Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science

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Summary 1-

The prion family comprises of Prion, Shadoo and Doppel proteins, encoded by *Prnp*, *Sprn* and *Prnd*, respectively.Expression pattern of these genes in early mouse embryogenesis was investigated using RT-PCR experiments on FVB/N mice embryosand by in situ hybridization (HIS) at 3 early developmental stages. They were found to be expressed at all stages, in both placenta and embryo.Ubiquitous hybridization was observed for the three lociby HIS. These results are compatible with potential biological overlapping roles of these proteins during mouse embryogenesis.

We also analyzed the transmission of an LS1.06 transgenic mice line, characterized by a 70% downregulationof*Sprn*gene expression.Only 5% of offspring from FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ with FVB/N *Prnp*$^{-/-}$crosses were of a FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$ genotype, suggestive ofa lethal-associated embryonic phenotype. However, crossing FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$with *Prnp*$^{-/-}$ showed a Mendelian transmission rate. This suggested a physical linkage between the transgene integration site and *Prnp* locus. Indeed, the transgene integration site was found to be located within an intron of the mouse *Api5* gene, at 37.5 Mbof the*Prnp* locus. It thus likely explains the observed transmission rates.

Keywords- Prion-family, Shadoo, Transgenesis, Mouse, Embryogenesis

Summary 2-

Prions are infectious proteinaceous particles responsible for transmissible spongiform encephalopathies (TSE).The pathology and clinical disease associated with TSE are brought about by conversion of the cellular form of prion proteinPrP^{C}, encoded by *Prnp,* to an infectious isoformPrP^{Sc}. Along with PrP^{c}, two other structurally-related proteins, called Doppel and Shadoo, encoded by *Prnd* and *Sprn*, havebeen identified as its paralogs. These three genes constitute 'the prion family'. However, the biological roles and possible biological redundancy between these proteins remain mostly enigmatic although a potential involvement during early embryogenesis was suggested. The first aim of my study was to investigate the expression pattern of the Prion gene family in early mouse embryogenesis to look for potential overlaps between them and to assess their putative role. Expression analysis of the three genes was done by RT-PCR experiments on FVB/N andFVB/N *Prnp*$^{-/-}$ embryosand by *in situ* hybridization (HIS) at three developmental stages. RT-PCR experiments showed lower expression pattern of *Prnd* and *Sprn*compared to*Prnp*. Expression was observed in all studied stages, in both placenta and embryo, except in *Prnp*$^{-/-}$ for *Prnp.*Ubiquitous hybridization was observed for the three lociby HIS.These results are compatible with potential biological overlapping roles and expression of these proteinsand also point out a potential role of Doppel in early embryonic stages. It would be of interest to assess the phenotypic consequences of the invalidation of *Sprn* and *Prnd*, and of the three loci. Comparative histological analysis was done between E7.5 FVB/N and FVB/N *Prnp*$^{-/-}$ mouse embryos. The phenotype was similar except for the observation of hemorrhagic foci in front of ectoplacental cone in FVB/N.This could be due to the involvement of PrP^{C} in biological pathways like angiogenesis, inflammation and cell mobility.

The second aim of my study was the analysis ofthe LSI.06 transgenic line and of thetransmission of its transgene onto *Prnp*$^{-/-}$genetic backgrounds. This line expresses ubiquitously a ShRNA against *Sprn* and has 70% downregulation of *Sprn* expression as observed in the adult brain. Transmission studies weredone by crossing FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ with *Prnp*$^{-/-}$ mice. FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$and LSI.06$^{-/-}$-*Prnp*$^{+/-}$offsprings were less than 5% and 16%, respectively. LSI.06$^{+/-}$-*Prnp*$^{+/-}$ mice were also crossed with C57/129/Sv *Prnp*$^{-/-}$animals to analyze the incidence of the genetic background associated with *Prnp*$^{-/-}$. There was again a non-Mendelian transmission with deficiency in LSI.06$^{+/-}$-*Prnp*$^{-/-}$ and LSI.06$^{-/-}$-*Prnp*$^{+/-}$ offsprings.FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$were crossed with FVB/N *Prnp*$^{-/-}$ mice to analyze secondary transmission rate of the LSI.O6 transgene. It showed a Mendelian

transmission rate. This data suggested a physical linkage between theLSI.06 transgene integration site and the*Prnp* locus rather than a lethal-associated embryonic phenotype. However, assessment of the fecundity of such mice, including growth rate and robustness of pups might give us a possible clue for what are the further effects of such introgression.The transgene integration site was cloned and found to be located within the last intron of the ORF of the mouse *Api5* (apoptosis inhibitor 5) gene,at 37.5 Mbof the*Prnp* locus. It thus likely explains the observed transmission rates.

INTRODUCTION

1. The Prion: a non-conventional transmissible agent

Prions are highly infectious particles which are composed entirely of protein and lack nucleic acid, making them different from other infectious agents. In humans and in animals they are responsible for a range of neurodegenerative diseases collectively called as transmissible spongiform encephalopathies (TSE's). However,the role of prions in TSE's remained unidentifiedfor a long time. The agent responsible for TSE's was initially considered to be a slow virus (Sigurdsson 1954). Later it was proposed that it lacks nucleic acid, besides being too small in size to be a virus (Alper *et al.,* 1966). Also the agent was found to be inactivated by treatment with phenol, urea, proteinase K (i.e. the procedures that inactivate proteins) and was stable or resistant to UV irradiation, treatment with nucleases, divalent cation hydrolysis, heat inactivation(i.e. the procedures that intervene nucleic acids). Thus, this 'viral hypothesis' could not be proved. Subsequently in 1982, the term 'Prion' was coined by Prusiner and 'Protein only' hypothesis was accepted much later with much debate and attempts to disapprove it (Prusiner 1982, 1998) In animals TSE's include scrapie in sheep, which has been prevalent in Europe for more than 200 years and is also recognized in countries worldwide. Other important diseases are transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, bovine spongiform encephalopathy (BSE), feline spongiform encephalopathy in domestic cat, exotic ungulate encephalopathy. Human Prion diseases comprise of Creutzfeldt-Jakob disease (CJD) which includes iatrogenic CJD, variant CJD, probably arising from BSE-contaminated food, familial CJD, and sporadic CJD. Other human prion diseases are Gerstmann Straussler Sheinker disease, fatal familial insomnia, fatal sporadic insomnia syndrome and Kuru.

All these diseases are of genetic, sporadic (via spontaneous mutations or due to higher genetic susceptibility to the infection) or infectious origins (via ingestion of prion contaminated food, scarred skin or iatrogenic). These infections typically remain asymptomatic for years, with the disease being always progressive and fatal once the clinical signs develop (Prusiner, 1998). The diseases are characterized commonly by neurological symptoms like ataxia in animals and progressive dementia in human commonly (Wells *et al*., 1987).

The histopathological description includes spongiform vacuolization of brain grey matter, neuronal loss, astrogliosis, and accumulation of amyloid plaques, which is common to both animal and human conditions (Collinge, 2001). These abnormal changes are brought about by post translational conversion of cellular form of prion protein (PrP^{C}) to an infectious isoform called PrP^{Sc}.Abnormal isoform of PrP, i.e. PrP^{Sc},has higher proportion of β sheets rather than α helical coiled sheets,that is normally present in PrP^{C} specially in the region between residues 90 and 140, leading to aggregations in the form amyloid fibres and plaques (Huang *et al.,* 1996, Prusiner, 1998).

The mechanism of conversion of PrP^{C} to PrP^{Sc} is incompletely understood. Amongst the several mechanisms proposed, one called 'heterodimer mechanism' elucidates that PrP^{Sc}acts as a template to guide or catalyse the conversion and misfolding of PrP^{C} to PrP^{Sc} (Cohen *et al*., 1994). The second proposition states that a mixed aggregation of PrP^{C} and PrP^{Sc} leads to an auto catalytic self propagation of $PrP^{Sc.}$(Bieschke*et al.*,2004).Another mechanism for the replication of prions is the nucleation-polymerisation model. According to this, PrP^{Sc} is actually a polymorphic form ofmonomeric PrP^{C}. Monomeric form of prion protein polymerizes to oligomers forming an unstable nucleus. This process remains at an equilibrium until a critical concentration of monomers is outreached. Following this event, along with addition of an 'infectious seed', another monomeric prion protein unit amplifies the polymerisation process leading to stable aggregates and amyloid plaques in brain (Harper & Lansbury 1997).

The exact nature of the neurotoxicity associated with PrP^{Sc} remains poorly understood. Mice lacking PrP^{C} expression in their entire body or only in their brain demonstrate no neuro-degeneration even in the presence of exogenous PrP^{Sc}, indicating an absolute requirement of PrP^{C} for pathological lesions associated with the prion diseases (Bueler *et al.,* 1993, Brandner *et al.,* 1996). Similarly, neuronal PrP^{C} depletion in adult TSE-inoculated mice induces the disappearance of the clinical signs and a partial recovery despite accumulation of large amount of PrP^{Sc} and of infectivity (Mallucci *et al.,* 2002), highlighting the specific neuronal PrP^{C} involvement in the pathology. Moreover, accumulation of atypical PrP^{Sc} and little clinical manifestation of the disease were shown in mice expressing relatively moderate levels of PrP^{C} without GPI anchor rather than the normal PrP^{C} with GPI anchor (Chesebro *et al.,* 2005). At moderate expression levels, scrapie infection induced a new fatal disease with unique clinical signs and altered neuropathology in these PrP-anchorless expressing

transgenic mice (Chesebro *et al.*,2010). At even higher levels of expression, transgenic mice expressing anchorless-PrP develop spontaneous neurologic dysfunction characterized by widespread amyloid deposition in the brain (Stohr *et al.*, 2011). Thus, both the cellular location of PrP and its expression level appears to influence its potentiality to sustain or induce neuropathologies.

