Non-neuronal functions of the prion protein

Insights from a unique animal model

Philosophiae Doctor (PhD) Thesis

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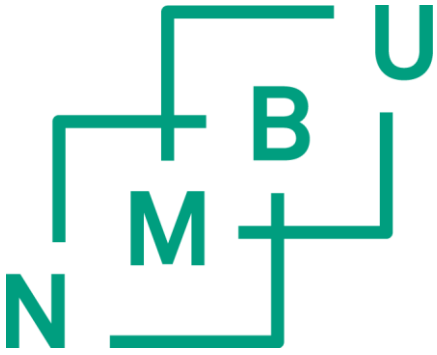

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"Nothing in biology makes sense except in the light of evolution"

Theodosius Dobzhansky

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Malin Rokseth Reiten
Oslo, September 2017

Abbreviations

7-m(dG)	7-methyl guanosine
ADAM	ADAM Metallopeptidase Domain
ATP	Adenosine triphosphate
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt Jakob disease
CNS	Central nervous system
CWD	Chronic wasting disease
DEG	Differentially expressed gene
DC	Dendritic cell
Dpl	Doppel
ER	Endoplasmic reticulum
GPI	Glycosylphosphatidylinositol
HSC	Hematopoietic stem cell
IF	Immunofluorescence
IFN	Interferon
IFNAR	Interferon-α/β receptor
IHC	Immunohistochemistry
IL	Interleukin
ISG3	Interferon-stimulated gene 3
ISG15	ISG15 ubiquitin-like modifier
Jak	Janus kinase
LPS	Lipopolysaccharide
MCHC	Mean corpuscular haemoglobin content
MCV	Mean cell volume
MMS	Methyl methanesulfonate
MX	MX dynamin like GTPase
NK cell	Natural killer cell
OAS	2'-5'-Oligoadenylate synthetase
ORO	Oil-red-O
PBMC	Peripheral blood mononuclear cell
PRNP	Prion protein gene
PrP^{C}	Cellular prion protein
PrP^{Sc}	Prion protein scrapie
RBC	Red blood cell
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
SOCS	Suppressor of cytokine signaling
SOD	Superoxide dismutase
Tyk	Tyrosine kinase
USP18	Ubiquitin specific peptidase
WB	Western blot
Wt	Wildtype

List of papers

Paper I

Hematological shift in goat kids naturally devoid of prion protein

Authors: Malin R. Reiten, Hege Brun-Hansen, Anna M. Lewandowska-Sabat, Ingrid Olsaker, Michael A. Tranulis, Arild Espenes and Preben Boysen

Published: Front. Cell Dev. Biol. (2015), **3**:44

Paper II

Loss of prion protein induces a primed state of type I interferon-responsive genes

Authors: Giulia Malachin, Malin R. Reiten, Øyvind Salvesen, Håvard Aanes, Jorke H. Kamstra, Kerstin Skovgaard, Peter M. H. Heegaard, Cecilie Ersdal, Michael A. Tranulis, Maren K. Bakkebø

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Paper III

Stress resilience of spermatozoa and blood mononuclear cells without prion protein

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Manuscript

Summary

The physiological function of the cellular prion protein (PrP^C) remains enigmatic. A misfolded, infectious conformer of the protein, known as the scrapie isoform PrP^{Sc}, is able to aggregate in brain tissue and cause a group of fatal transmissible neurodegenerative disorders in humans and animals. Amongst them are Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in cervids.

PrP^C is evolutionary conserved and abundantly expressed in neurons, but also widely in other non-neuronal tissues, depicting a central role in cellular physiology. Several methods have been used for studies of PrP^C function, and the development of *Prnp* knockout mice during the 1990s, provided researchers with a new and valuable tool for studies of *in vivo* consequences of PrP^C ablation. Interestingly, apart from being completely resistant to prion disease, *Prnp* knockout mice displayed no major aberrant phenotypes; rather they developed normally and exhibited normal lifespans. Further studies have, however, attributed several phenotypes to the loss of PrP^C, including inhibition of apoptosis, protection against oxidative stress, a role in synapses, NMDA-receptor signaling and modulation of behavior, amongst others.

Recently, a line of Norwegian Dairy Goats was found to carry a nonsense mutation at codon 32 of the *PRNP* reading frame that completely blocks PrP^C synthesis. A non-transgenic, non-rodent mammalian PrP^C-null model represents a new and valuable resource for prion research that could, in combination with other model systems, shed light on PrP^C physiology. The studies going into this thesis represent the first sets of analyses and experiments characterizing this unique line of goats.

Firstly, we set out to characterize goats with (*PRNP*$^{+/+}$) and without (*PRNP*$^{Ter/Ter}$) PrP^C with regard to standard health parameters. Hematological analyses revealed that *PRNP*$^{Ter/Ter}$ goats presented with an increased number of red blood cells (RBCs) with a smaller mean cell volume (MCV) as well as a tendency for increased levels of neutrophils, an alteration we collectively coined a "hematological shift". This observation suggests that PrP^C may serve a role in bone marrow hematopoiesis. The bone marrow morphology, however, did not deviate between the two genotypes and further studies are needed to clarify PrP^C's role in RBC development and/or physiology. Based on the moderate, but dynamic expression of PrP^C in mononuclear cells and previous research showing that PrP^C might play a role in modulating basic immune cell functions and immune responses, we pursued this by

investigating basal immune cell traits. Numbers of peripheral blood mononuclear cells (PBMCs) were similar between the *PRNP* genotypes. Moreover, basal immune cells functions such as monocyte phagocytosis and lymphocyte proliferation were also similar between the genotypes, suggesting that PrP^C deficiency had no major effects on these important processes, which is contrast to some observations in mouse models.

A deeper analysis of PrP^C loss was undertaken by investigating the transcriptome of PBMCs from $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ animals. Analysis of differentially expressed genes (DEGs) revealed a significant upregulation of type I interferon (IFN)-responsive genes in $PRNP^{Ter/Ter}$ cells, which could not be attributed to differences in cell populations or altered expression of genes encoding major components of the type I IFN-signaling pathway, indicating that PrP^C somehow downregulates tonic type I INF signaling. By using several clones of human neuroblastoma SH-SY5Y cells, stably expressing different levels of PrP^C, we were able to demonstrate that mock-transfected cells with very low levels of PrP^C responded with increased transcription of the IFN-responsive gene *MX2* after treatment with IFN-α, compared with clones expressing moderate or high levels of PrP^C. Although, providing support to the observations from the PBMCs, the data from the SH-SY5Y cells did not reveal a simple dose-response relationship between PrP^C levels and apparent sensitivity to INF-α. However, an independent dataset from an *in vivo* lipopolysaccharide (LPS) challenge of goats with and without PrP^C, showed a similar gene expression signature in circulating leukocytes both at basal level and after LPS exposure, demonstrating that the phenotype is also present and functional *in vivo*. Further studies are needed to reveal the molecular mechanisms behind these observations and to clarify at which level PrP^C impacts type I IFN signaling.

Goats naturally devoid of PrP^C can have significant breeding value. Knowing that PrP^C is normally present at high levels in the male genital tract, including ejaculated spermatozoa, we wanted to investigate if lack of PrP^C could influence spermatozoa stress resilience, as suggested by mouse studies. Our data confirmed the prominent presence of PrP^C in the testicle and epididymis as well as lower levels in spermatozoa in $PRNP^{+/+}$ animals. However, analysis of freeze tolerance, viability, motility, adenosine triphosphate (ATP) levels and acrosome intactness at rest and after acute stress, induced by Cu^{2+} ions, as well as levels of reactive oxygen species (ROS) after exposure to $FeSO_4$ and H_2O_2 revealed no differences between the *PRNP* genotypes. Since cytoprotective roles have been assigned to PrP^C, we wanted to broaden our analysis by inclusion of PBMCs with and without PrP^C in our *in vitro* analysis of oxidative and genotoxic stress. Similar to the observations from spermatozoa, PrP^C

appeared dispensable for *in vitro* stress resilience of PBMCs. Expression levels of genes involved in DNA damage repair and ROS scavenging in PBMCs were also unaffected by PrP^C loss. In conclusion, no *PRNP* genotype-related differences in stress resilience were detected concerning viability and global accumulation of DNA damage in PBMCs after treatment with H_2O_2, doxorubicin or methyl methanesulphonate (MMS). These observations were corroborated by data from SH-SY5Y cells expressing very low or moderate levels of PrP^C, again revealing no direct cytoprotective function of PrP^C, under these *in vitro* conditions.

Altogether, the degree to which PrP^C is able to confer cytoprotection during stressful situations *in vitro* is questioned, as we were unable to detect any differences between cells with and without PrP^C in our studies. However, PrP^C could still execute cytoprotective functions in neuro-immune crosstalk at rest and during inflammatory stress, possibly protecting immune-privileged tissues.

Taken together, this thesis has provided unique new knowledge concerning PrP^C's normal physiological function by the use of a non-transgenic animal model. Animals without PrP^C displayed a hematological shift, but no effects were observed in circulating leukocytes or basal immune-cell functions. The finding of an immunological signature dominated by increased levels of type I IFN-responsive genes in PBMCs is a previously unrecognized phenotype in cells without PrP^C, which can function as a gateway for further studies needed to dissect the pathway in which PrP^C might be involved. No stress-protective properties could be found *in vitro* when cells with PrP^C were assessed in this work; however, it remains to be investigated if these effects are better explored *in vivo*.

Sammendrag (summary in Norwegian)

Det cellulære prionproteinets (PrP^C) fysiologiske funksjon er, til tross for årevis med forskning, fortsatt ukjent. En feilfoldet, infeksiøs isoform av prionproteinet, bedre kjent som scrapievarianten, PrP^{Sc}, har evne til å aggregere i hjernevev og forårsake en rekke fatale, overførbare nevrodegenerative sjukdommer hos mennesker og dyr. Blant disse er Creutzfeldt Jakobs sjukdom hos menneske, skrapesjuke hos sau og geit, bovin spongiform encefalopati eller kugalskap hos storfe, og skrantesjuke hos hjortedyr.

PrP^C er evolusjonært bevart og særdeles høyt uttrykt i nevroner, men er også rikelig uttrykt i flere ikke-nevrale vev, noe som kan tyde på at proteinet spiller en viktig rolle i cellenes fysiologi. Et bredt utvalg metoder har blitt brukt for å studere PrP^Cs funksjoner, men få metoder har vært så viktig som introduksjonen av *Prnp* knockout-mus på 90-tallet. Metoden revolusjonerte arbeidet innen prionforskningen, og ga forskere et verdifullt verktøy for å studere hvordan PrP^C-tap påvirket det levende dyret (*in vivo*). Bortsett fra at disse musene var totalt motstandsdyktige mot prionsjukdom ble få tydelig unormale fenotyper oppdaget; musene utviklet seg heller normalt og hadde normale livsløp. Fenotyper som etter nærmere undersøkelser har blitt satt i sammenheng med PrP^C inkluderer motstand mot apoptose, beskyttelse mot oksidativt stress, normal synapsefunksjon, NMDA-reseptorsignalering og atferdsendringer, blant annet.

Nylig ble det oppdaget at en linje av norsk melkegeit innehar et prematurt stoppkodon i genet som koder for PrP^C, *PRNP*, og dermed blokkeres PrP^C-syntese fullstendig hos dyr som har mutasjonen i begge alleler. En ikke-transgen dyremodell representerer en ny og verdifull ressurs for prionforskning som sammen med andre modellsystemer kan belyse PrP^Cs fysiologi. Studiene i denne avhandlingen er de første analysene og eksperimentene som er utført med sikte på å karakterisere denne unike geitelinjen.

