7.5 MGs. Different individual samples were used for each mixture. Three biological replicates per tissue and genotype were performed. Differentially expressed genes between FVB/NJ and FVB/NJ Sprn−/− were identified following normalization of the raw data, with fold change ratios and p values determined as described in the results section. Data were analyzed by Gene set enrichment analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp). Differentially expressed genes were clustered and classified in pathways and networks by using Ingenuity (http://www.ingenuity.com/).

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Conception of the Work: V.B., A.D., K.M.G., P.C., B.P. and J.L.V. Design of the work: B.P., S.M., J.C., O.A., A.D., K.M.G., J.L.V. Acquisition, analysis and interpretation of the data: B.P., J.C., S.M., S.T., A.V., S.F., C.B., A.L.G., L.H., Ma.B., O.A., D.V., M.V., S.M.R., P.C., M.M., N.D.C., D.L., Mo.B. Drafted the work: V.B., J.L.V., D.V., S.M.R., A.D., K.M.G.

Competing interests

The authors declare no competing interests.

Additional information

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