2. The biological functionof PrP remains enigmatic

Despite its well documented involvement in spongiform encephalopathies, the biological role of PrP^C remains mostly unknown.PrP^C is coded by a conserved gene *Prnp* in mammals, birds, fish and has also been identified in *Xenopus laevis* (Aguzzi and Calella, 2009). Located on chromosome 20 in humans and chromosome 2 in mice, it is normally expressed on the cell membranes throughout the body and at higher levels in neurons, lymphocytes (*Cashman et al,. 1990)* and stromal cells of lymphoid organs (*Kitomotoet al.*, 1991). One difficulty for deciphering the biological function of PrP comes from the observation that the *Prnp*($Prnp^{-/-}$) knockout mice live normally and don't exhibit any immunological defects (Bueler *et al.*, 1992). Knockout cattle and goat were similarly obtained with no obvious associated phenotype. Careful phenotyping of the knockout mice revealed some subtle neuronal alterations. Amongst the many subtle defects in PrP null mice is disruption of calcium and potassium ion channel in hippocampus (Collinge *et al.*, 1996). Additionally, mice post natally ablated for prion protein are found to be having electrophysiological defects like decreased in after hyperpolarization in hippocampal CA1 cells (Mallucci *et al.*, 2002). PrP^C knockout mice have also been shown to exhibit an altered circadian rhythm. The behavioral change in fatal familial insomnia might indicate that PrP^C has a possible role in maintaining normal circadian rhythm (Tobler *et al.*, 1996).However, while PrP^C was reported to aid a normal synaptic transmission (Collinge *et al.*, 1994), *Prnp* null mouse have normal synaptic transmission in CA1 hippocampal region (Lledo *et al.*, 1996).

PrP^C appears to be involved in adult mammalian neural development and neurogenesis in dentate gyrus and olfactory bulb. But other factors are also involved in neurogenesis, since regardless of expression of PrP^C the final number of neurons in the dentate gyrus remained the same (Steele *et al.*, 2005). Lethal irradiation of PrP knockout mice impaired the self renewal of haemopoetic stem cells and made them more sensitive to depletion following the treatment with cell cycle specific myelotoxic agent. Exogenous expression of PrP^C through reteroviral

infection rescued this effect (Zhang *et al.,* 2005). This and other data suggest a role of PrP in stem cell biology (Lopes and Santos, 2012). However, its precise role remains uncertain and appears to differ between the tissue and/or the species origin of the stem cells. It has been shown that PrP^C is involved in the transduction of specific signals via for example the activation of the tyrosine kinase Fyn (Mouillet-Richard *et al.,* 2000) and the negative regulation of the signaling activity of β1 integrins (Loubet *et al.,* 2012), both involved in neuritogenesis.

PrP^C has also neuroprotective properties. In human neurons, PrP^C counteracts Bax induced cell apoptosis. Provided surface localization of PrP^C and its transport are adequate, this anti-apoptic activity of PrP^C is retained in its secretory form (i.e. PrP^C lacking GPI anchor).
Moreover, following a cerebral hypoxia and ischemia in humans and in rodents, PrP^C expression is up-regulated. In case of induced cerebral hypoxia, the PrP null mice had a greater infarct size, while in case of induced ischemic damage the severity of ischemia affects the amount of PrP up-regulation, consequently the severity of lesion and neuronal damage. (McLennan *et al.*, 2004, Weise *et al.*,2004). This ischemic injury was later found out to be decreased on over expression of PrP^C by adenovirus mediated gene targeting (Shyu *et al.,* 2005). It indicates an important protective action of PrP^C and its possible involvement in stroke biology.

A role of PrP^C in the modulation of the calcium dependent embryonic cell adhesion and of efficient gastrulation has been demonstrated in zebrafish (Malaga-Trillo *et al.*,2009). Transcriptomic alteration during early embryogenesis in PrP null mice highlighted similar biological pathways, although with no adverse visible phenotype (Khalife *et al.,* 2011). The possible role of PrP^C during embryogenesis, suggested by its developmental regulation (Tremblay *et al.,* 2007, Miele *et al.,* 2003), is perhaps a clue to why *Prnp* is a evolutionary conserved gene

To explain how a protein such as PrP is evolutionary conserved, putatively implicated in embryogenesis and neuritogenesis, is neuroprotective while PrP^{Sc} is neurotoxic, and that *Prnp* genetic invalidation has little phenotypic consequences in mammals, it was hypothesized that

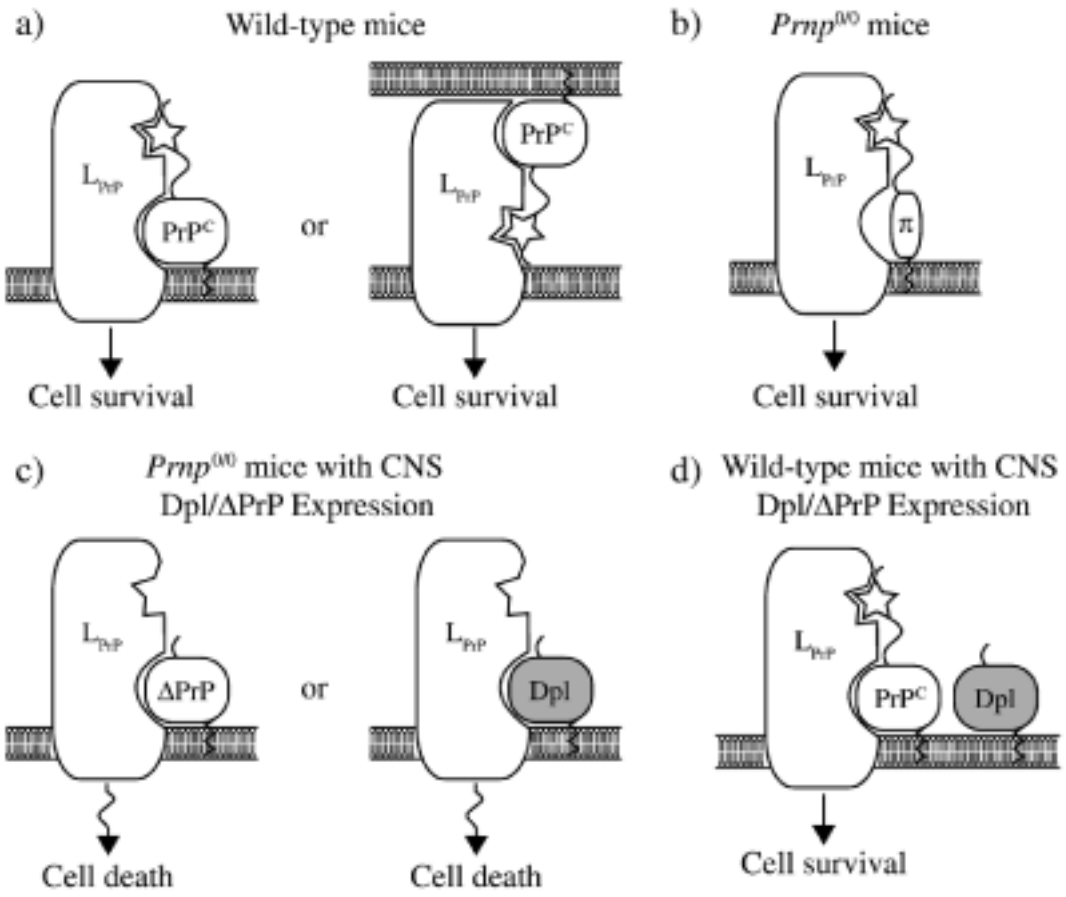


Figure 1. The LPrP model of functional interactions between prion proteins in transgenic mice. (a) In wild-type (Prnp+/+) mice, initiation of unidentified signaling event following PrP^c binding (in cis or trans configuration) to a hypothetical ligand (LPrP) favours cell survival. A C-terminal anchoring site and an N-terminal effectors' site are the two implicated binding sites of PrP^C for LPrP which enable signaling. (b)π , a hypothetical PrPC-like protein shares common N-terminal effectors' domain with PrP^C thar binds to LPrP initiating the favorable signaling event in the absence of PrP^C in *Prnp*$^{0/0}$ mice. (c) Since N terminal effectors' domain is required for pro-survival signaling, following Doppel or ΔPrP binding to LPrP in *Prnp*$^{0/0}$ mice initiates 'improper' signaling leading to cellular death. (d) PrPC possesses higher affinity for LPrP which reduces Doppel/ΔPrP binding in *Prnp*$^{+/+}$ mice (This figure was taken from Shmerling *et al.*, 1998)

another host-encoded protein, called π, can bind to a putative PrP-receptor at the cell membrane and induces similar transducing pathways in the absence of PrP (Figure 1, Shmerling *et al.,*1998).

3. PrP belongs to a gene family.

The prion gene phylo-genetically derives from a ZIP-like ancestral molecule of metal ion transporters (Schmitt-Ulms *et al.,* 2009). Besides PrP^C, two other closely structurally-related proteins called Doppel and Shadoo have been identified as its paralogs comprising what is called the 'prion gene family'. The biological functions of these two proteins and their interactions remain equally elusive. Like PrP^C, both Doppel and Shadoo are GPI anchored proteins.