En generell karakterisering av geiter med ($PRNP^{+/+}$) og uten ($PRNP^{Ter/Ter}$) PrP^C ble initiert for å undersøke om dyr uten PrP^C har normal helsestatus. Hematologiske analyser viste at $PRNP^{Ter/Ter}$-geiter hadde et økt antall røde blodceller med et mindre cellevolum, i tillegg til en tendens til et økt antall nøytrofile. For å beskrive denne endringen laget vi begrepet «hematologisk skifte». Observasjonen tyder på at PrP^C kan inneha en rolle i beinmargshematopoiese. Siden beinmargsmorfologien ikke avvek mellom genotypene er det tydelig at videre studier kreves for nærmere å belyse PrP^Cs funksjon i

fysiologien og/eller utviklingen til de røde blodcellene. Basert på det moderate, men dynamiske uttrykket av PrP^C på mononukleære celler og tidligere forskning som viser at PrP^C muligens spiller en rolle i modulering av basale immunecellefunksjoner og –responser, fulgte vi opp med studier av basale immuncelleegenskaper. Antallet av mononukleære celler i perifert blod (PBMCer) var likt mellom genotypene. I motsetning til tidligere studier i andre modellsystemer viste studier av monocyttfagocytose og lymfocyttproliferasjon at PrP^C-mangel ikke hadde en vesentlig effekt på disse basale immuncellefunksjonene.

En dypere analyse av PrP^C-tapets ringvirkninger ble ble gjenomført ved hjelp av en transkriptomundersøkelse av PBMCer fra *PRNP*$^{+/+}$- og *PRNP*$^{Ter/Ter}$-dyr. Analyse av differensielt uttrykte gener avdekte en signifikant oppregulering av type I interferon(IFN)responsive gener i *PRNP*$^{Ter/Ter}$-celler som ikke kunne forklares med forskjeller i cellepopulasjoner eller økte nivåer av gener som koder for viktige komponenter av type I IFN-signalveien, noe som indikerer PrP^C har evne til å nedregulere tonisk type I IFN-signalering. Ved bruk av ulike kloner av humane neuroblastoma SH-SY5Y-celler som stablit uttrykker ulike nivåer av PrP^C kunne vi demonstrere at mock-transfekterte celler med veldig lave nivåer av PrP^C responderte med økt transkripsjon av det type I IFN-responsive genet *MX2* etter behandling med IFN-α, sammenlignet med kloner som uttrykte moderate eller høye nivåer av PrP^C. Selv om dataene fra SH-SY5Y-cellene ikke avdekket et enkelt dose-responsforhold mellom PrP^C-nivåer og den tilsynelatende sensitiviteten til IFN-α, ga de støtte til observasjonene som ble gjort ved transkriptomundersøkelse av PBMCene. Et uavhengig datasett fra en *in vivo* LPS-studie av geiter med og uten PrP^C viste en lignende genekspresjonssignatur i sirkulerende leukocytter både ved basalnivå og etter LPS-eksponering, noe som indikerer at fenotypen også er tilstede og er funksjonell *in vivo*. Videre studier kreves for å avdekke mekanismene bak dette funnet og for å undersøke hvor og hvordan PrP^C påvirker type I IFN-signalveien.

Geiter uten PrP^C kan ha signifikant avlsverdi. Med bakgrunn i kunnskap om at PrP^C er normalt til stede i høye nivåer i hanndyrets genitaltraktus, inkludert i ejakulerte spermier, ønsket vi å undersøke om mangel på PrP^C kunne påvirke spermienes motstandsdyktighet ovenfor stress, noe som har blitt indikert i musestudier. Våre data bekreftet den prominente tilstedeværelsen av PrP^C i testikkelvev og epididymis så vel som i spermier, dog i lavere nivåer, hos *PRNP*$^{Ter/Ter}$-dyr. Til tross for dette var det ingen tydelig beskyttende effekt av PrP^C når det gjelder frysetoleranse, DNA-integritet, viabilitet, motilitet, ATP-nivåer og akrosomintakthet, verken ved hvile eller etter akutt stress indusert av Cu^{2+}. Ingen målbare forskjeller ble funnet i mengden ROS etter eksponering for $FeSO_4$ og H_2O_2 hos spermier

med og uten PrP^C. På grunnlag av at PrP^C er beskrevet å ha en cellebeskyttende rolle ønsket vi å utvide analysene ved å inkludere PBMCer med og uten PrP^C i en *in vitro* analyse av oksidativt og genotoksisk stress. Likt som i observasjonene av spermier virket PrP^C unnværelig for *in vitro* stressmotstandsdyktighet. Ekspresjonen av gener involvert i reparasjon av DNA-skade og fjerning av ROS hos PBMCer var upåvirket av PrP^C-tap ved en normal fysiologisk tilstand. Videre ble det ikke påvist *PRNP*-genotypeforskjeller i viabilitet og global akkumulasjon av DNA-skade i PBMCer etter behandling av PBMCer med H_2O_2, doxorubicin og MMS. Disse observasjonene ble underbygget av data fra SH-SY5Y-celler som veldig lave eller moderate nivåer av PrP^C, igjen funn som indikerer at tilstedeværelse av PrP^C ikke har en beskyttende funksjon under disse omstendighetene.
Resultatene stiller spørsmål ved om PrP^C er cellebeskyttende under stressende situasjoner *in vitro*, men PrP^C kan fortsatt tenkes å inneha funksjoner i samspillet mellom immunceller og nervesystemet, både ved hvile og inflammatorisk stress, og muligens beskytte immunprivilegerte vev.

Kort oppsummert har denne avhandlingen bidratt til ny kunnskap angående PrP^Cs normale fysiologiske funksjon ved bruk av en unik, ikke-transgen dyremodell. Dyr uten PrP^C viste et hematologisk skifte, men ingen effekter ble funnet hos sirkulerende leukocytter eller knyttet til basale immuncellefunksjoner. Oppdagelsen av en immunologisk signatur dominert av et økt nivå type I IFN-responsive gener hos PBMCer er en tidligere ukjent fenotype i celler uten PrP^C og kan fungere som en plattform for videre studier. Ingen stressbeskyttende egenskaper av PrP^C ble funnet hos celler *in vitro* i dette arbeidet, men det gjenstår å undersøke om disse resultatene lar seg reprodusere i *in vivo* studier.

1. Introduction

1.1. BACKGROUND: From prion diseases to the prion protein

In 1982, American scientist Stanley B. Prusiner proposed that transmissible neurodegenerative diseases like scrapie in sheep and Creutzfeldt Jakob disease (CJD) in humans were caused by infectious proteins he denoted prions. Due to the pathological similarities and transmission properties, the group of diseases had hitherto been named transmissible spongiform encephalopathies (TSEs, Prusiner 1982) but from this point on, prion diseases gradually became a common designation. Parts of the amino acid sequence of the infectious prion protein (PrP^{Sc}) was soon determined (Prusiner, Groth et al. 1984) and when researchers led by Charles Weissmann reported that the sequence of PrP^{Sc} was identical to an endogenously encoded protein, more precisely the cellular prion protein (PrP^{C}, Basler, Oesch et al. 1986), an entire new field of research was opened. This was an important discovery because the key event in development of prion disease is the misfolding of PrP^{C} into PrP^{Sc}. This connection between PrP^{C} and prion diseases has made PrP^{C} one of the most studied molecules in biological sciences, and as of August 2017, a search on "prion protein" in the medical database Pubmed produced 15837 hits.

As a background for the studies of PrP^{C} presented in this thesis, a short overview of prion diseases and their history is warranted.

1.1.1. Prion diseases in a global perspective

Prion diseases (Table 1) are fatal neurodegenerative diseases affecting several mammalian species, most notably humans and ruminants. Although distributed worldwide, the prevalence of each disease differs between continents and countries, explained by the fact that even if all prion diseases are associated with PrP^{C} abnormalities, they differ in etiology ranging from spontaneous and genetic (familial) forms to forms that can spread as natural infection or via artificial transmission (iatrogenic or through the food chain). Common features are incubation periods that range from months to years and neurodegeneration with a lack of immune response.

Globally, sporadic Creutzfeldt Jakob disease (sCJD) constitutes about 85 % of the total CJD cases, 10-15 % of the cases are caused by variant CJD (vCJD), and iatrogenic CJD (iCJD) accounts for about 1 % of the cases (Jackson and Clarke 2000). sCJD causes 1-2 deaths per million people per year, and the

risk of developing the disease increases with age (WHO 2017). PrP^{Sc}'s resistance to radiation, nucleases, and standard sterilization and disinfection agents, and inactivation by procedures that modify proteins pose a large economic challenge as well as potential risk to contract iCJD. Humans undergoing surgeries with instruments used in undiagnosed CJD patients are in the risk of contracting iCJD (Brown, Preece et al. 1992). Specifically, contaminated human growth hormones and dura mater grafts (Brown, Preece et al. 2000), as well as ophthalmic surgery (P, Ward et al. 2004) have been identified as situations where CJD has been found to transmit CJD from one patient to another.

Table 1. Overview of prion diseases in humans and animals

Prion disease	**Host species**	**Mechanism/source**
Sporadic CJD	Humans	Mutation in *PRNP* or spontaneous conversion
Familial CJD		Mutations in *PRNP*
Variant CJD		Foodborne infection of classical BSE
Iatrogenic CJD		Infection from medical procedures
Kuru		Infection through ritual cannibalism (eradicated)
Fatal familial insomnia		Mutations in *PRNP*
Gerstmann-Sträussler-Scheinker syndrome		Mutations in *PRNP*
Scrapie - Classical - Atypical/Nor98	Sheep and goats	Classical: Infectious Atypical/NOR 98: Probably sporadic
Bovine spongiform encephalopathy - Classical - Atypical BSE-H - Atypical BSE-L	Cattle	Classical: Foodborne Atypical forms H and L are probably sporadic
Chronic wasting disease	Cervids	Infection
Transmissible mink encephalopathy	Mink	Foodborne infection, unknown prion source
Feline spongiform encephalopathy	Cats	Foodborne infection of classical BSE

Historic archives, dating back more than 200 years, from France, Germany and Great Britain provide convincing documentation of sheep scrapie being a significant problem for sheep farmers. Considerable co-grazing and other elements of habitat overlap between cattle and scrapie-infected sheep had taught farmers and governments that cattle were not at risk of developing sheep scrapie. The diagnosis of a spongiform encephalopathy in cattle in the mid-1980s in Great Britain was therefore highly unexpected and a problem of considerable scientific interest. Within a few years of its discovery, bovine spongiform encephalopathy (BSE) developed into a massive epidemic resulting in culling of nearly 750.000 cattle with dramatic economic consequences for the livestock industry and farmers; it also had severe impacts on animal welfare. The disaster was the result of new regulations regarding the rendering processes in the production of cattle-derived meat and bone meal, which was an important protein supplement in certain cattle feedstuffs (Wilesmith 1993). The use of meat and bone meal was banned in 1988 and the ban was further reinforced in 1996. This has been the single most important component in the combat of BSE, reducing the number of BSE cases in the United Kingdom from 37.280 in 1992 to fewer than five cases per year from 2012 onwards (OiE 2017).

Similar to sheep scrapie, BSE was initially considered harmless to humans. This, however, changed dramatically when the occurrence of a new variant of CJD, later known as vCJD, was announced in 1996, a decade after BSE was identified in the UK. Strong evidence pointed towards transmission of BSE to humans (Will, Ironside et al. 1996, Bruce, Will et al. 1997, Hill, Desbruslais et al. 1997).
So far, vCJD has been diagnosed in around 225 people (WHO 2012), suggesting a relatively protective species barrier considering that around 10 million people had ingested potentially BSE-infected meat (Chen and Wang 2014). However, the vCJD epidemic may not have reached its potential. A new case with the PrP^{C} genotype 129MV, different from the genotype 129MM that has been linked to all vCJD cases so far (Diack, Head et al. 2014), may, in a worst case scenario, be the first in a second wave of the epidemics, affecting people with more resistant PrP^{C} genotypes (Mok, Jaunmuktane et al. 2017). In addition, around 500 per 1 million people in the UK, a high proportion with PrP^{C} genotype VV129, was found to have accumulated PrP^{Sc} in their appendices at the time of removal (Gill, Spencer et al. 2013). The incubation period of prion disease in humans could be several decades, and only time will show if these people with more resistant PrP^{C} genotypes will eventually succumb to vCJD.