Comparable to PrP^C, Doppel has 3 α helices, shares 24% sequence identity and has a similar C terminal domain to that of PrP^C. In contrast, Shadoo has a similar N terminal half and hydrophobic tract to that of PrP^C. Both the proteins lack octarepeat regions.(Figure 2, Watts and Westaway 2007)

Doppel which is encoded by the gene *Prnd* is located 16 kb dowstream to *Prnp.* In adult male humans, rodents, and boars it is mainly expressed in testis, ejaculated spermatozoa, seminal plasma, semeniferous tubules, sertoli cells, with there being a variation in level of expression. Also during embrygenesis and neonatal life it is reported to be expressed at low levels in brain and circulating lymphoid cells, B cells, neutrophils and in follicular dendritic cells. (Moore *et al.,* 1999, Serres *et al.,* 2006)

Doppel is important for maintaining male fertility. $Prnd^{-/-}$ and $Prnd^{-/-}$- $Prnp^{-/-}$ double knockout mice both develop normally, but, due to an absence of acrosome reaction, their spermatozoides are incapable of fertilizing oocytes noramally *in vivo*. At a decreased rate *in vitro* fertilization could the carried out. There is an elevation in the oxidative DNA damage of spermatozoa (Paisley *et al.,* 2004) The importance of Doppel for the maintenance of fertility was confirmed by another group as well in which the *Prnd* null mice had low number of spermatoza, were morphologically abnormal and immotile (Behrens *et al.,* 2002).
Due to its stuctural similarity and close distance from *Prnp* gene, it could appear logical to assume that Doppel, along with PrP^C, is also involved in TSE pathogenesis. However, in a

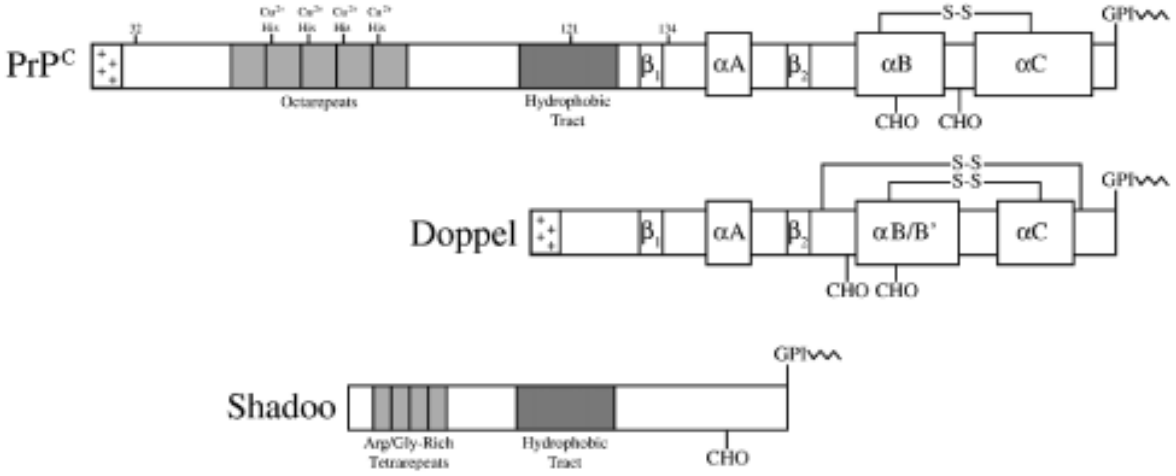


Figure 2. Schematic representation of the domain architecture of the prion protein family members. Doppel and PrP^{C} have structured C-terminal domains consistingof 3 α-helices and 2 short β-strands and basically-charged N-terminal regions. Disulfide bridges are indicated above the proteins (–S–S–) and N-glycosylationsites (CHO) are denoted below the proteins. $PrP^{C}$and Shadoo share a common hydrophobic tract. PrP has octarepeats that can bind copper, while Shadoo arginine and glycine richtetrarepeats (This figure was taken from Watts & Westaway 2007).

mice affected by TSE the PrP^{Sc} mass and dissemination, incubation period along with extent of vacuolization is not affected by the degree of expression of *Prnd* in the CNS, nor does this degree of expression of *Prnd*has been shown to have any impact on the course and pathogenesis of TSE (Tuzi *et al.,* 2002). This has been found to hold true in case of CJD patients too, where the disease pathogenesis is unaffected by the presence or absence of Doppel expression (Peoc'h *et al.,* 2003).

Doppel was also shown to be neurotoxic causing neurodegeneration. Although normally Doppel was shown to be expressed in low amounts in adult brain, its expression gets up-regulated in some PrP^{C} null mice leading to development of late onset ataxia and purkinje cell loss (Moore *et al.,* 1999). It has been demonstrated that the higher the expression of Doppel is in the brain, higher is the extent of purkinje cell loss and earlier is the onset of ataxia (Rossi *et al.,* 2001). Interestingly, this Doppel induced neurotoxicity could be rescued by PrP^{C} (Masterangelo and Westaway, 2001).

The gene encoding the second PrP^{C} paralog Shadoo is on chromosome 7 in mice. It is expressed in adult CNS in rodents, sheep, bovines and humans. However the degree and exact location might vary between the species (Daude and Westaway 2011). It has also been shown to be expressed in testicle and ovary in mice (Young *et al.,* 2011). Interestingly, Shadoo's (Sho) expression is higher in dendrites than in cerebellar granular neurons which is exactly the contrary to PrP^{C} suggesting of functional redundancy. Sho also shows functional overlaps and share common protein binding partners with PrP^{C}. Amongst these common functions is the neuroprotective properties against Doppel and N terminal truncated PrP^{C} (Figure 3, Watts *et al.,* 2007). Sho has further been displayed to be down regulated in disease specific manner during TSE and accumulation of PrP^{Sc} in CNS, pointing towards the fact that it might act as a stand by substrate for PrP^{Sc}. Additionally α-Sho antibodies generated against Sho could be used as a tool to measure it's expression and serve as a diagnostic tool earlier in the course of disease (Westaway *et al.,* 2011).

Knockdown of *Sprn* gene expression by RNA interference using lentivirus vectors revealed early embryonic lethality in FVB/N $PrP^{-/-}$ but not in FVB/N mice suggesting that either PrP or Sho is required for normal mouse early development (Young *et al.,* 2009). Furthermore, origin of this lethality could be correlated with a failure of the trophoectoderm cell lineage by

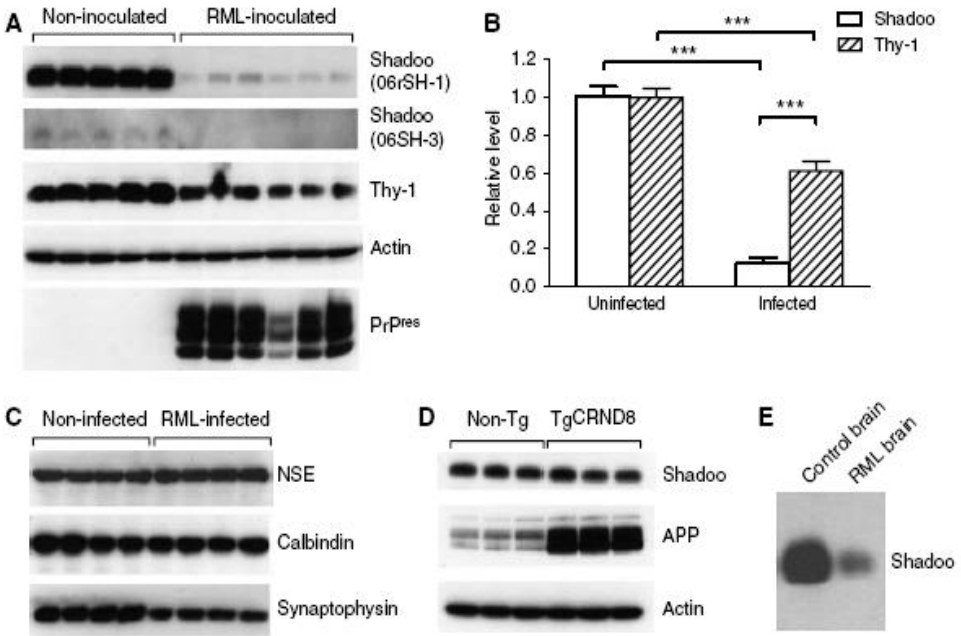


Figure 3. Reduced Sho levels in clinically ill prion-infected mice. (A) Western blot of homogenates prepared from the brains of non-inoculatedor clinically ill (average of 172 days post-inoculation) RML prion-inoculated mice (C3H/C57BL6 background). Remarkable reduction of Sho protein levels in prion-infected brains. For comparison purpose levels of the GPI-anchored protein Thy-1 are shown. (B) Quantitation of Sho(06rSH-1) and Thy-1 blot signals in panel A by densitometry. Sho levels in prion-infected brains are reducedto 12.172.8% (Po0.001) the levels observed in non-inoculated mice. ***P<0.001. (C) Western blot analysis to assessexpression of neuronal markers in prion-infected andcontrol mouse brains. In prion-infected brains, neuron-specific enolase (NSE) or calbindin levels are unchanged, and synaptophysin levels are decreased moderately. (D) Brain homogenates of clinically ill (8 months old) Tg mice (TgCRND8) exhibiting familial Alzheimer's disease-associated variant of the amyloidprecursor protein and control non-Tg littermates show no change in Sho levels. (E) Normalized brain homogenates in a second cohort of RMLinoculatedmice show reduced Sho expression versus control mice injected with a brain homogenate from healthy mice (C57BL6 background, 154 days post inoculation). (This figure was taken from Watts *et al.*, 2007)

targeted delivery of the lentivirus (Passet *et al.,* 2012). Transcriptomic analysis highlighted synergetic and convergent roles of PrP and Sho at these early developmental stages. These data could suggest that Sho is the hypothesized π protein.

The knockdown of *Sprn* suggested that the prion protein family members play a crucial role in mouse embryogenesis during the developmental window E7.5 – E11 (Young *et al.,* 2009). Although expression of different members of the prion gene family has been reported during mouse embryogenesis, these studies either focused on later developmental stages (Miele *et al.,* 2003) and/or were indirect, using reporter transgenes (Tremblay *et al.,* 2007, Young *et al.,* 2011). Furthermore, very little information, if any, describes the developmental regulation of *Prnd* during mouse embryogenesis.

The first aim of my study was to further study the expression pattern of the prion protein gene family in the early stages of mouse embryonic development which remains poorly describe, especially for Sho and Doppel (Watts *et al.,* 2007, Young *et al.,* 2011). Such data could provide information on whether or not these genes have redundant function during embryonic development.

The second aim of my work was to study a specific mouse transgenic line LS1.06. Mouse from this line expresses a shRNA targeting *Sprn*. On FVB/N adult mice; it results in a 70% down-regulation of the brain level of expression of the *Sprn* gene (Young *et al.,* 2009 and unpublished data). It was observed that this transgene was difficult to transmit to FVB/N $PrP^{-/-}$ mice. Origin of these difficulties was further assessed to determine if it could be associated with an embryonic lethal phenotype.

MATERIALS AND METHODS

Classical molecular biology manipulations, such as plasmid and genomic DNA purifications, restriction enzyme digestions, agarose gel electrophoresis were done according to Maniatis *et al.,* 1982. In this section, we will only mention experiments that involved using specific kits or adapted procedures.

I. Histological analysis-

Collected mouse FVB/N embryos at 7.5 days post coitum (dpc) alongside their deciduas and uterine tissue were fixed in 4% PFA, dehydrated in ethanol before being embedded in paraffin and 5µm sections cut on a microtome. Sections were stained by hematoxylin, eosin, and

saffron then photographed using the Nanozoomer (Hamamatsu). On average, 50 sections per embryos were made and analyzed.

II. Expression analysis of the prion gene family at early embryonic stages

II.I RT-PCR analyses :

Reverse transcriptions were performed on total RNAs extracted from pools of embryos and/or of placenta tissues, using the SuperScript First-Stand Synthesis System for RT-PCR (InVitrogen, Cat.11904-018) according to the manufacturer's instructions. For the reverse-transcription reaction, either the random hexamers or specific primers (SprnR1 for *Sprn* and PrndR1 for *Prnd*) were used. Around 5 µg of total RNA were used per reaction.PCR analyses were performed on 2 µl of RT reactions. Unless specified, PCR were performed using the GoTaq Flexi DNA polymerase kit (Promega, M8306), following the manufacturer's instructions. PCR were made in a volume of 100 µl, using 2 µl of purified genomic DNA (~20 ng) or of RT reaction mixture. The reaction conditions were as follows: 40 cycles with 30s denaturation step at 92°C, 30s hybridization step at 60°C and 30s elongation step at 72°C. Following a final elongation step of 2 min at 72°C, the PCR reactions were stored at 4°C before analysis of a sample by agarose gel electrophoresis. Nested PCR were made similarly using 2 µl of the first PCR reaction as template.

II.II In Situ Hybridization

a. Synthesis of riboprobes:

Mouse *Prnd*, *Sprn* and *Prnp* cDNA ORF (Open Reading Frame) were PCR-amplified from mouse genomic DNA. The gel purified DNA fragments (using GenEluto Agarose Spin Columns (Sigma 56500-70 EA)) were cloned in the pGEM-T vector (Figure 4), using the corresponding cloning kit (Promega, A1360). After electro-transformation of E. Coli DH5α, recombinant plasmid containing colonies were selected and identified by plating the bacteria suspension into ampicillin, IPTG and X-Gal agarose plates, as described in Maniatis *et al.*, 1982. Recombinant plasmid DNAs were amplified and their inserts sequenced. It allowed to confirm the identity of the amplified cDNAs and to orientate the insert in the vector. Using the restriction sites present in the vector at the edges of the insert, the recombinant plasmids were linearized. According to the restriction site used and to the orientation of the insert, it then allowed to synthesize a sense or an antisense probe using the Sp6 or T7 polymerases, recognition sites of with are present in the pGEM-T vector (figure 4 derived from Promega)

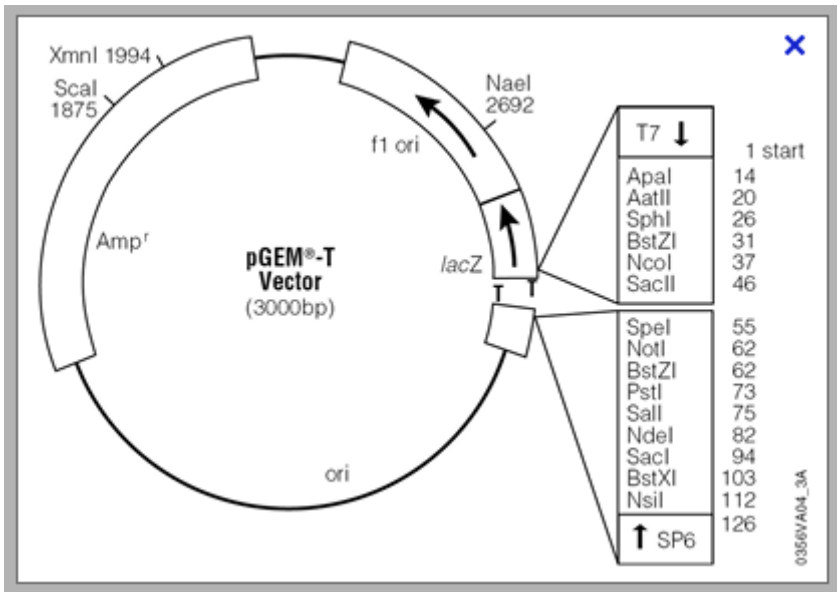


Figure 4.pGEM-T vector map used to clone Mouse *Prnd*, *Sprn* and *Prnp* cDNA from gel purified DNA fragments.

Riboprobe synthesis was performed as follows:

The following reagents were 13 µl H_2O, 2 µl of 10x transcription buffer (roche,10810274001 or fermentas EP0113), 1 µl of 0.2 M DTT, 2 µlof 10x DIG RNA Labeling Mixture (Roche, 11277073910), 1 µlof linearised plasmid (1µg), 0.5 µlof RNase Inhibitor (20u, Roche, 3335399001) and1 µlof either SP6 (20u, Roche,10810274001) orT7 RNA Polymerase (200u,Fermentas, EP0113) were mixed in an eppendorf tube at room temperature. The tube was incubated 2h at 37°C and 1 µl aliquot was removed and ran on an 1% agarose gel to

estimatethe amount of RNA synthesized. An RNA band ~10-fold more intense than the plasmid band indicates that ~10 µg of probe had been synthesized. Then, 2 µl of DNase I (40u, Turbo Dnase, Life technologies, AM2238) was added to the mix and the tube incubated at 37°C for 15 min. The RNA probe was then ethanol-precipitated, collected by centrifugation, air dried and resuspended in 100 µl TE and stored at -20C.

b. In situ Hybridization:

The protocol adapted from David Wilkinson, 1999 was used. Only a summary of it is given below.

The embryos were collected at 7.5, 8.5 and 9.5 dpc, dissected in (Phosphate Buffer Saline) PBS and fixed in 4% para-formaldehyde in PBS at 4°C overnight. Later they were washed twice in PBT (PBS+.1% Tween 20) at 4°C followed by washings with 25%, 50%, 75% Methyl alcohol (MeOH) in PBT and then twice with 100% MeOH. Embryos could be kept at -20°C in this solution.

DAY 1

Embryos are rehydrated by several washings in PBT solutions, treated with 6% hydrogen peroxide, proteinase K and prehybridized in the presence of yeast RNA. Then the prehybridization solution is replaced by the hybridization buffer in the presence of the heat-denaturated riboprobe and the samples incubated at 70°C overnight.

DAY 2

Post-hybridization washes were performed and binding of the Anti- Dig- AP antibody was allowed to occur overnight at 4°C in TBST (Tris buffered saline+ Tween 20) in the presence of sheep serum.

DAY 3

Several washes were performed to remove the excess of the antibody.

DAY 4

Antibody detection was performed using BM purple (Roche, 11442074001). Following washes with PBT, the embryos pictures were taken. Afterwards they were stored in 4% Paraformaldehyde.

III. Analysis of the LSI.06 transgenic line

III.I Transmission rate on Prnp$^{-/-}$ genetic backgrounds:

Genotyping of offspring from FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$crossed with *Prnp*$^{-/-}$**,** FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ with C57/129/Sv *Prnp*$^{-/-}$and from FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$ mice with FVB/N *Prnp*$^{-/-}$ mice was carried out using primers 5A and 3B for *LSI 06* while mPrP A and mPrP B for *Prnp*locus (Table 1).

III.II Cloning of the LSI.06 transgene integration site:

The general strategy used is shown in figure 5. It is based on the use of a 6 bp-cutter restriction enzyme known to cut once near the 5' or 3' end of the integrated transgene. In the first step, genomic DNA from several (2 in our case) transgenic mice and from non-transgenic control animals are digested to completion with the chosen restriction enzyme. The second step involved a circularization of the obtained linearized fragments by ligation. Then two consecutive PCRs, including a nested second PCR, are performed using set of primers located within the transgene sequence in order to amplify the surrounding mouse genomic DNA (steps 3 and 4). In these PCRs, the elongation time is of 5 min in order to potentially amplify fragments of several kbp. A DNA fragment was then specifically amplified from the transgenic genomic DNA. This DNA is then gel purified using GenEluto Agarose Spin Columns (Sigma 56500-70 EA), sequenced and analyzed (step 5).

RESULTS AND DISCUSSION

I. Histological analysis of E7.5 embryos

Comparative histological analyses of E7.5 embryos between FVB/N *Prnp*$^{-/-}$ and FVB/N *Prnp*$^{-/-}$ embryos injected at the zygotic stage with either a FG12 lentiviral solution, used as a control as it only encodes GFP (http://www.addgene.org/14884), or an shRNA targeting *Sprn* LS2-lentiviral solution (Young *et al.,* 2009) were already performed (Passet *et al.,* 2012).