Today, prion diseases have again become the center of attention, as prion diseases continue to adapt to new hosts and spread to new areas. CWD in deer and elk has been spreading markedly throughout the United States and Canada during the past few decades (Saunders, Bartelt-Hunt et al. 2012), and recently, the first moose and reindeer were diagnosed with CWD in Norway (Benestad, Mitchell et al. 2016). The consequences for the wild animal populations and on the cervid ecosystems are hitherto unknown; however, considering these species' role in hunting traditions, tourism and agriculture, the economic and cultural consequences are significant. As of today, the Norwegian government has decided to eliminate the Nordfjella reindeer population of approximately 2200 animals and subsequently quarantine the area for five years in an attempt to eradicate CWD from the reindeer herd. Effective therapies and management practices in endemic CWD areas are highly needed. Although, the scientific data suggest that CWD is not transmissible to humans, efforts to minimize human exposure to CWD prions are prudent.

1.1.2. A brief history of prion diseases and development of the prion hypothesis

First described by British shepherds or their employers, scrapie made its way into the history books already by the 1750s, with large outbreaks in the sheep population on British soil (Schneider, Fangerau et al. 2008). In many flocks, the outbreaks were kept secret to avoid sanctions against the flock and economic losses, resulting in a disease that was largely unknown to veterinarians for a long time. Due to long incubation periods, the discussions were vivid as to whether the disease was infectious or hereditary (Schneider, Fangerau et al. 2008). However, in 1899, the idea that scrapie constituted a viral disease was put forward, but inoculation experiments were not successful (Besnoit 1899), later revealed to be caused by too short an observation period. The unusually long incubation period of 2-3 years was noted in 1913 by Steward Stockman (Stockman 1913).

In 1936, the transmissibility of scrapie was confirmed by experimental transmissions, demonstrating that the disease was infectious to both sheep and goats, albeit with longer incubation periods in goats. In the experiments, brain and spinal cord from an affected sheep were used in intraocular, epidural, subcutaneous and intracerebral inoculations, demonstrating a variety of infectious pathways (Cuillé and Chelle 1936). The disease was thought to originate from the infection of a slow virus (Cuillé and Chelle 1936), although viral particles or nucleic acids and histopathological changes corresponding to viral encephalitis were absent. Simultaneously, the transmissibility of scrapie was clearly demonstrated

in an accidental experiment where a vaccine against Louping Ill made from formalin-inactivated brain, spinal cord and/or spleen from an infected animal were subcutaneously inoculated into thousands of sheep, after which 7 % went on to develop scrapie (Gordon 1946). Conclusions from use of the infectious vaccine batch were that the scrapie agent is not inactivated by formalin, is different from conventional viruses, confirming what earlier experiments had suggested regarding the incubation period (Gordon 1946). However, unsuccessful attempts to cultivate bacteria and viruses further nourished the belief that scrapie was a slow virus (Sigurdsson 1954). In addition to the experimental transmissions, it was reported that natural transmission could occur to healthy sheep by grazing a pasture alternately with affected sheep (Greig 1940, Greig 1950).

Although the first case of Creutzfeldt-Jakob disease (CJD) in a human being was described by scientists as early as 1920 (Creutzfeldt 1920, Jakob 1921), its etiology was to remain unsolved for many years to come. The first report describing a mysterious disease spreading through cannibalism (Mathews, Glasse et al. 1968) among the Fore tribe in Papua New Guinea, called kuru, came in the 1950s (Gajdusek and Zigas 1959). However, the neuropathological similarities between kuru and scrapie were not recognized before 1959 when a veterinary pathologist, by chance, was introduced to a display about humans in Papua New Guinea suffering from a hitherto unknown brain disease. Hadlow recognized that kuru and scrapie shared similarities in brain pathology (Hadlow 1995), microscopically characterized by vacuolation of neurons, extensive neuronal loss and astrogliosis.

After extensive experimental inoculations in a range of animal hosts over many years, transmission of kuru to chimpanzees succeeded in 1966 and thus confirmed its transmissibility (Gajdusek, Gibbs et al. 1966). Likewise, CJD was established as a transmissible disease after successful transmission to chimpanzees (Gibbs, Gajdusek et al. 1968).

Following these major discoveries, the researchers began studying physical and chemical traits of the scrapie agent. An important basis for the work with scrapie pathogenesis and characterization of the agent was that scrapie was adapted to laboratory mice (Chandler 1961). In 1966, irradiation experiments showed that inactivation of scrapie infectivity only occurs after very high doses. This led to the suggestion that the scrapie agent could replicate without nucleic acid (Alper, Haig et al. 1966) and perhaps consists of a small protein (Alper, Haig et al. 1966, Pattison and Jones 1967), although alternative theories such as a virus, carbohydrate and membrane still circulated (Gibbons and Hunter 1967). However, experiences from Iceland with eradication through destruction of sheep in large areas and restocking after 1-3 years indicated long-term persistence of the agent (Palsson and

Sigurdsson 1958). Griffith was one of the first to postulate a "protein only" theory of scrapie pathogenesis (Griffith 1967).

After several years of studying experimental scrapie in hamsters with purification of infectivity from infected brains, the prion concept was presented by Prusiner et al. in 1982 (Prusiner 1982), for which he in 1997 would win the Nobel Prize in Medicine. Prusiner put forward that the infectious agent of prion diseases consisted solely of a single protein that was named the prion protein, a summary of the caption "*pro*teinaceous *in*fectious particle". After purification of a protease-resistant protein of 27 to 30 kDa (later designated PrP 27-30), the amino acid sequence of a small part of the protein could be determined (Prusiner, Groth et al. 1984). Subsequently, the gene for PrP^C, *PRNP*, was identified as a single-host gene expressed at high levels in mammalian neuronal cells and responsible for production of endogenous PrP^C (Chesebro, Race et al. 1985, Oesch, Westaway et al. 1985). The implication of this finding was that PrP^C exists in two forms, one normal cellular form (PrP^C) and one related to scrapie infectivity (PrP^{Sc}), and co-purification studies later established that the concentration of PrP^C was proportional to its infectivity (Bolton, McKinley et al. 1982). The two isoforms were shown to differ in their 3D structure, solubility, tendency to aggregate and resistance to protease degradation. Included in the prion hypothesis is that PrP^{Sc} triggers misfolding of endogenous PrP^C through an auto-catalytic process (Prusiner 1982).

1.2. THE CELLULAR PRION PROTEIN: A closer look

1.2.1. Structure and cellular localization

PrP^C (Figure 1) is encoded by the *PRNP* gene located on chromosome 20 in humans, chromosome 2 in mice and chromosome 13 in sheep, goats and cattle. The gene is highly conserved across species (Harrison, Khachane et al. 2010), and shows a high level of sequence identity among mammals, birds, reptiles and amphibians (Wopfner, Weidenhöfer et al. 1999, Calzolai, Lysek et al. 2005). In mice, sheep, cattle, and rats, the *PRNP* gene is composed of three exons, whereas only two are present in humans (Yoshimoto, Iinuma et al. 1992, Saeki, Matsumoto et al. 1996, Lee, Westaway et al. 1998).

The entire open reading frame of *PRNP* resides within one exon and encodes a primary transcript of 253 amino acids prior to proteolytic maturation of the protein. The majority of PrP^C translocate into the endoplasmic reticulum (ER) lumen where the N- and C-terminal signaling peptides are removed and a glycosylphosphatidylinositol (GPI)-anchor is attached (Stahl, Borchelt et al. 1987), leaving a 208

amino acids-long protein. Studies of the three-dimensional structure of the mature PrP^C show that the C-terminal two-thirds of the protein is a well-structured and globular domain dominated by three α-helices and a stabilizing disulfide bridge. Two glycosylation sites reside in this part of the protein. The N-terminal domain of the protein appears unstructured and is often referred to as a flexible domain, which contains a variable number of octapeptide-repeated sequences that have been shown to bind Cu atoms (Brown, Qin et al. 1997).

Within the ER, PrP^C may be glycosylated at two asparagine residues (N181 and N197) (Haraguchi, Fisher et al. 1989). Both unglycosylated and monoglycosylated forms of the protein are observed in addition to the dominating diglycosylated form, however the functional consequences of different glycosylation patterns is unknown.

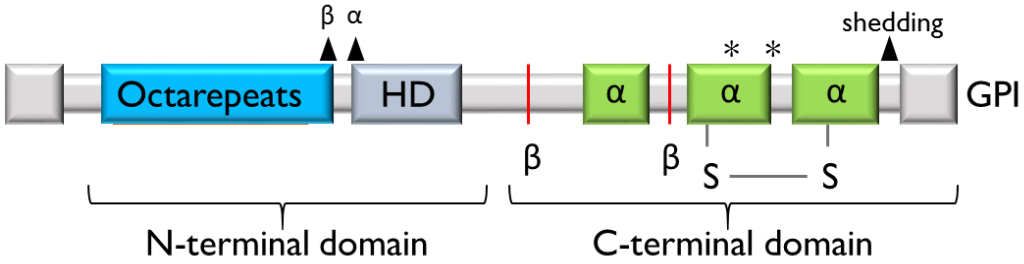

*Figure 1: Schematic illustration of the structure of PrP^C. Grey box to the left represents an ER signaling peptide, its role being the guidance of the protein into the ER. HD = hydrophobic domain, α = alpha helix, β = beta sheet. * indicates possible glycosylation sites and pyramids indicate cleavage sites, namely α and β cleavage (left) and shedding (right). The grey box represents the GPI-signaling sequence.*

Proteolytic processing of PrP^C is a common phenomenon with unclear physiological consequences. A varying amount of the total PrP^C is enzymatically cleaved (α-cleavage) between the residues 110 and 111 (human numbering) during its transport to the cell membrane, or during endocytic recycling, generating the C_1 and N_1 fragments (Harris, Huber et al. 1993). Whereas the N_1 fragment is released from the cell, the C_1 fragment resides at the cell membrane. Another form of cleavage, commonly referred to as β-cleavage, occurs within the octapeptide sequence and is reported to be a result of oxidative stress (McMahon, Mange et al. 2001, Watt, Taylor et al. 2005). Enzymatic processing by ADAM8 is also a possibility (McDonald, Dibble et al. 2014). The generated C_2 fragment is found in small amounts in healthy brain tissue (Mange, Beranger et al. 2004, Campbell, Gill et al. 2013). Shedding occurs when PrP^C is enzymatically cleaved by ADAM10 between the residues 227 and 228, removing the GPI anchor and three additional amino acids (McDonald, Dibble et al. 2014). Recently, a γ-cleavage

was presented (Wulf, Senatore et al. 2017); mainly occurring in unglycosylated PrP^C within the C-terminal part, generating N_3 and C_3 fragments (Linsenmeier, Altmeppen et al. 2017).

On the cell membrane, PrP^C is confined to lipid rafts (Taylor and Hooper 2006) rich in cholesterol and sphingolipids, and can cycle between the cell surface and endosomal compartments through entering clathrin-coated pits (Taylor, Watt et al. 2005) or caveolin-dependendt endocytic pathways (Peters, Mironov et al. 2003). Since cell-signaling proteins often are localized in lipid rafts, it has been postulated that PrP^C's presence here confirms its role as a protein involved in signal transduction. Accordingly, a wide range of interaction partners have been described for PrP^C (Nieznanski 2010); however, the functional importance of these is not fully clear.