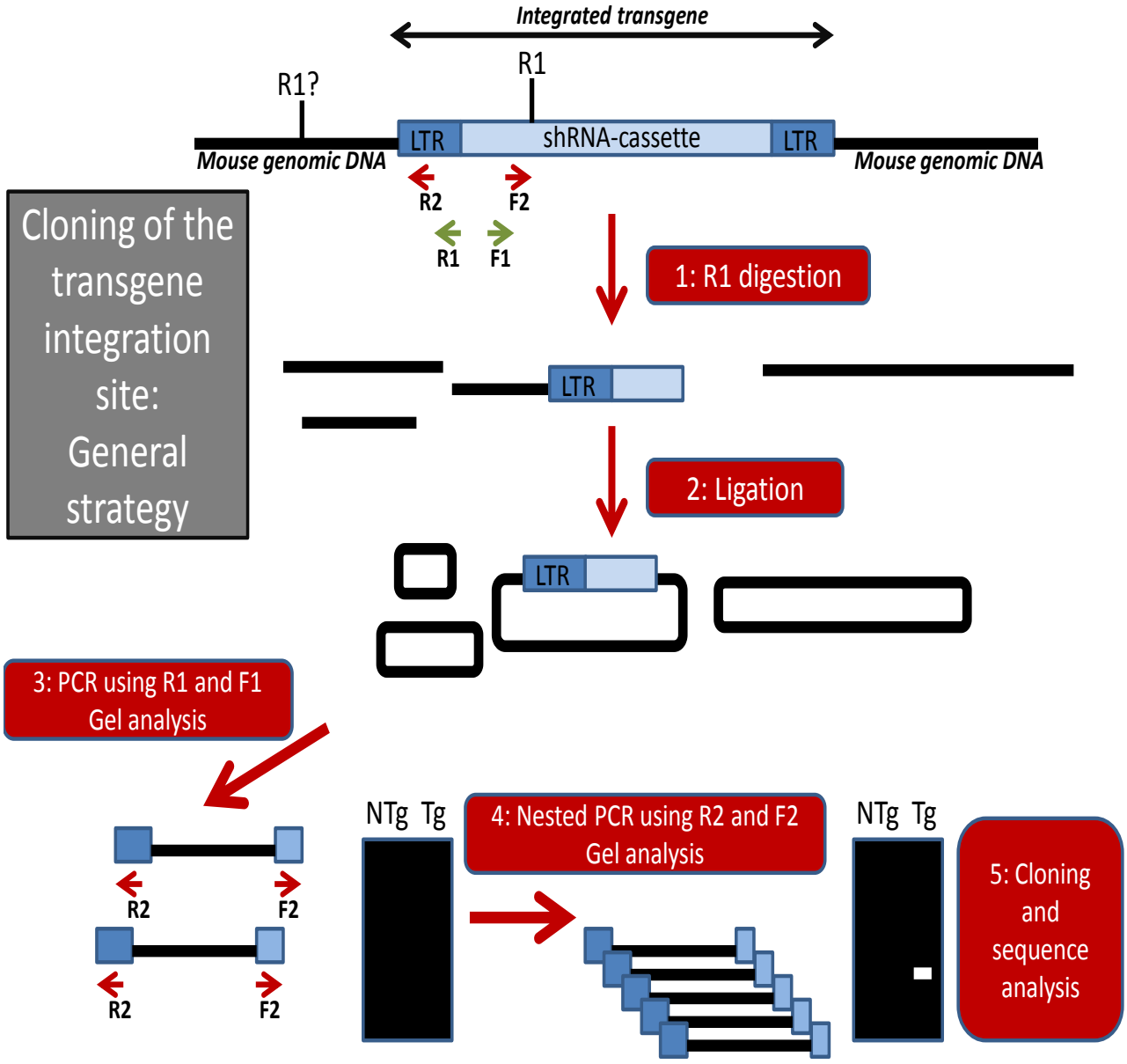


Figure 5. Strategy of Cloning Transgene integration site

To summarize this study, *in vitro* manipulation of the eggs induced a developmental delay. Furthermore, LS2-injected embryos were characterized by reduced and even disorganized ectoplacental cones, with a notably reduced invasive trophoblast cell layer.

The aim of this part of my study was to complete this observation by similarly analyzing FVB/N mouse embryos.

The comparative analysis was done between mouse 7.5 dpc FVB/N embryos, in which PrP, Sho and Doppel are expressed normally, and FVB/N *Prnp*$^{-/-}$ embryos at the same developmental stage. The development and size of the embryos were similar for both genotypes. The ectoplacental cones were also comparable in size and development. However, a region of hemorrhagic foci located just in front of ectoplacental cone was evidenced in all the FVB/N embryos (5/5). This lesion was never detected in any of the 6 analyzed FVB/N *Prnp*$^{-/-}$embryos (Figure 6 and data not shown).

PrP was suggested to be involved in various biological processes like cell migration and death, protease inhibition transcriptomic analysis of early mouse embryos (Khalifé *et al.*, 2011). Such biological functions might explain the observed phenotype. However, the number of embryos analyzed remains relatively low and further experiments would be needed to sustain this preliminary observation.

II. Expression analysis of the prion gene family at early embryonic stages

To assess the expression of *Prnp*, *Prnd* and *Sprn* during the developmental window that appeared important in Young et al., 2009 both in the embryonic body and the extra-embryonic tissue since this latter compartment is suspected to be responsible of the lethal phenotype in *Prnp*$^{-/-}$, *Sprn*$^{-/-}$knockdown embryos. The RNASeq analyses performed in the above-mentioned study also revealed that the three genes were expressed in E6.5 and E7.5 FVB/N embryos and that the knockout of *Prnp* did not apparently affect the observed low expression of *Prnd* and *Sprn* at these developmental stages (Passet *et al.*, 2012, and unpublished observations). We thus focused on later developmental stages. Two complementary approaches were used, RT-PCR and In Situ Hybridization (HIS).

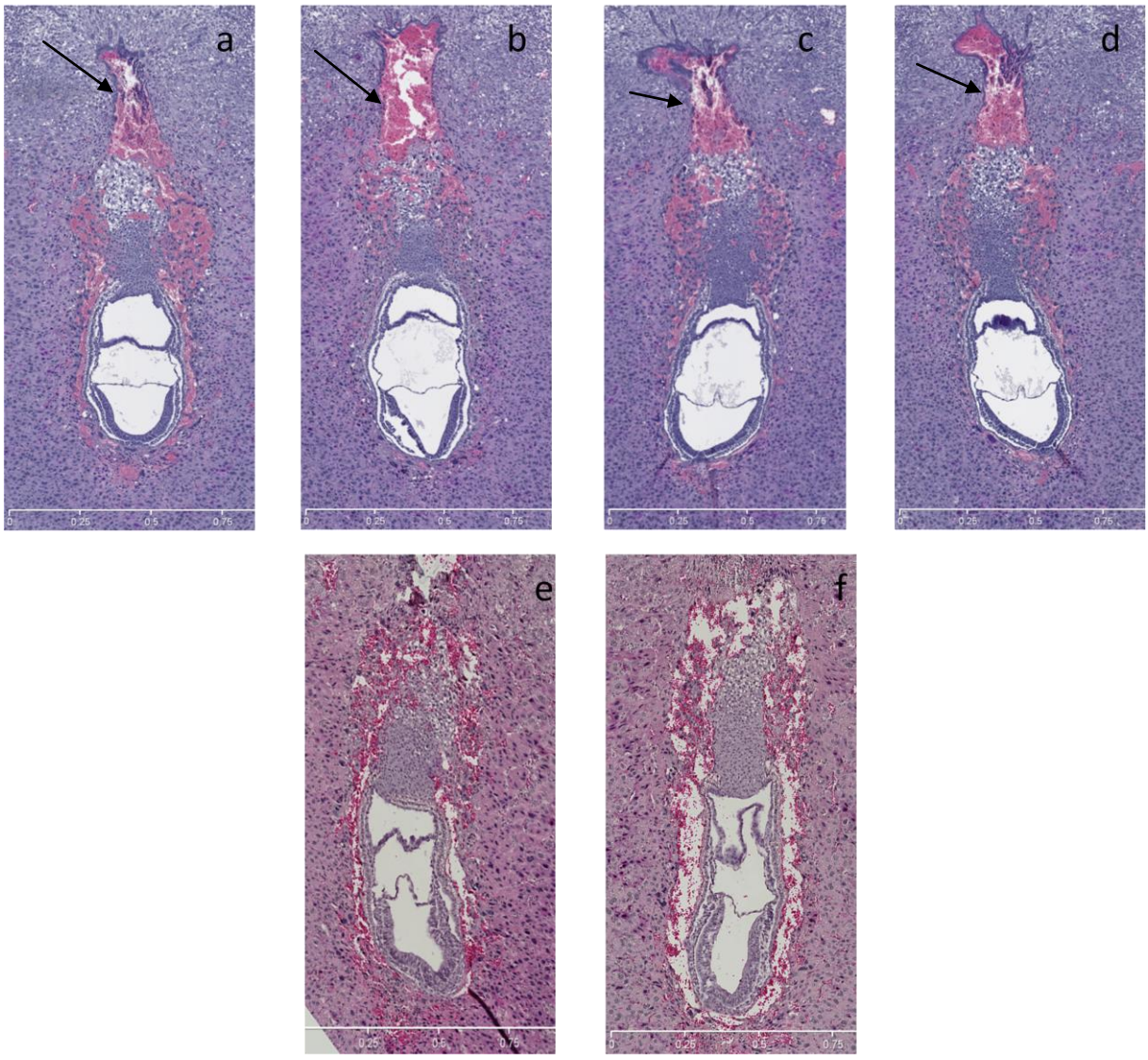


Figure 6. Histological section of FVB/N WT embryos (*a,b,c& d*) at 7.5 dpc. The arrows show area of hemorrhagic foci in front of ectoplacental cone. *e* and*f* are FVB/N *Prnp*$^{-/-}$embryo 7.5 dpc(Taken from Passet *et al.*, 2012,). The development, size and ectoplacental cones of embryos of both the genetic backgrounds are similar, but no hemorrhagic foci in *Prnp*$^{-/-}$ embryos is observed.