Two structurally related proteins have later been invited into the prion-protein family, namely Doppel (Dpl) and Shadoo, encoded by the *PRND* (downstream prion protein-like gene) and *SPRN* (shadow of the prion protein gene) genes, respectively. *PRND* is located downstream of *PRNP*, whereas *SPRN* can be found on a different chromosome. The functions of the proteins are hitherto unknown, although Dpl appears to be testicle-specific and thus widely expressed in this organ after puberty (Paisley, Banks et al. 2004, Allais-Bonnet and Pailhoux 2014). Male mice devoid of Dpl are completely sterile (Behrens, Genoud et al. 2002). A more thorough introduction to these proteins can be found in (Watts and Westaway 2007).

1.2.2. PrP^C expression and proposed functions-general aspects

Although PrP^C is abundantly expressed in the peripheral and central nervous system (CNS), particularly on the surface of neurons in CNS (Manson, West et al. 1992, Moser, Colello et al. 1995, Ford, Burton et al. 2002), the protein is present in a wide range of non-neuronal tissues, including intestine, dental lamina, heart, lung, liver and kidneys (Manson, West et al. 1992, Ford, Burton et al. 2002, Tremblay, Bouzamondo-Bernstein et al. 2007, Peralta and Eyestone 2009). The expression pattern may depict that PrP^C's presence is important also in cells and tissues outside the CNS, and, if PrP^C's function is universal across tissues, investigating these can yield valuable information on PrP^C physiology.

Accordingly, the broad expression pattern is in accordance with the description of the plethora of phenotypes. Several studies have suggested that PrP^C plays a role already at the embryonal stage, particularly in embryonic stem cell regulation and differentiation. PrP^C was found to be co-regulated with the neuroectodermal stem cell marker Nestin, suggesting that PrP^C contributes to the development of embryonic stem cells into neural progenitor cells (Peralta, Huckle et al. 2011). A role for PrP^C in neurogenesis was supported in another study where mice overexpressing PrP^C had more proliferating cells in certain brain areas than *Prnp* knockout mice (Steele, Emsley et al. 2006). Perhaps was this due to PrP^C's proposed involvement in cell-cycle dynamics (Lee and Baskakov 2013). Furthermore, more compelling evidence connecting PrP^C and cell differentiation was provided by Miranda et al., showing a relationship between *PRNP* and pluripotency genes during early embryonic stem cell-differentiation stages (Miranda, Pericuesta et al. 2011, Miranda, Pericuesta et al. 2011). PrP^C was acknowledged as necessary for normal synaptic transmission, indicating an essential role within cell signaling (Collinge, Whittington et al. 1994); however, a following study did not confirm these results (Herms, Kretzchmar et al. 1995).

Furthermore, a postulated role in cell-cell contacts came from the observation that PrP^C was detected in cell-cell junctional domains in human enterocytes (Morel, Fouquet et al. 2004), and PrP^C induced cell adhesion between N2a neuroblastoma cells (Mange, Milhavet et al. 2002). A follow-up of the former study showed that PrP^C was important for normal epithelial barrier function in the intestine, since *Prnp* knockout mice showed increased permeability over the intestinal epithelium (Morel, Fouquet et al. 2008). PrP^C appeared to interact with several cytoskeleton-associated proteins, and was necessary for normal cell architecture and junction sizes (Morel, Fouquet et al. 2008). Irregularities in tight junctions after PrP^C ablation was also found in a subsequent study of intestinal cells, the functional importance of this finding was shown by the increased sensitivity to dextran sodium sulfate-induced colitis in *Prnp* knockout mice (Petit, Barreau et al. 2012).

Within behavioral studies, altered circadian rhythms and sleep continuity were noted in Edinburgh and Zürich I knockout mice (Tobler, Gaus et al. 1996, Tobler, Deboer et al. 1997), whereas cognitive (Criado, Sanchez-Alavez et al. 2005) and memory-formation deficits (Coitinho, Roesler et al. 2003, Coitinho, Freitas et al. 2006) have been reported from other mice models. In contrast to previous

studies (Lipp, Stagliar-Bozicevic et al. 1998, Roesler, Walz et al. 1999), changes in behavioral responses towards stress were observed in Zürich I knockout mice.

Further *in vivo* studies showed that PrP^C could be involved in the protection against oxidative stress induced by focal brain ischemia (McLennan, Brennan et al. 2004, Weise, Crome et al. 2004, Sakurai-Yamashita, Sakaguchi et al. 2005, Shyu, Lin et al. 2005). *Prnp* knockout mice also displayed higher levels of oxidized proteins and lipids in their brains at basal level, and decreased superoxide dismutase (SOD) activity was found in brain and muscle (Klamt, Dal-Pizzol et al. 2001). This was not supported in another study where no differences in enzymatic activity of Cu-Zn SOD could be detected (Waggoner, Drisaldi et al. 2000, Hutter, Heppner et al. 2003).
In this regard, PrP^C was found to bind Cu (Brown, Qin et al. 1997), which could contribute to its suggested antioxidant properties. It has been suggested that PrP plays an important role in Cu homeostasis, since it was observed that Prnp knockout mice had significantly lower Cu levels in brain (Brown, Qin et al. 1997), however this was not found in a subsequent study (Waggoner, Drisaldi et al. 2000).

Finally, an important role for PrP^C in maintenance of myelin integrity has been proposed, since an age-dependent demyelinating peripheral neuropathy has been observed in several different lines of *Prnp* knockout mice, including the newly developed ZH3/ZH3 line (Nishida, Tremblay et al. 1999, Baumann, Tolnay et al. 2007, Bremer, Baumann et al. 2010, Nuvolone, Hermann et al. 2016). Neurophysiological and morphological analysis of peripheral nerves of goats without PrP^C are currently ongoing. Interestingly, at the age of 7 years no clinical signs of neuropathy were present.

1.2.3. PrP^C in hematopoiesis and immune cell functions

Comprehensive studies of immune cells, with regard to their role in prion propagation in peripheral tissues and as carriers and distributors of prion infectivity, have contributed massively to our understanding of peripheral prion pathogenesis. Notably, only immune cells expressing PrP^C on their cell surface take part in this, therefore, levels of PrP^C on the surface of immune cells have been characterized. Indeed, PrP^C is distinctly present on the surface of most circulating immune cells and their bone marrow precursors. In the bone marrow, PrP^C is present on the cell surface of murine long-term hematopoietic stem cells (HSCs) (Zhang, Steele et al. 2006), as well as $CD34^+$ multi-

potential stem cells in humans (Dodelet and Cashman 1998) and c-kit$^+$ bone marrow cells (Kubosaki, Yusa et al. 2001). Whether PrP^C can be considered a marker for HSCs at certain developmental stages is unknown. Giving rise to all mature blood cells, HSCs are required throughout life to secure a constant refill of hematopoietic precursors. Notably, no morphological differences in PrP^C-deficient bone marrow were observed (Kubosaki, Yusa et al. 2001, Zhang, Steele et al. 2006), indicating that lack of PrP^C expression does not result in abnormal bone marrow development, at least at a normal physiological state.

Also murine erythroblasts express PrP^C (Panigaj, Glier et al. 2011), with expression levels declining during erythroid maturation (Griffiths, Heesom et al. 2007) reaching a level far below detection in their mature form (Antoine, Cesbron et al. 2000, Panigaj, Brouckova et al. 2011).

More conflicting results have been obtained concerning PrP^C's expression on megakaryocytes and platelets, since this expression seems to vary according to species (Holada and Vostal 2000, Herrmann, Davis et al. 2001, Starke, Harrison et al. 2005).

Whereas the expression of PrP^C is maintained throughout the differentiation of the lymphoid and monocyte lineages in humans, a downregulation at both protein and mRNA levels was reported in the granulocyte and erythroid lineages (Dodelet and Cashman 1998). Among the circulating blood cells, PrP^C is highly expressed on mononuclear cells such as lymphocytes and natural killer (NK) cells, albeit at lower levels in B cells. This expression seems to increase following the cells' maturation and/or activation (Cashman, Loertscher et al. 1990, Mabbott, Brown et al. 1997, Li, Liu et al. 2001, Nitta, Sakudo et al. 2009, Mabbott 2015), also valid for monocytes and dendritic cells (DCs) (Durig, Giese et al. 2000, Ballerini, Gourdain et al. 2006). Similarly, although granulocytes have been shown not to express PrP^C on their surface (Antoine, Cesbron et al. 2000), neutrophils upregulate PrP^C following activation with LPS, glucocorticoids and transforming growth factor-β (Mariante, Nobrega et al. 2012). Confirmation of these results in other animal models, such as the goat, would be very valuable since significant species differences could occur.

Another PrP^C-expressing cell type developing from a HSC in the bone marrow are mast cells, which circulate in blood at precursor stages and become stationary in tissues as they mature. Mast cell differentiation or homeostasis appears not to depend on PrP^C expression; however, interestingly, the protein is released upon degranulation (Haddon, Hughes et al. 2009). Considering the longevity of

mast cells, understanding PrP^C's role in this cell type, particularly during immune reactions would be of great value. Unfortunately, the current lack of protocols for obtaining mast cells from goats, prevented such investigations in the present work.

Based on the presence of PrP^C on the surface of immune cells, the role of PrP^C in specific immune cell functions has been explored *in vitro*. Indeed, PrP^C was attributed a role as an activating signaling molecule in T cells (Mattei, Garofalo et al. 2004), particularly in antigen-presenting cell-driven T-cell responses (Ballerini, Gourdain et al. 2006), and in monocytes (Krebs, Dorner-Ciossek et al. 2006).
Studies of T cells from *Prnp* knockout mice have proposed a role for PrP^C in T-cell pathways leading to proliferation. Whereas a reduced proliferative response and altered cytokine production were reported from mice (Mabbott, Brown et al. 1997, Bainbridge and Walker 2005), no differences were found in the proliferative capacity of T cells from *PRNP* knockout cattle (Richt, Kasinathan et al. 2007). PrP^C-ablated peritoneal macrophages showed increased apoptosis compared to wildtype macrophages (de Almeida, Chiarini et al. 2005), and similar results were reported from a study using bone marrow-derived macrophages (Wang, Zhao et al. 2014). Another study utilizing bone marrow-derived macrophages observed reduced phagocytosis after knockout of *PRNP* (Uraki, Sakudo et al. 2010). PrP^C's role in modulating phagocytosis is controversial since polymorphisms in the PrP^C-flanking gene *SIRPA* was found to influence the results (Nuvolone, Kana et al. 2013).
A clarification of PrP^C's role in T-cell proliferation and phagocytosis would be beneficial in the further investigations of PrP^C physiology.

1.2.4. PrP^C's role in inflammation

The dynamic expression of PrP^C in immune cells (Mabbott, Brown et al. 1997, Dodelet and Cashman 1998, Durig, Giese et al. 2000, Li, Liu et al. 2001, Ballerini, Gourdain et al. 2006, Mariante, Nobrega et al. 2012), described in detail in the previous section, suggests that the protein may have the ability to modulate immune responses. Indeed, in an autoimmune encephalomyelitis (EAE) model, PrP^C knockout mice presented with earlier symptoms than their wildtype counterparts, and also suffered from prolonged and more severe neuroinflammation dominated by T-cell and microglial infiltrates, and increased pro-inflammatory cytokine gene expression in the brain (Tsutsui, Hahn et al. 2008).