II.I RT-PCR analyses

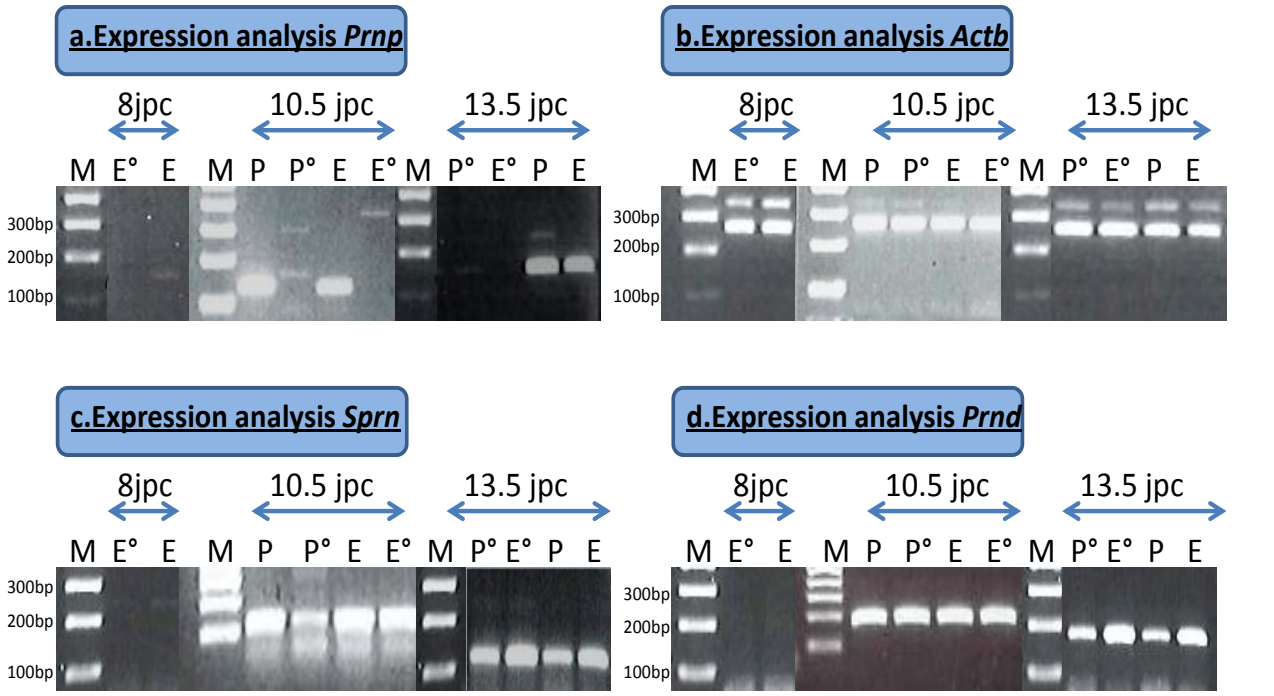
Total RNAs were isolated from pooled of 4 to 6 i) E8 total embryos, ii) dissected E10.5 and E13.5 trophectoderms and iii) embryonic bodies from FVB/N and FVB/N *Prnp*$^{-/-}$ mice. At each developmental stage, two different pools were analyzed.

Randon-primed RT-PCR experiments were performed using PrnpF/PrnpR , ActbF/ActbR, PrndF1/PrndR1, PrndF2/PrndR2, SprnF1/SprnR1, SprnF2/SprnR2 sets of primers (Table 1). All primer sets were chosen so that both primers are located on two different exons to avoid amplification of potential genomic DNA that could have contaminated the RNA preparations. Actb primers target the β-actin cDNA, detection of which was used for i) validation of the RT step and ii) internal normalization of the RT amount. The results obtained for *Prnp* and *Actb* indicated that both genes are expressed in the three developmental stages, both in the placenta (or trophectoderm) and in the embryonic compartment of FVB/N mice (Figure 7). As expected, no *Prnp*-expression signal could be detected in *Prnp*$^{-/-}$ samples (Figure 7). No or very faint and non-reproducible signals were obtained when expression of *Prnd* and *Sprn* were similarly assessed. We could exclude the non-recognition of the targeted cDNAs by the oligonucleotides since they were successfully used in RT samples derived from adult tissues known to express *Sprn* and *Prnd* (data not shown). Thus, these results could suggest either that i) these genes were not expressed at these developmental stages or ii) they were expressed at much lower levels compared to *Prnp* and *Actb*. This latter hypothesis was indirectly sustained by the RNASeq data obtained at E6.5 and E7.5 that indicated a 100 or more fold difference between the expression levels of *Prnp* and *Actb* and that of *Prnd* or *Sprn* (Passet *et al.*, 2012 and unpublished results).

To further assess *Prnd* and *Sprn* expressions, we performed i) nested PCR using PrndF2/PrndR2 and SprnF2/SprnR2 sets of primers on PrndF1/PrndR1, SprnF2/SprnR2 RT-PCR, respectively and ii) RT experiments using PrndR1 and SprnR1 oligonucleotides followed by PCR using PrndF2/PrndR2 and SprnF2/SprnR2 sets of primers. Again, nested PCR gave inconsistent results for the detection of *Sprn* expression, while it allowed that of *Prnd* in all analyzed samples but E8 (data not shown). Specific RT experiments allowed detecting the expression of both genes in placentas and embryos at E10.5 and E13.5 of FVB/N and FVB/N *Prnp*$^{-/-}$ mice (Figure 7). Only very faint signals could be observed at E8. These results are consistent with i) expression of these genes during the assessed developmental stages, both in the embryonic body and extra-embryonic tissues and ii) a much

LS1-EcoR1: F1	GGCAGGGATATTCACCATTATC
LS1-EcoR1: R1	GCTAGAGATTTTCCACACTGACTA
LS1-EcoR1: F2	CTTGGGTAGTTTGCAGTTTTAAAAT
LS1-EcoR1: R2	ATCTCTAGTTACCAGAGTCACACA
LS1-EcoR1: F2'	CTTGTGGAAAGGACGAAACACC
LS1-EcoR1: R2'	AGAGCTCCCAGGCTCAGATCTGGT
PrnpF	CAACCGAGCTGAAGCATTCTG
PrnpR	GGACATCAGTCCACATAGTC
PrndF1	TCCAAGCTTCAGAGGCCACAGTA
PrndR1	AGCTACCCGAGCTTCGGTGATCT
PrndF2	CCACAGTAGCAGAGAACCGA
PrndR2	TTCGGTGATCTGGCCGCCGCT
SprnF1	CAGTCGTGAGCTCTGCCTAA
SprnR1	GCCTTACGCGTACTCAAGATG
SprnF2	CACGGCCCCTAAATCGCTCA
SprnR2	GGAACAGCTGTCACAGAGGA
ActbF	GCTGTATTCCCCTCCATCGTG
ActbR	CACGGTTGGCCTTAGGGTTCAG

Table 1. Sequence of Primers used for RT PCR and Genotyping of LS1 06



M: 1kb plus DNA ladder (InVitrogen)
P: Placenta FVB/N
E: Embryo FVB/N
P°: Placenta FVB/N *Prnp*$^{0/0}$
E°: Embryo FVB/N *Prnp*$^{0/0}$

Figure7.Prion protein family gene expression in developing embryos:RT-PCR analysis. 8,10.5 & 13.5 dpc are embryo stages. *a*, *b*, *c*, and *d* show the expression analysis of *Prnp*, *Actb*, *Sprn and Prnd* respectively. *(a& b)*Random-primed RT-PCR experiment for expression analysis of *Prnp* and *Actb* show both genes are expressed in the three developmental stages and in both the placenta and embryo of FVB/N WT mice. *(a)* No expression of *Prnp* in *Prnp*$^{-/-}$ mice.*(c& d)* Specific RT experiments for expression analysis of *Sprn* and *Prnd* show expression of both genes in placentas and embryos at E10.5 and E13.5 of FVB/N and FVB/N *Prnp*$^{-/-}$ mice.

level in the embryonic body (Figure 7). This observation could reflect either a lower transcriptional activation and/or an expression restricted to specific cellular lineages, as suggested by the data recently published on Sprn-LacZ transgenic mice at E13.5 (Young *et al.*,2011). HIS experiments were performed in parallel to further assess this point and to define more accurately potential overlapping expressing profiles of these three genes.

II.II In Situ Hybridization

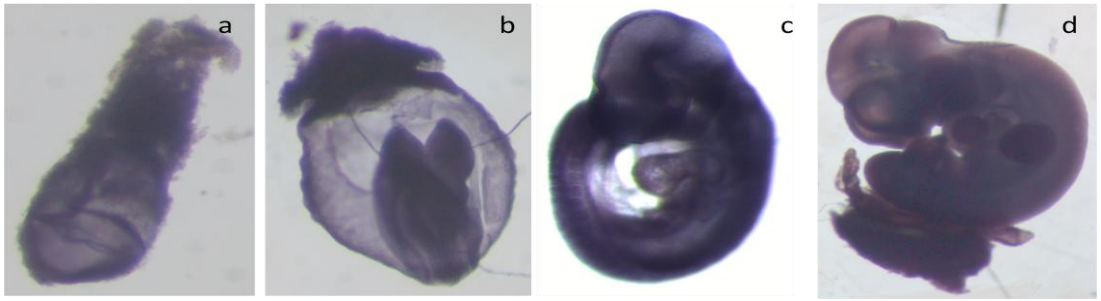
In situ hybridization using anti sense probe was carried out for *Sprn, Prnp* and *Prnd* at embryo stages 7.5 (a), 8.5(b), 9.5(c) and 11.5(d) dpc while the same was carried out for *Prnd* using sense strand as negative control in mouse embryo stages 8.5(e) and 9.5(f) dpc (Figure 8).

Hybridization reaction signals in the form of blue stain were observed throughout the embryo and also in the ectoplacenta in each of the embryonic stage and for each of the gene when antisense probe was used (except for E8.5/b of *Prnp*, that gave poor signals for technical reasons. All the other embryos regardless of their developmental stage showed poor signals in that particular round of HIS. Since it was the only intact embryo obtained after 3 rounds of HIS, we decided to include it in the results). For *Prnd*, when the sense probe was used at E8.5 and E9.5 no hybridization or signal were detected.

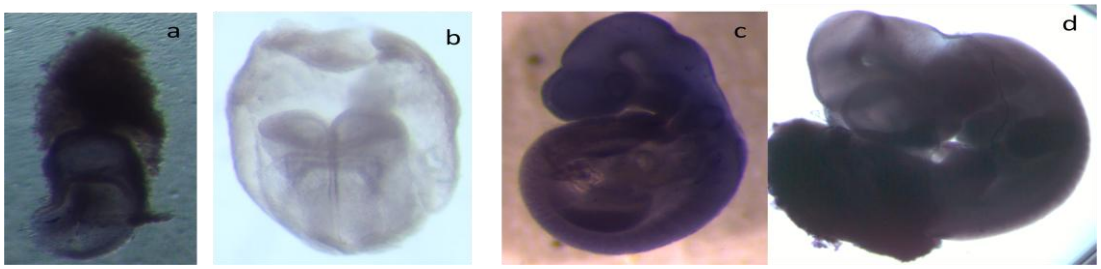
This indicates that all the three genes are expressed in mouse embryos from E7.5 to E11.5 and their expression appears ubiquitous, although some embryonic regions could be seen as less labeled such as the heart. These results are in accordance with the results shown by RT PCR analysis and demonstrate the potential overlapping expression of these genes. More precise locations of the genes' expression could perhaps be achieved on histological examination of sections of these embryos on which HIS has been performed. Such experiments are currently performed.