To analyze the importance of PrP^C expression on immune cells in the EAE model, immunopathology following knockout of *PRNP*, either in brain tissue or in lymphocytes, was investigated (Gourdain, Ballerini et al. 2012). The disease was most severe with a PrP^C-depleted CNS and normal lymphocytes than the reciprocal situation. A combined CNS- and lymphocyte-*Prnp* knockout did not worsen the brain pathology compared with lack of PrP^C in CNS solely, and it was concluded that *Prnp* expression in the CNS is important for neuroprotection during stress, especially in neuroinflammation. In a subsequent study of autoimmune disease in the CNS; however, PrP^C depletion of lymphocytes affected T-cell activation, survival and differentiation. There was an increased tendency for T cells to develop a pro-inflammatory phenotype, and the *Prnp* knockout mice displayed increased disease severity (Hu, Nessler et al. 2010). The latter study indicated that the impact of PrP^C deficiency is related to modulation of immune cell activity causing a more severe neuroinflammation.
Other inflammatory models such as experimentally induced colitis have shown similar results with increased PrP^C levels in inflamed colon tissue and increased severity of disease after PrP^C ablation (Martin, Keenan et al. 2011).

The *in vivo* role of PrP^C in cytoprotection has been studied in models inflicting ischemia and hypoxia in tissues. In murine stroke models where ischemic challenge was applied to an area of the brain, PrP^C ablation had a profound effect on the disease progression and outcome, resulting in larger infarcts in the *Prnp* knockout mice. (McLennan, Brennan et al. 2004, Weise, Crome et al. 2004, Spudich, Frigg et al. 2005). Interestingly, PrP^C levels increased in the surrounding brain tissue of wildtype mice post-infarction, supporting an important role for PrP^C in reducing tissue damage. Ischemia triggers generation of free radicals (Iadecola 1997) that could be directly damaging to cells, but a wide range of cellular pathways are initiated during stroke, including pro-inflammatory pathways (George and Steinberg 2015). Whether the results from studies of ischemia in *Prnp* knockout mice are a direct reflection of PrP^C's proposed protective role during oxidative stress, or if they are more related to its immunomodulatory role, is unclear. PrP^C's role in protecting cells during oxidative stress will be further discussed in section 1.2.5.

Taken together, *in vivo* studies have shown that PrP^C might exhibit immune-dampening effects during inflammatory processes. In this context, it is interesting to note that the expression pattern of PrP^C in the human body largely overlaps with organs considered to be "immunologically quiescent", specifically

organs where immune pathology and inflammation can potentially have dramatic consequences for the body as a whole (Bakkebo, Mouillet-Richard et al. 2015). Immune privilege is an evolutionary protective trait developed to protect vulnerable tissues with limited capacity for regeneration after trauma or inflammatory damage (Niederkorn and Stein-Streilein 2010). The mechanisms behind PrP^C's immunomodulatory functions are largely unknown.

1.2.5. PrP^C and oxidative stress

Cells are normally exposed to chemically unstable oxygen-containing molecules, collectively known as reactive oxygen species (ROS). These are generated at physiological levels in various organelles of the cell, such as the endoplasmic reticulum, peroxisomes and mitochondria; however, also from exogenous sources including ultraviolet light, environmental toxins, metals, various chemicals and radiation. In order to keep levels of ROS tightly controlled and protect cellular macromolecules from ROS-induced damage, cells have evolved a sophisticated and complex antioxidant network involving both enzymatic (Figure 2) and non-enzymatic antioxidants. If the antioxidant pool diminishes, or if enhanced ROS levels accumulate, cells will get into a state of oxidative stress, which, unless neutralized, can have injurious consequences for the cell including lipid peroxidation, mitochondrial dysfunction, protein degradation, DNA damage and ultimately autophagy and apoptosis.

Notably, low to moderate levels of ROS are crucial for the cell and contributes to normal physiological processes. H_2O_2 is described as an important signaling molecule, and ROS is involved in modulating the activity of several signaling pathways (Zhang, Wang et al. 2016) and transcription factors such as nuclear factor-kappaB and activator-protein 1 (Giordano 2005). Immune cells utilize ROS to assist their functions; activated monocytes or macrophages release superoxide upon encounter with microbes, and neutrophils and eosinophils utilize oxidants in antibacterial defense through a process called the "oxidative burst" (Hensley, Robinson et al. 2000).

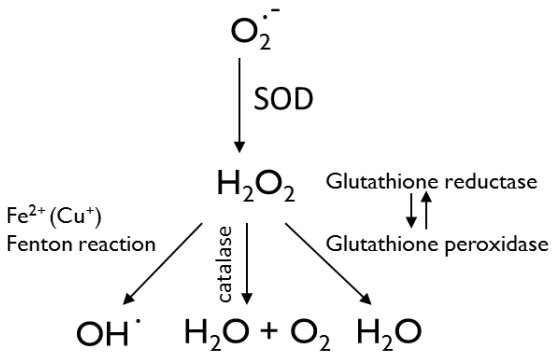

Figure 2: Cellular sources of ROS and main pathways of neutralization. Superoxide anion (on the top) is neutralized by SOD into H_2O_2. Through the Fenton reaction, H_2O_2 can be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$, left). By catalase and glutathione peroxidase, H_2O_2 can be converted to H_2O and O_2. Glutathione peroxidase cycles to glutathione reductase and back.

Several studies have reported that the presence of PrP^C is protective during a cellular state of oxidative stress, predominately reflected as poorer cell survival in *PRNP* knockout cells after induction of oxidative stress with xanthine oxidase (Brown, Schulz-Schaeffer et al. 1997, Brown, Nicholas et al. 2002), paraquat (Senator, Rachidi et al. 2004) and H_2O_2 (White, Collins et al. 1999, Oh, Choi et al. 2012, Bravard, Auvre et al. 2015). While limited mechanistic insights have been gained from these *in vitro* studies, some have explained the increased vulnerability to oxidative stress in *PRNP* knockout cells by changes in enzymatic antioxidant activity. Decreased basal levels of SOD activity (Brown, Schulz-Schaeffer et al. 1997, Klamt, Dal-Pizzol et al. 2001) were reported in brain tissue of *Prnp* knockout mice, however no changes in glutathione peroxidase, catalase or Cu/Zn-SOD activity in brain were found in a subsequent study (Brown, Nicholas et al. 2002). Xanthine oxidase-induced oxidative stress increased SOD-1 activity in cells from cerebellum and cortex from wt mice, but not in the cells derived from PrP^C-deficient mice (Brown, Schulz-Schaeffer et al. 1997). However, in contrast to previous studies, it has been demonstrated that PrP^C lacks SOD activity (Hutter, Heppner et al. 2003, Steinacker, Hawlik et al. 2010). Furthermore, reduced glutathione reductase activity was found in H_2O_2-exposed cerebellar granule neurons (White, Collins et al. 1999). Clearly, PrP^C's role in antioxidant protection is an unsettled subject and whether PrP^C affects cellular antioxidant activities directly or indirectly is yet unknown.

Several studies in *Prnp* knockout mice have shown that in the absence of PrP^C the oxidative load in brain and certain peripheral tissues appears to be increased, evident by measurement of higher levels

of lipoperoxidation and protein oxidation in these tissues (Klamt, Dal-Pizzol et al. 2001, Wong, Liu et al. 2001). As previously discussed, in murine stroke models, *Prnp* knockout mice generated a larger dissemination of the stroke area compared to wt mice, with a higher proportion of neurons succumbing to cell death (McLennan, Brennan et al. 2004, Spudich, Frigg et al. 2005), indicating that PrP^C is protective during stressful circumstances where oxidative stress is generated.

1.2.6. PrP^C and male reproduction

We know from several reports that PrP^C is expressed in the testicles, epididymis and spermatozoa (Shaked, Rosenmann et al. 1999, Ford, Burton et al. 2002, Peoc'h, Serres et al. 2002, Fujisawa, Kanai et al. 2004). Conflicting data exist concerning whether spermatozoa contain C-terminally (Shaked, Rosenmann et al. 1999) or N-terminally truncated (Peoc'h, Serres et al. 2002) PrP^C. *PRNP* mRNA was detected in several developmental spermatogenic stages by (Ford, Burton et al. 2002), but immunostaining showed the strongest staining in Sertoli cells and spermatozoa, suggesting an inverse relationship between *PRNP* mRNA levels and expressed PrP^C. Another study found *PRNP* mRNA in early developmental stages, but not in late spermatids and spermatozoa (Fujisawa, Kanai et al. 2004). In contrast to the previous study by Ford et al., Sertoli cells were negative (Fujisawa, Kanai et al. 2004). Peoc'h et al. detected PrP^C in germinal cells, spermatocytes, spermatids and spermatozoa, but spermatogonia were negative (Peoc'h, Serres et al. 2002).

During spermatogenesis, Sertoli cells, connected by tight junctions, create a physical barrier known as the blood-testis-barrier. The barrier prevents leukocytes and macromolecules from crossing the seminiferous epithelium. In addition, Sertoli cells can modulate immune responses by producing anti-inflammatory cytokines such as transforming growth factor-β and interleukin (IL)-10, apoptosis and complement inhibitors, as well as other chemokines. As a result, the whole testicle is immune privileged (Reviewed in Chen, Deng et al. 2016, Franca, Hess et al. 2016). The expression of PrP^C in the testicle could indicate that the protein inhabits an indispensable role here, perhaps as a contributor to the immunosuppressive milieu. A clarification of the PrP^C expression pattern in the testicles is thus needed.

A putative role for PrP^C in protection against Cu^{2+}-induced stress in spermatozoa was presented (Shaked, Rosenmann et al. 1999), indicating that PrP^C can display protective properties in various cellular settings. In the presented study, *PRNP* knockout spermatozoa derived from the epididymis

from mice of various ages had a significantly faster loss of motility during the course of experiment as compared to wt spermatozoa (Shaked, Rosenmann et al. 1999). This is interesting since mature spermatozoa retain only the minimal components required for their unique function, and they are mostly transcriptionally inactive due to compaction of chromatin (Sassone-Corsi 2002). Therefore, spermatozoa are particularly prone to stress, particularly that induced by oxidative stressors. ROS-induced motility loss correlated well with lipid peroxidation levels in human spermatozoa (Aitken and Baker 2006), and it appears that the major ROS involved in peroxidation-induced motility loss is H_2O_2 (Aitken and Baker 2006). Further investigations of PrP^C's role in the highly specialized testicular tissue and in spermatozoa could yield valuable information about PrP^C's normal physiology.

1.2.7. Conversion of PrP^C to PrP^{Sc} and initiation of neurodegeneration

Since an overview of both PrP^C and prion diseases is given, and as a final aspect of the introduction of PrP^C's possible function in the organism, a brief introduction to some theories connecting PrP^C functions and prion disease is relevant.

Based on the knowledge that PrP^{Sc} originates from a host-encoded protein, detailed mechanistic studies of prion disease pathogenesis commenced. It has become clear that the conversion of PrP^C to PrP^{Sc} depends upon direct interaction between GPI-anchored PrP^C and incoming PrP^{Sc} either at the cell surface or in endosomal compartments (for a review see Poggiolini, Saverioni et al. 2013), provoking a decrease in α-sheet content and an increase in β-sheets (Pan, Baldwin et al. 1993). While it is known that the β-sheet-rich PrP^{Sc} is prone to aggregation and acts as a template for further pathogenic conversion of PrP^C, the events in this cascade of PrP^{Sc} accumulation, eventually culminating in neurodegeneration, is incompletely understood.

Since PrP^{Sc} cannot be detected in healthy neuronal tissue, a "gain-of-function" theory was proposed suggesting that PrP^{Sc} aggregates or break-down products of PrP^{Sc} is directly toxic to neurons (theories outlined in Figure 3). However, by grafting PrP^C-expressing brain tissue into brains of *Prnp* knockout mice and subsequently infecting the mice, Brandner et al. showed that PrP^{Sc} only induces pathology in the PrP^C-expressing brain graft, meaning that PrP^C somehow is involved in mediating the possible toxicity of PrP^{Sc} (Brandner, Isenmann et al. 1996). Also, depletion of PrP^C during the preclinical stages of prion infection reversed the pathological changes in neuronal tissue and prevented progression of disease (Mallucci, Dickinson et al. 2003), corroborating the obligatory presence of PrP^C.