Our data thus suggest that the expression of *Sprn, Prnp* and *Prnd* is ubiquitous. However,Young et al, (2009)reported expression of *Sprn* in 10.5–14.5 dpc embryos to be more restricted to specific embryonic and extra-embryonic cell lineages. This might be due to the fact they used an *Sprn*-LacZ transgene. Such a transgene might have a more restricted expression pattern compared to the endogenous gene, at least at these early developmental stages and/or its level of expression might only allow detection of the LacZ in cells that express *Sprn* at high levels.

Sprn



Prnp



Prnd

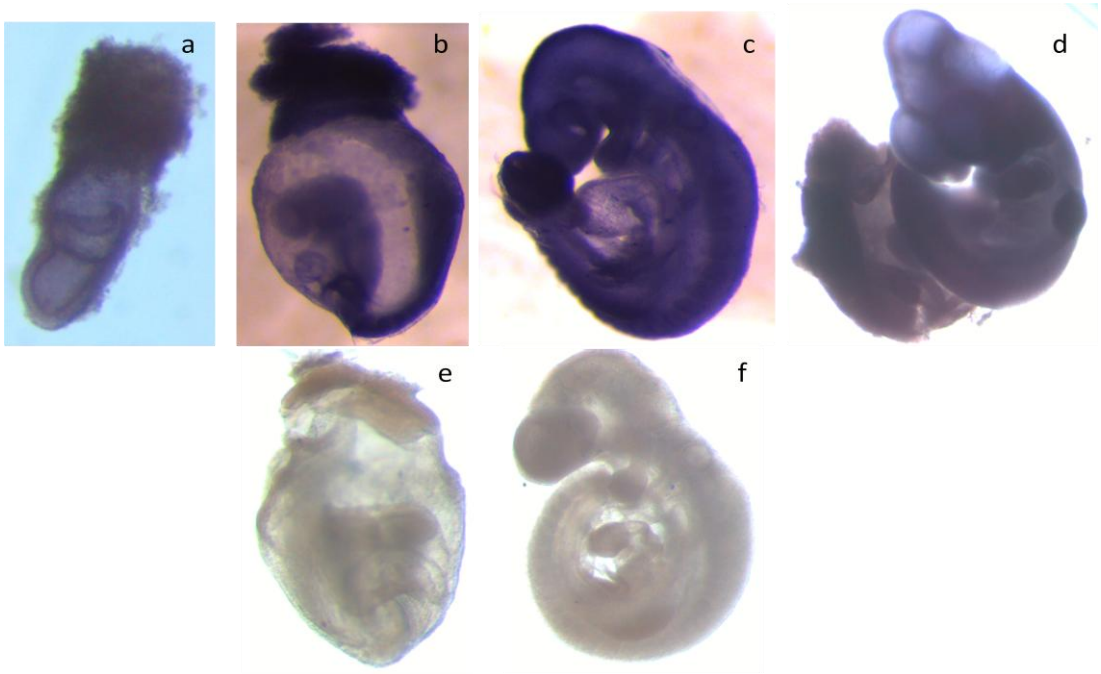


Figure 8.In situ Hybridization on FVB/N WT embryos for *Sprn, Prnp and Prnd* expression analysis.(*a-d)* Embryo stages 7.5, 8.5, 9.5 and 11.5 dpc respectively for each of the protein; HIS is carried out using antisense probe show ubiquitous hybridization and signal. *(e&f)* Embryo stages 8.5 and 9.5 dpc; HIS is carried out using sense probe for *Prnd* expression show no hybridization or signal.(The size of all embryos was similar within the stages but varied between the stages).

Alternatively, it is also possible that some of the observed signals in our HIS experiments correspond to false signals. The results obtained with the sense *Prnd* probe make this hypothesis less likely. Immuno-histochemical analyses of embryos at these developmental stages would be interesting to perform to help clarifying this uncertainty.

III. Further analysis of the LSI.06 transgenic line

LSI-06 is a mouse transgenic line established on an FVB/N genetic background. It contains a single integration site encompassing a lentivirus-based insert that expresses a ShRNA targeting the mouse *Sprn* transcript (Young *et al.*, 2009). In the adult brain, the level of expression of the *Sprn* gene was found to be down-regulated by 60 to 70% in LSI-06 transgenic mice, at the RNA level. We wanted to assess the potential phenotypic impact of such a down-regulation in an FVB/N *Prnp*$^{-/-}$ genetic background.

III.I Transmission rate on Prnp$^{-/-}$ genetic backgrounds

LSI.06$^{+/-}$-*Prnp*$^{+/+}$ mice were crossed with FVB/N *Prnp*$^{-/-}$ animals. Genotyping was done using primers 5A and 3B for *LSI 06* while mPrP A and mPrP B for *Prnp*locus (Table 1). Fifty percent of their progeny were found to be LSI.06$^{+/-}$-*Prnp*$^{+/-}$, as expected (data not shown). These mice were again crossed with FVB/N *Prnp*$^{-/-}$ mice. The observed transmission rate did not statistically follow a Mendelian ratio, as judged by X_2 analysis (Table 2 and data not shown).

Indeed, less than 5% of the offspring were of an FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$ genotype instead of the 25% expected. The percentage of FVB/N LSI.06$^{-/-}$-*Prnp*$^{+/-}$ was close from the expected ratio, although also slightly lower, 16%. We first suspected an embryonic lethality in the FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$ associated with an incomplete penetrating phenotype. We thus analysed i) the potential incidence of the genetic background associated with the *Prnp* null allele and ii) the secondary transmission rate of the LSI.O6 transgene on an FVB/N *Prnp*$^{-/-}$ environment. For the first point, LSI.06$^{+/-}$-*Prnp*$^{+/-}$ mice were crossed with C57/129/Sv *Prnp*$^{-/-}$ animals (Table 3). The obtained results indicated a non-Mendelian transmission rate with this time a clear deficiency in both the LSI.06$^{+/-}$-*Prnp*$^{-/-}$ and LSI.06$^{-/-}$-*Prnp*$^{+/-}$ genotypes.

Analysis of the transmission rate of the LSI.O6 transgene on an FVB/N *Prnp*$^{-/-}$ was made by crossing FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$ mice with FVB/N *Prnp*$^{-/-}$ mice. Out of the 38 offspring obtained, 19 (50%) were found to be of an FVB/N LSI.06$^{+/-}$-*Prnp*$^{-/-}$ genotype. It suggested

Genotype of offspring from FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ X *Prnp*$^{-/-}$	*Prnp*$^{+/-}$	*Prnp*$^{-/-}$
LSI.06$^{+/-}$	29	3
LSI.06$^{-/-}$	10	19

Table 2: Analysis of FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ X *Prnp*$^{-/-}$ crossing.

Genotype of offspring from FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ X C57/129/Sv *Prnp*$^{-/-}$	*Prnp*$^{+/-}$	*Prnp*$^{-/-}$
LSI.06$^{+/-}$	20	1
LSI.06$^{-/-}$	3	18

Table 3: Analysis of FVB/N LSI.06$^{+/-}$-*Prnp*$^{+/-}$ X C57/129 *Prnp*$^{-/-}$ crossing.

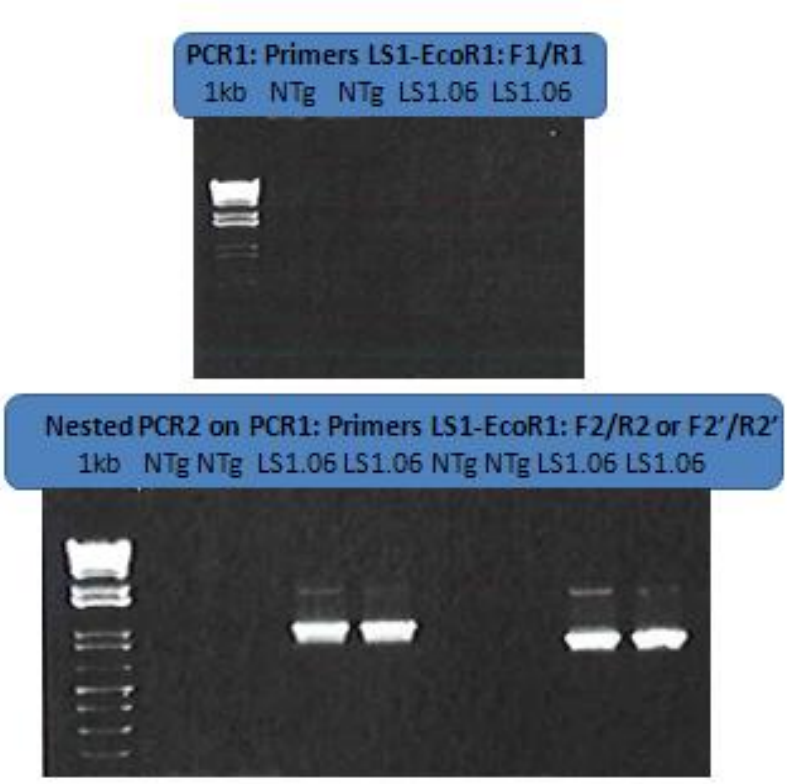


Figure 9. Amplification of the LS1.06 transgene integration site. Using *Eco*R1 a specific band is generated from the transgenic genomic DNA samples following Nested PCR. 1kb: DNA ladder (GeneRuler, InVitrogen).

that once established on an FVB/N *Prnp*$^{-/-}$ genetic background, the LSI.06 transgene was transmitted at a normal rate.

Altogether, these transmission analyses of the LSI.06 transgene favored a physical link between its integration site and the *Prnp* locus rather than a lethal-associated embryonic phenotype on a *Prnp*-knockout genetic background. To further assess this new hypothesis, we decided to clone its integration site.