A second theory questions the potential consequences that the loss of PrP^C's normal, physiological function upon conversion to PrP^{Sc} may play in the initiation of neuronal dysfunction. If PrP^C, as part of its normal physiological function, sends a protective signal and this is lost, pathways involved in apoptosis or other stress-related mechanisms could be activated. However, this theory does not fully explain prion disease pathogenesis, as mice devoid of PrP^C show no major phenotypes and have normal life expectancy (Bueler, Fischer et al. 1992).

Furthermore, it has been suggested that in prion disease pathology, there is a combined loss of neuroprotective signaling mediated by PrP^C with a gain of toxic signaling driven by intermediary, possibly oligomeric, prion protein complexes. Whereas this theory will not be further outlined here, more information can be found in (Westergard, Christensen et al. 2007).

To date, the molecular mechanisms underlying prion disease neurodegeneration are an area of active research. Consensus is lacking regarding which physiological roles PrP^C truly occupy, and evidently, detailed knowledge of PrP^C's normal physiological function is necessary and important in the ongoing work.

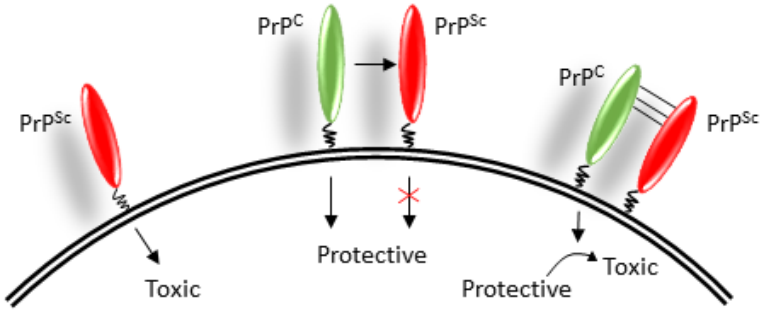

Figure 3: Proposed theories of how PrP^C and PrP^{Sc} contributes to neurodegeneration. Left: Gain-of-function, middle: loss-of-function, right: subversion-of-function. Based on a figure by (Westergard, Christensen et al. 2007).

1.3. THE CELLULAR PRION PROTEIN: Models to study PrP^C

1.3.1. Models to study PrP^C functions

A wide range of techniques have been employed in the ongoing studies of PrP^C physiology:

1) identification of interaction partners, 2) genetic studies of the *PRNP* gene, 3) studies of phenotypes following ectopic and overexpression of PrP^C in various animals and cell lines, and 4) deletion of *PRNP* in animals and cell lines.

The successful knockout of *Prnp* in mice in 1992 undoubtedly revolutionized the work within prion research (Bueler, Fischer et al. 1992), and deletion, or knockout, of *Prnp* quickly became one of the most extensively used methods in the studies of PrP^C physiology. Although, considering PrP^C's well-preserved expression in several species and the idea therefore that PrP^C was essential to sustain life, it came as a major surprise that the main phenotype of *Prnp* knockout mice was inability to develop prion disease (Bueler, Aguzzi et al. 1993).

The original Zürich I and Edinburgh *Prnp* knockout-mice lines turned out to develop and behave normally (Bueler, Fischer et al. 1992), although a myriad of phenotypes have later been attributed to PrP^C (recently reviewed in Castle and Gill 2017, Wulf, Senatore et al. 2017). A full review of the knockout methods is outside the scope of this text (reviewed in Weissmann and Flechsig 2003), but it should be mentioned that the chosen knockout strategy has limitations that can influence the phenotype. Whether an observed phenotype is the result of a mixed genetic background explained by locus heterozygosity or flanking genes, or the deleted gene itself, can be falsely interpreted unless the researchers are aware of this scenario (Steele, Lindquist et al. 2007).

Late-onset ataxia, caused by loss of cerebellar Purkinje cells, was observed in several lines of *Prnp* knockout mice (Sakaguchi, Katamine et al. 1996, Weissmann, Fischer et al. 1998). The phenotype was originally attributed to the loss of PrP^C since the phenotype could be rescued by reintroduction of PrP^C; however, erroneously expressed Dpl in brain tissue later explained the phenotype, a consequence of fusion of PrP^C's promoter to Dpl during the *Prnp*-deletion process (Moore, Lee et al. 1999, Li, Sakaguchi et al. 2000).

Ectopic Dpl expression is also an issue in several of the cell lines derived from the mouse lines listed in Table 2. The Dpl expression in these mouse lines could hamper the interpretation of phenotypes discovered in these models (Castle and Gill 2017) and yield phenotypes that are not attributed to PrP^C loss, particularly in the neuronal cell lines where Dpl is normally not expressed. One example is the phenotype observed in hippocampal neurons isolated from Rikn mice, where protection of PrP^C against serum withdrawal and apoptosis was observed (Kuwahara, Takeuchi et al. 1999, Kim, Lee et al. 2004). Other cell lines without Dpl expression have later been used for similar purposes. For an overview of *Prnp* knockout cell lines, see (Sakudo and Onodera 2014). Whether Dpl expression can

explain all the proposed phenotypes in *PRNP* knockout cell lines with ectopic Dpl expression is hitherto unknown.

Interestingly, the Npu and Zürich III mouse lines are presumably free from gene linkage. The Zürich III mouse line was created with an entirely new knockout technology, thus creating a whole new platform to study PrP^C-related phenotypes. Recently, a phenotype of demyelinating peripheral neuropathy was presented, suggesting that PrP^C's normal function is peripheral myelin maintenance (Nuvolone, Hermann et al. 2016). It will be interesting to follow the studies on this mouse line in the future to see if other phenotypes are presented.

Table 2. Overview of mouse lines with ectopic *Dpl* expression

Mouse line	Ectopic *Dpl* expression
Wildtype	-
Zürich I	-
Edinburgh	-
Nagasaki	+
Rcm0	+
Zürich II	+
Rikn	+
Zürich III	-
Npu	-

Furthermore, altered phagocytosis, a phenotype originally attributed to PrP^C, has been questioned due to the finding of polymorphisms in the *PRNP*-flanking gene encoding SIRP-α, a cellular "don't eat me signal", able to influence the rate of phagocytosis in the established cell lines (Nuvolone, Kana et al. 2013).

In addition to mice, alternative transgenic PrP^C-free ruminant models have been produced such as cattle and goats. In the goat model, few aberrant phenotypes were found and the animals have not been characterized further (Yu, Chen et al. 2009). *PRNP* knockout cattle have been more extensively investigated and growth and health data from *PRNP* knockout cattle were generated from birth until

20 months of age (Richt, Kasinathan et al. 2007). Reproductive data were normal, as were clinical examinations, serum chemistry and routine macro and micro pathological investigations, although an increased nervous reaction was found in a few knockout cattle. Hematological analysis revealed that *PRNP* knockout cattle displayed slightly aberrant RBC sizes (lower mean cell volume, MCV) and hemoglobin content (MCH); however, other RBC characteristics were similar. Furthermore, higher white blood cell number and neutrophil counts were found. Evaluation of the immune system, including B- and T-cell populations, T-cell proliferation, IFN-γ production and humoral immune responses revealed no differences between the two genotypes (Richt, Kasinathan et al. 2007).

Taken together, it is evident that confirmation of a phenotype in several animal models or more than one line of *Prnp* knockout mouse is important to build confidence in a proposed phenotype.

1.3.2. A new model for PrP^C research–goats naturally devoid of PrP^C

In 2012, a study of *PRNP* polymorphisms in a unique line of Norwegian Dairy Goats led to the accidental discovery of a point mutation in *PRNP* (Benestad, Austbo et al. 2012). The mutation introduces a premature stop codon in codon 32 (Figure 4), hence terminating *PRNP* translation. Animals homozygous for the mutation are devoid of PrP^C. The genotype appears to be the only one of its kind in the world, at least that has been reported so far, considering the vast amount of screening programs in both humans and other mammals which have failed to identify other naturally PrP^C-deficient animals or humans.

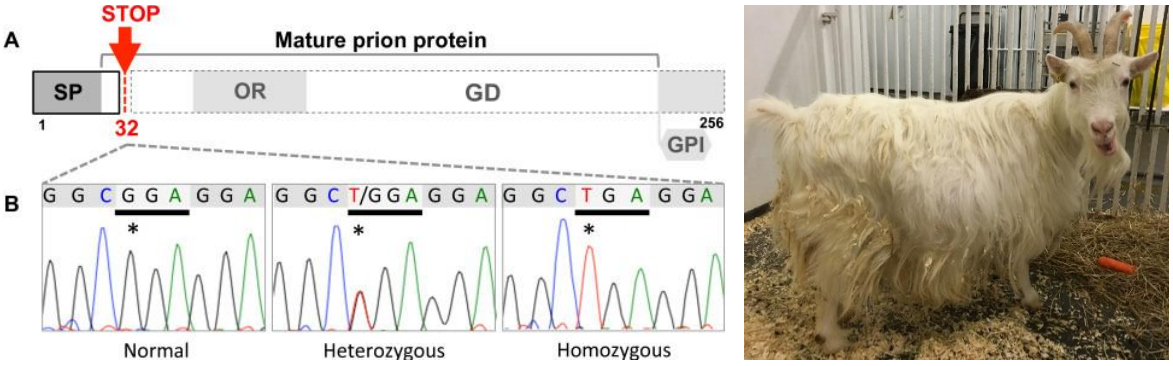

*Figure **4**: Left: the full-length PrP^C is shown and the stop codon is marked in red color. Right: Priona, one of the research goats devoid of PrP^C, eating a carrot. Photo by Liv-Heidi Nekså. Figure from (Benestad, Austbo et al. 2012).*

To date, 2927 goat bucks have been screened for the mutation and the *PRNP*^{Ter}-allele frequency is 5.89 %, suggesting that approximately 11 % of the Norwegian Dairy Goat population carry the *PRNP*^{Ter} allele and 0.6 % are homozygous. Given that the total number of dairy goats in Norway is about 38.000 animals, a couple of hundred animals are expected to be homozygous for the nonsense mutation.
The goats display no obvious phenotypic differences compared to normal goats, and we have no data to support that the animals suffer from any abnormalities or are overrepresented in the disease statistics. Both bucks and does are capable of breeding normally, since homozygous animals have been used for breeding within the flock and goats have given birth normally.

Whether these goats hold the key to the secrecy of PrP^{C} physiology requires further investigation, but the absence of some of the confounding factors, namely those that may be introduced by genetic engineering, make them a useful and appealing resource worth exploring.

2. Aims

The overall aim was to investigate the non-neuronal effects of PrP^C deficiency in *PRNP*$^{Ter/Ter}$ goats with respect to fundamental physiological and immunological parameters.

The specific objectives were to:

1. Investigate if basal blood parameters in PrP^C-deficient goats deviate from normal goats
2. Identify if PrP^C contains a role in basal immune functions of blood leukocytes derived from goats with and without PrP^C
3. Describe the transcriptomic effects of PrP^C loss in circulating blood mononuclear cells to reveal the effects of PrP^C loss on molecular pathways and cell functions that may or may not be associated with findings derived from objective 1 – 2
4. Study the stress-protective effects of PrP^C in PBMCs and spermatozoa *in vitro*

3. Summary of papers

Paper I

Hematological shift in goat kids naturally devoid of prion protein

A nonsense mutation in the *PRNP* gene was recently described (Benestad, Austbo et al. 2012), terminating *PRNP* translation and rendering animals devoid of PrP^C. This paper aimed at performing a basal hematological and immunological characterization of PrP^C-deficient animals. In accordance with the literature, PBMCs expressed moderate levels of PrP^C while granulocytes were negative, suggesting PBMCs to be interesting cells to study of PrP^C functions. Clinical chemistry and complete blood counts of the animals were investigated. While only minor deviations in clinical chemistry were detected, the hematological analysis presented a hematological shift in PrP^C-deficient animals, dominated by an increased number of RBCs characterized by a smaller cell volume, and possibly an increased number of neutrophils. The results suggested that PrP^C exhibits a role in RBC production and/or maintenance, however no morphological changes could be detected in the bone marrow. All values were within reference range. Immunophenotyping showed no genotype effects on the levels of circulating immune cells, and basic immune functions such as monocyte phagocytosis and T-cell proliferation were similar in both genotypes, showing that PrP^C loss does not impair these important functions *in vitro*.