III.II Cloning of the LSI.06 transgene integration site

The general strategy for cloning the LSI.06 integration site has been described in Figure 5. It is based on the use of a restriction enzyme that should cut within the transgene. Two such restriction sites were used *Bam*HI and *Eco*RI. With *Bam*HI, we could not isolate specific bands generated only from the transgenic mouse genomic DNA (data not shown). It is possible that no *Bam*HI site exists in the mouse genomic DNA at sufficiently short distance from the LSI.06 integration site to allow efficient PCR amplification. We did not re-assed this point. Using EcoR1, following nested-PCR with either LSI-EcoRIF2/R2 or LSI-EcoRIF2'/R2' set of primers (Table 1), a specific band was generated from the transgenic genomic DNA samples (Figure 9). This DNA fragment was gel-purified and sequenced. Its sequence was than aligned, using the NCBI Blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), against the mouse genome and against the transgene backbone sequence (http://www.addgene.org/8453/sequences/#addgene_seq). As expected, part of the sequence (78 bp) was 100% identical with the edge of the vector backbone (Figure 10). Most of the remaining of the sequence (600 bp) was 99% (599/600) homologous with a unique mouse genomic sequence located on mouse chromosome 2 within the transcription unit of the apoptosis inhibitor 5 gene. More precisely, the transgene appears to be integrated within the last known intron of the *Api5* gene. This Api5 gene seems to be expressed at early mouse developmental stages according to recorded data (http://www.ncbi.nlm.nih.gov/sites/geo), and is thus a potential target for lentivirus that are known to favor integration in active transcription units (Ciuffi *et al.,* 2008).

The *Prnp* locus is also located onto mouse chromosome 2 (Figure 1) and the distance between the LSI.06 integration site and this gene appears to be of 37.5 Mb. Thus, this physical distance could explain the observed non-Mendelian transmission rate of the LSI.06 transgene. The genetic distance between the two loci, LSI.06 and *Prnp*, is of 17 cM, according to Table 1 and 2, and is thus probably underestimated according to the observed physical distance.

Figure 10. Blast of the cloned LS1-integration site sequence against mouse genomic DNA

```
>    ref|NT_039207.8|  Mus musculus strain C57BL/6J chromosome 2 genomic contig, GRCm38
C57BL/6J MMCHR2_CTG5
Length=116378660


 Features in this part of subject sequence:
apoptosis inhibitor 5

 Score = 1101 bits (596),  Expect = 0.0
 Identities = 599/600 (99%), Gaps = 1/600 (0%)
 Strand=Plus/Minus

Query  147       TGAATTCCTCTGACCTTTGACAGTGGTCAAATCCCTTTTGACCAGACAGGTTGGAAAAAG  206
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296502  TGAATTCCTCTGACCTTTGACAGTGGTCAAATCCCTTTTGACCAGACAGGTTGGAAAAAG  35296443

Query  207       CCAGTTTTAGACTGAGAAATGAACCCTCCCATATAAATGAGCTCTTACAGCCTTCTTTAA  266
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296442  CCAGTTTTAGACTGAGAAATGAACCCTCCCATATAAATGAGCTCTTACAGCCTTCTTTAA  35296383

Query  267       GGAGTGCACCGAATCTTGGTACTCATATAACTGCATATACATGTGCATATGCACATTTGC  326
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296382  GGAGTGCACCGAATCTTGGTACTCATATAACTGCATATACATGTGCATATGCACATTTGC  35296323

Query  327       TTATGTGAAAAATAGGGATAGCAGAGTTCTTAGGAACTTAGGTAAGAATGGTTTTGAGAG  386
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296322  TTATGTGAAAAATAGGGATAGCAGAGTTCTTAGGAACTTAGGTAAGAATGGTTTTGAGAG  35296263

Query  387       GATTTGGTAATGAAAAGCAGTCTTTGTTGTTTTATTACATCAAATACTATCTTCTGATAT  446
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296262  GATTTGGTAATGAAAAGCAGTCTTTGTTGTTTTATTACATCAAATACTATCTTCTGATAT  35296203

Query  447       TTTTTGACTTAATGTGATATCTGTAAAATCTGAAAATCAGCAGTTAGATGCTATGAGGAA  506
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296202  TTTTTGACTTAATGTGATATCTGTAAAATCTGAAAATCAGCAGTTAGATGCTATGAGGAA  35296143

Query  507       ATAGCATGAACGAAAGTAATCATGATATTTTGCACAGCACATAAAGAAATGTATTTCTCT  566
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296142  ATAGCATGAACGAAAGTAATCATGATATTTTGCACAGCACATAAAGAAATGTATTTCTCT  35296083

Query  567       GTAACTTGAATAGTTACATGGTGAGTATGAGTTTCGAAACATAAAGATTTTGTCTCACAG  626
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296082  GTAACTTGAATAGTTACATGGTGAGTATGAGTTTCGAAACATAAAGATTTTGTCTCACAG  35296023

Query  627       AACCCACAGCTTGATATAAGGTGGAGAACAAATGCAGAAAATGTAACACATAGTTTGTAT  686
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35296022  AACCCACAGCTTGATATAAGGTGGAGAACAAATGCAGAAAATGTAACACATAGTTTGTAT  35295963

Query  687       GCCAGTAAACTAGGATGTAAATATATTTTAATTTTAAA-GCTGCAAAAAAAAAAAAAAAA  745
                  |||||||||||||||||||||||||||||||||||| |||||||||||||||||||||
Sbjct  35295962  GCCAGTAAACTAGGATGTAAATATATTTTAATTTTAAAAGCTGCAAAAAAAAAAAAAAAA  35295903
```

Alignment with the pLKO.1-puro vector sequence

(vector: http://www.addgene.org/8453/sequences/#addgene_seq)

```
>lcl|40691
Length=7032

 Score =  147 bits (79),  Expect = 4e-38
 Identities = 79/79 (100%), Gaps = 0/79 (0%)
 Strand=Plus/Plus

Query  15    ACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGT  74
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6742  ACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGT  6801

Query  75    GGAAAGGACGAAACACCGG  93
             ||||||||||||||||||
Sbjct  6802  GGAAAGGACGAAACACCGG  6820
```

Location of the *Prnp* exon 1 on mouse chromosome 2

```
 Score =  143 bits (77),  Expect = 1e-30
 Identities = 77/77 (100%), Gaps = 0/77 (0%)
 Strand=Plus/Plus

Query  1         CCCCTTTCCACTCCCGGCTCCCCCGCGTTGTCGGATCAGCAGACCGATTCTGGGCGCTGC  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72789287  CCCCTTTCCACTCCCGGCTCCCCCGCGTTGTCGGATCAGCAGACCGATTCTGGGCGCTGC  72789346

Query  61        GTCGCATCGGTGGCAGG  77
                 |||||||||||||||||
Sbjct  72789347  GTCGCATCGGTGGCAGG  72789363
```

Deduced major features :

- The integration site of LS1 is located within the last intron of the ORF of the mouse *Api5* (apoptosis inhibitor 5) gene
- The LS1 integration site is located at 37.5 Mb (37.493.025 nt) of the *Prnp* locus:

CONCLUSIONS

Histological examination demonstrated that the phenotype of 7.5 dpc FVB/N mouse embryos is similar to that of FVB/N *Prnp*$^{-/-}$at the same stage except for the observation of hemorrhagic foci just in front of ectoplacental cone in FVB/N WT embryos. By increasing the number of samples analyzed perhaps we can validate this phenotype and associate it with involvement of PrP^{C} in biological pathways such as angiogenesis, inflammation, cell mobility and gastrulation. Besides this, it could be interesting to compare FVB/N mouse embryos which have been down regulated for only *Sprn* with FVB/N and FVB/N *Prnp*$^{-/-}$embryos to ascertain if these findings are consistent with potential overlapping roles of Sho and PrP and/or biological redundancy.

To analyze the expression the prion gene family RT-PCR and In Situ Hybridization were carried out. RT PCR analysis was done on E8 total embryos, dissected E10.5 and E13.5 trophoectoderms and embryonic bodies from FVB/N and FVB/N *Prnp*$^{-/-}$ mice. We observed expression of *Prnp* in all the developmental stages and in both the placenta and embryo except in *Prnp*$^{-/-}$, while we found low expression pattern of *Prnd* and *Sprn*.

In situ hybridization of FVB/N WT embryos at 7.5 to 11.5 dpc for the 3 genes using antisense probes confirmed the expression of these genes in all the stages. Both the experiments thus could be considered as another clue suggesting overlapping expression profiles of these genes at early embryonic stages. The obtained results are thus compatible with biological overlapping roles of these proteins.

The difficulty to introgress LSI 06 on *Prnp* null mice was demonstrated likely to be due to the physical linkage between the transgene and *Prnp* locus rather than an embryonic lethality. This would suggest that 70 % down regulation is not enough to induce lethality in *Prnp* null background (if at all it exists). Also an assessment of the fecundity of such mice, including growth rate and robustness of pups might give us a possible clue for what are the further effects of such introgression.

However, the recent publication of the knockout of *Sprn* with no embryonic lethality associated in a *Prnp* knockout genetic background (Daude *et al.,* 2012) appears to question the data obtained with the RNA interfering approach (Young *et al.,* 2009, Passet *et al.,* 2012). Several hypotheses could explain this apparent discrepancy; i) the use of a similar but not identical genetic background, ii) a specific susceptibility of the double-knockout embryos to lentiviral infection and iii) an off-target effect of the ShRNAs. This latter hypothesis is unlikely as two different shRNAs were used and the off-targeted locus (or loci) would have to

induce lethality only in the absence of PrP. The use of ZFN will allow to inactivate *Sprn* in the precise genetic background used in Young *et al.,* 2009 and such experiments are currently underway.

Our results also point out a potential role of Doppel in early embryonic stages. Although the double knockout of *Prnp* and *Prnd* has been reported and gave a phenotype similar to that of the single *Prnd* invalidation, it would be of interest to assess the phenotypic consequences of the invalidation of *Sprn* and *Prnd*, and of the three loci. Again, such experiments are currently performed using a ZFN approach.

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