Altogether, $PRNP^{Ter/Ter}$ animals displayed a generally healthy appearance. In this study, PrP^C loss was associated with subtle hematological changes, indicating a role for PrP^C in maturation and release of selected cell lineages from the bone marrow. However, no effects of PrP^C could be detected in relation to the relative ratio of circulating immune cells and basal immune cell functions.

Paper II

Loss of prion protein induces a primed state of type I interferon-responsive genes

Previous gene expression studies of *PRNP* knockout animals have revealed subtle expression differences with divergent pathways involved. In this paper we investigated the transcriptomic responses to PrP^C deficiency in $PRNP^{Ter/Ter}$ goats compared to normal $PRNP^{+/+}$ goats. By mRNA-sequencing (RNA-seq) a modest, but distinct, expression profile was revealed, showing an upregulation of type I IFN-responsive genes in $PRNP^{Ter/Ter}$ cells, a result which was confirmed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) of selected genes. The profile could not be explained by changes in health status or altered levels of the components involved in type I IFN

signaling. The finding suggested that PrP^C is involved in the fine-tuning of the expression levels of type I IFN-responsive genes. To clarify whether cells without PrP^C respond faster to IFN-α exposure, human neuroblastoma SH-SY5Y cells transfected with PrP^C were used to mimic the response. Indeed, expression of the type I IFN-responsive gene *MX2* was suppressed in hu-PrP SH-SY5Y cells. The finding was further confirmed by an independent data set generated through analysis of circulating leukocytes isolated from goats intravenously injected with LPS.

Evidently, direct or indirect stimulation of type I IFN signaling elicited a slightly stronger response in PrP^C-deficient cells, strongly suggesting that PrP^C harbors an immune-dampening effect in cellular type I IFN responses. Further analyses are warranted to fully uncover mechanisms where PrP^C is involved.

Paper III

The prion protein and genotoxic stress

Various stress-protective roles have been acclaimed to PrP^C, however the direct stress-protective function remains unproven. With this background we aimed to investigate stress resilience in spermatozoa and PBMCs from *PRNP*$^{Ter/Ter}$ goats compared to *PRNP*$^{+/+}$ goats. Western blot (WB) confirmed PrP^C expression in spermatozoa with a band pattern slightly different from the pattern in the testicles. By immunofluorescence (IF) and immunohistochemistry (IHC), we confirmed that PrP^C is widely expressed in the testicles, particularly in Sertoli cells and Oil-red-O (ORO)-positive lipid vacuoles, and in the interstitial epididymal tissue. In spermatozoa, the PrP^C level was likely below the detection limit of these techniques. Investigations of freeze tolerance, viability, motility, ATP levels and acrosome intactness in spermatozoa at rest and after acute stress, induced by Cu^{2+} ions, as well as levels of ROS after exposure to $FeSO_4$ and H_2O_2 were similar among the genotypes, showing no evident protective trait, such as ROS scavenging, of PrP^C in spermatozoa. Similarly, no stress-protective effects of PrP^C could be detected in PBMCs and SH-SY5Y cells when cells were exposed *in vitro* to doxorubicin, H_2O_2 and the genotoxin MMS. Based on the lack of detectable differences in the accumulation of 7-m(dG), a critically important role for DNA repair was not supported. Furthermore, results from RNA-seq showed that PBMCs during steady-state express similar levels of mRNA transcripts encoding enzymes involved in DNA repair and antioxidant activity.

These data, acquired from several different cellular models, suggest that PrP^C is not directly protective against these stressors *in vivo*, but does not rule out a protective role *in vivo*.

4. Materials and methods

A general workflow that was followed throughout the experiments can be seen in Figure 5.

Necessary details on materials and methods are presented in each of the papers. In this part of the thesis, an overview of the materials and supplementary information to some of the most important and frequently used methods will be given.

A short discussion on some of the advantages and pitfalls by use of these methods will also be included.

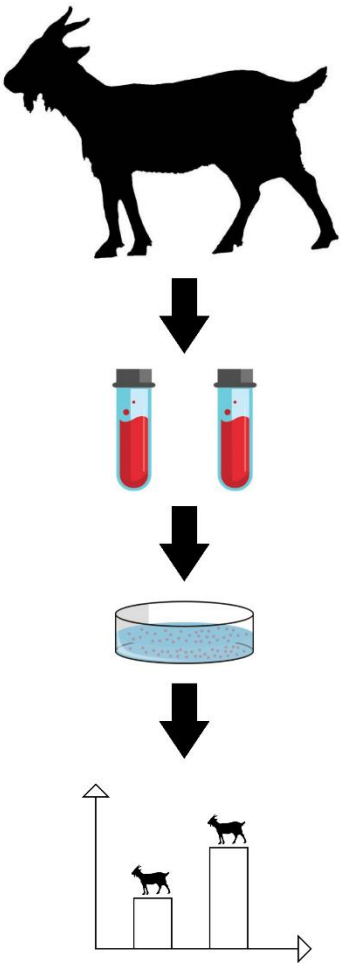

Figure 5: Experiments were initiated after isolation of PBMCs (or spermatozoa) from goats of both genotypes, and continued with in vitro experiments culminating in read-outs where phenotypes were compared.

<u>The material and methods highlighted in **bold** will be described:</u>

- **Animals**
- **Cell culture (papers I-III)**
- Functional immune-cell assays: lymphocyte proliferation test, phagocytosis assay (paper I)
- **Protein detection: flow cytometry, IF, IHC, WB (papers I and III)**
- **RT-qPCR (papers I-III)**
- **RNA-sequencing (paper II)**
- Basal spermatozoa parameters and induction of oxidative stress (paper III)
- ROS analysis of spermatozoa (paper III)
- DNA-damage measurements by LC-MS/MS (paper III)

4.1. Animals

The animals included in this study were exclusively goats of the Norwegian Dairy Goat Breed obtained from a research herd of approximately 100 winter-fed goats at the Norwegian University of Life Sciences, located in Ås, Norway.

A number of animals in the flock harbor a mutation in *PRNP* and were genotyped as either $PRNP^{Ter/Ter}$, $PRNP^{+/Ter}$ or $PRNP^{+/+}$. In all studies, animals were age- and gender-matched if possible. Details concerning the age and number of animals included in each experiment can be found in the respective papers.

All animals were subjected to careful observation and handling in accordance with the national animal welfare laws and regulations for research animals. The principle of the three R's in animal research (replacement, reduction, refinement) were considered prior to experiments.

Throughout the study, all animals were housed in a natural farm environment to minimize stress and discomfort. Goat kids were born during early spring (Feb-April) and followed normal farm procedures such as dehorning and castration, including a normal fertility cycle with estrus and pregnancy. The flock has regular visits by veterinarians and the flock health is generally good, free from CAE, caseous lymphadenitis and paratuberculosis (Nagel-Alne, Asheim et al. 2014).

Within prion research, transgenic rodents, mostly mice and to a certain extent rats, have traditionally been used as experimental model organisms. Based on this, a few aspects concerning the suitability of transgenic mice as opposed to a non-transgenic goat model in research are worth mentioning.

While studies of *Prnp* knockout mice have been very useful in the understanding of PrP^C biology, one of the issues when using transgenic animals in research is the presence of genetic confounders that can influence the observed phenotype (Eisener-Dorman, Lawrence et al. 2009), as outlined in the *introduction*. A detailed analysis of the $PRNP^{Ter}$ haplotype has not yet been performed, but preliminary data indicate that a flanking gene problem in the goats is limited. For instance, comparisons of single nucleotide polymorphisms (SNPs) in the SIRP α gene revealed an even distribution between the genotype groups (data not shown). The result suggests that there is a low probability of linkage between certain *SIRP α* polymorphisms and the $PRNP^{Ter}$ haplotype. However, more research is needed to investigate the possible importance of genetic confounders, such as linked flanking genes or differences in genetic background, in the goat model.

To avoid the consequences of possible accumulation of certain genetic variants, breeding plans have aimed to avoid as much inbreeding as possible.

In paper II, we were able to assess the relatedness of the animals after transcriptomic analysis of PBMCs. The map clearly separated the animals from each other, indicating that the degree of

inbreeding was low. Genes found to be differentially expressed in the two genotypes were mapped on different chromosomes, making it less likely that a flanking gene problem was affecting our analyses.

Environmental factors profoundly influence the natural development and maturation of the innate and adaptive immune system. Animals kept in natural environments have more mature immune systems, exemplified by increased resistance to infection and increased T-cell differentiation (Beura, Hamilton et al. 2016) and priming of NK cells (Boysen, Eide et al. 2011). By keeping research animals in a natural farm environment, animals are able to develop a varied and functional immune system, as opposed to mice living in sterile environments, which is particularly important during studies where immunological homeostasis is to be assessed.
A final aspect to mention is that the use of a large animal creates tissue limitations since each goat is highly valuable (based on the statements above), but on the other side, this can be advantageous as tissue sampling is considerably easier and more material can be obtained from each animal.

4.2. Cell culture

Culture of cells in vitro provides a controlled and stable environment to study cellular mechanisms and responses without interference. However, it is important to be aware of that culturing cells in a closed and artificial system lacks the complexity of an *in vivo* environment.
PBMCs were used in *in vitro* experiments in all three papers. In papers II and III, human neuroblastoma SH-SY5Y cells were included as a supplementary cell type and in paper III, culture of ejaculated spermatozoa was performed.

4.2.1. PBMCs

Blood sampling, isolation and culturing of PBMCs constitute the basis for all papers in this thesis. Sampling was performed by skilled personnel and blood was drawn into EDTA tubes. Isolation of PBMCs and further analysis took place within a few hours after sampling.
The PBMC population is dominated by lymphocytes (~75%), namely B and T cells, but monocytes and natural killer (NK) cells also constitute a significant number. Since PBMCs are easily obtainable, they are commonly used for different research purposes. Being in constant contact with blood and

peripheral tissues, PBMCs are suitable for assessment of systemic homeostatic changes. Particularly interesting is the finding of an 80 % overlap of gene expression in PBMCs as compared to other tissues, suggesting that PBMCs can function as a surrogate tissue (Liew, Ma et al. 2006). Indeed, this was shown in a model of multiple sclerosis (Achiron and Gurevich 2006), where gene expression in PBMCs from multiple sclerosis patients was clearly different from healthy patients, and genes involved in multiple sclerosis pathogenesis could be identified (Achiron, Gurevich et al. 2004).

The composition of PBMCs in peripheral blood, as well as the gene expression in these, can be influenced by several factors including health status, age, gender, season and stress (Whitney, Diehn et al. 2003). Regarding health status, a rise in white blood cells can occur in inflammation; however, all animals included in our analyses were assessed as healthy prior to sampling. Furthermore, the effect of age on PBMC composition in goats is not known, but to avoid this possible influence, we age-matched our samples whenever possible.

Stress is known to cause alterations in numbers and percentages of leukocytes in peripheral blood as soon as 10 minutes after stress induction (Dhabhar, Miller et al. 1995). Whether the stress influenced our animals negatively is unknown, but it should be taken into consideration that some animals are likely to be more prone to stress than others. The animals are used to handling, and to avoid prolonged stress, the blood sampling procedure was quick; the animal was only taken out of the flock for a very short period. Animals of different genotypes were also kept together in the same pens, and samples were taken randomly without knowing the genotype at sampling.

Isolation of PBMCs can influence cellular properties. If pathways involved in activation or apoptosis are induced, dysfunctional cell cultures can be created, which can result in altered gene transcription or metabolic activity, amongst other consequences. Expression levels for many genes in human peripheral blood cells are sensitive to *in vitro* incubation. Many of these genes are involved in basic cellular processes such as transcriptional regulation, cell cycle progression, and apoptosis (Baechler, Batliwalla et al. 2004). However, PBMCs were carefully isolated by gradient centrifugation and consistently had a viability above 90 %.

Whereas PBMCs normally do not proliferate during incubation, monocytes are known to adhere to wells. We experienced, however, that major monocyte adherence rarely was an issue when wells were investigated by light microscopy after suspension removal. A few cells will always remain in the

well; most likely, the majority of these are monocytes. In paper I, pure monocyte populations were cultured in non-adherent wells to avoid this issue.

4.2.2. SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells normally express low levels of PrP^C. In papers II and III, hu-PrP-transfected and non-transfected cells were used as a supplementary model system in addition to PBMCs. Since this cell line is well-established and commonly used within the research community, also within prion research, it will not be described further.

4.2.3. Spermatozoa

In paper III, ejaculates were used in the analysis of PrP^C's role in spermatozoa. Fourteen bucks (7 of each genotype) were housed at a goat sperm production facility (Hjermstad) belonging to the Norwegian sheep and goat breeder's association. Samples from 4 bucks of each *PRNP* genotype were included in the study. All spermatozoa used in our studies were produced at this facility according to validated protocols for sampling, dilution and freezing of buck semen for artificial insemination. After housing at Hjermstad, animals were necropsied in Oslo, Norway. Tissue sections from these animals were used in further analyses in paper III.

To obtain a representative sample of spermatozoa, several issues should be considered. Young bucks have smaller ejaculate volumes and lower spermatozoa number than older bucks. The number and volume can also be affected by the time between the collections; however, motility score after freezing will most likely remain similar (Furstoss, David et al. 2009). Age and seasonal effects, as well as stressful stimuli, can also influence spermatozoa characteristics (Söderquist, Janson et al. 1996). To avoid any negative influence on the spermatozoa all bucks were age-matched, and acclimatized to both the environment and people for 2 weeks.

4.2.4. Homogenous vs heterogeneous cell populations

As previously mentioned, PBMCs consist of several cell types. Accordingly, the subpopulations are likely to react differently to stimuli. For this reason, FACS sorting of specific cell types would have

been a better option than using a heterogeneous cell population, however; few antibodies exist for goat immune cells and FACS sorting is a very time-consuming and costly method. Furthermore, substantial amounts of blood would have had to be sampled to obtain an appropriate amount of cells for our experiments. Considering that 40 ml of blood yields approximately 100 mill PBMCs, and some of the immune cell populations are at low numbers, a considerably larger volume would have had to be collected to yield a sufficient number of cells. We experienced during the work with paper I that recovery of RNA from immune cells could be challenging. Using PBMCs rather than specific subsets of circulating immune cells was therefore a favorable solution. As such, analyses of PBMCs can be used as a way of screening and thus as a basis for further studies. In paper I we showed that the relative sizes of subpopulations within the PBMCs were stable among the two genotypes.

SH-SY5Y cells represent a homogenous cell population which we used for supplementary studies in papers II and III. As these cells are immortalized they have the advantage of being relatively resistant to freezing and thawing. Homogenous cell populations are also advantageous when cellular pathways are to be explored. However, cells in an organism are part of a larger network of different cell types, which could be an argument to rather study biological processes in heterogeneous cell populations

4.3. Protein detection

To detect specific proteins expressed in cells or tissues a variety of optimized indirect immunolabeling methods were used:

- Immunofluorescence (IF, papers I and III)
- Immunohistochemistry (IHC, paper III)
- Western blot (WB, paper III).

Even though these are different methods, detection of proteins is based on the same principle and the objective is to detect and visualize a protein (antigen) in a cell or tissue.

The indirect immunolabeling method is a two-step approach (see Figure 6) involving 1) binding of primary antibody to target antigen, and 2) binding of secondary antibody conjugated with tag.

The main objective is to find primary antibodies specific for the protein target. A detailed overview of primary and secondary antibodies used in the work included in this thesis can be found in papers I-III.

Some of the methods require certain pre-steps, such as fixation to preserve cell morphology (IF, IHC), homogenization or lysis of with disruption of cells and denaturation of proteins with SDS (WB), while cells in suspension needs to be permeabilized to detect intracytoplasmic or intranuclear antigens (flow). Importantly, the primary antibody should ideally not be produced in the same animal that the cell/tissue comes from to avoid cross-binding and false positive results.

Blocking of reactive sites such as Fc receptors with goat serum is important to avoid non-specific binding of primary antibody to other receptors/proteins.

Several markers can be assessed simultaneously by use of these methods as long as the primary antibodies are of different immunoglobulin (Ig) isotypes.

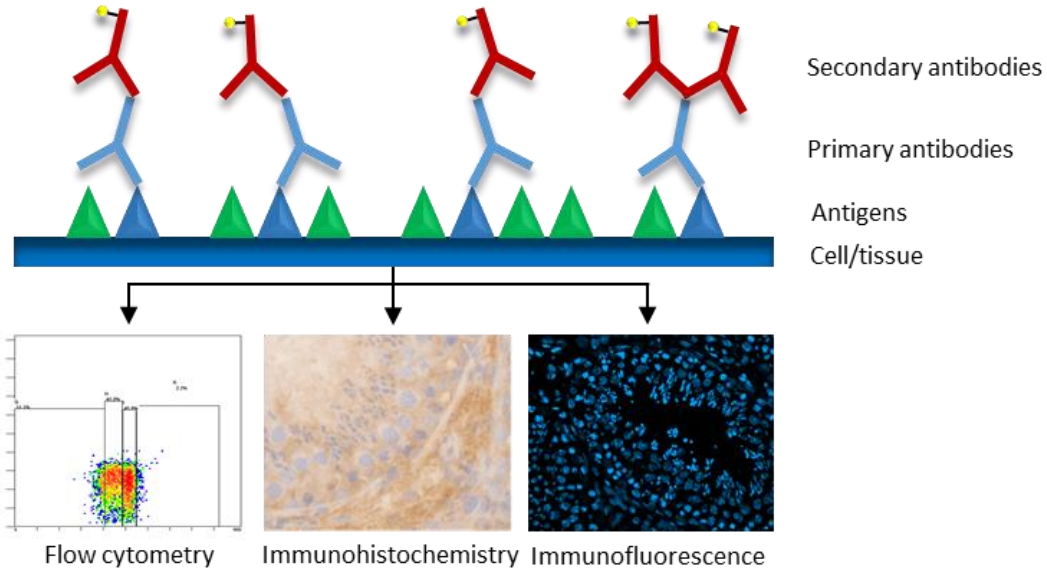

Figure 6: The principles of indirect immunolabeling with different read-outs

In IHC the secondary antibody is conjugated to an enzyme. A substrate is added to yield a coloured product and the cells/tissue are/is counterstained with haematoxylin to visualize the remaining cellular structures. IF applies the same principle, but the secondary antibody is connected to a fluorophore with a specific excitation and emission spectrum.

Target antigens were visualized in several ways:

- Flow cytometry: detects fluorescently tagged molecules (papers I and III)
- Fluorescence microscopy (paper I)
- Light microscopy (paper III)

Flow cytometry and fluorescence microscopy are methods in which a laser is used to detect biomarkers in/on cells. In, flow, the principle is to label cells in solution before passing them through the flow cytometer where the machine detects the fluorescent signal(s) in a single cell at a time. The cells are discriminated based on their size and granularity. Since the machine evaluates single cells, a whole body of knowledge can be obtained from a sample, such as cell counting, viability and cell phenotypes such as immune cell and activation markers.

In immunofluorescence as well as flow cytometry, it is very important to have samples where no antibodies have been applied, a negative control, to assess the level of autofluorescence. An important advantage with immunofluorescence is that you can combine different fluorochromes, and each fluorochrome emit light with different wavelength and color. However, laser light bleaches the fluorochromes leading to weakened signal over time.

Since the secondary antibody also can bind non-specifically, it is important to have controls where only the secondary antibody is applied. Fully negative controls are for this reason extremely valuable, but usually, rarely obtainable. In this thesis, PrP^C-deficient animals functioned as excellent negative controls for all experiments where PrP^C staining was performed.

4.4. RT-qPCR

In the work presented in this thesis, RT-qPCR was used to detect and relatively quantify the expression level of *PRNP* and other genes described in the respective papers (papers I-III).

The initial step is to generate good-quality cDNA from mRNA by reverse transcription, catalyzed by an enzyme, reverse transcriptase (RT). Accurate quantification later during the process demands that this step is carefully carried out, as the amount of cDNA produced must reflect the input amount of totRNA. In general, the RT reaction is highly reproducible as long as the same experimental protocol and reaction conditions are used; thus/therefore, results are comparable.

RT-qPCR is a high-sensitivity method in which it is possible to detect the expression level of a given gene in a sample, based on the amount of mRNA present. The main concept is real-time detection of the amplified target gene by the binding of a fluorescent reporter to the PCR product. After several cycles, the signal will exponentially increase, reflecting the reporter binding to the accumulating amount of products. Dyes such as SYBR Green I, as used in our experiments, have the advantage of

having no fluorescence when free in a solution, but being exclusively fluorescent when bound to DNA. When the signal exceeds the background threshold, the signal is detected, and this cycle number is known as the sample's crossing point, Cp. This is a logarithmic value directly comparable to the amount of template initially present in the sample. If the same amount of cDNA is used in the experiment, calculation of the initial expression level of PrP^C is possible based on the number of cycles necessary to reach the detection threshold.

In RT-qPCR, false positive signals may arise from amplification of the gene or pseudo-gene from genomic DNA. To avoid this problem, the two PCR primers are designed to hybridize to different exons, hence having at least one intron in between, or designing one of the primers to span across an exon-exon boundary. If there are no introns, as in the case of *PRNP*, DNase treatment can be used if background DNA is present. In addition, a control sample without RT is always used as a negative control.

To correct for differences between runs, it is common to relate the samples to a run calibrator sample that is included in every run.

The most common way to normalize the samples before comparing them is to divide the results for the target gene by an internal reference gene. This will correct for differences in quality and quantity caused by variations in sample preparation, nucleic acid quality, pipetting errors and variations in cDNA synthesis efficiency. To do so, the expression of the reference gene needs to be steadily expressed in the cell and unaffected by experimental factors. The latter is a difficult requirement to fulfil, as there is no universal gene with a constant expression in various cells and tissues. Usually a basic metabolism gene such as *GAPDH* is chosen, as its stable expression is essential to ensure cell survival.

In papers I and II, samples were normalized to the levels of *GAPDH*. In papers III, the results were normalized to the totRNA level.

4.5. RNA-sequencing

In paper II, RNA-sequencing (RNA-seq) was used to compare the transcriptomes of $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ PBMCs. The principle in RNA-seq is the application of next-generation sequencing to detect and quantify with high sensitivity and specificity all transcripts present in a sample. Comprehensive

transcriptomic studies have allowed a great amount of advances within research since its introduction, particularly in the understanding of the dynamic and complex state of the genome, including detailed understanding of biological pathways that operate during various physiological conditions.

Briefly, a typical workflow consists of
(1) preparation of a sequence library,
(2) sequencing and
(3) data analysis.

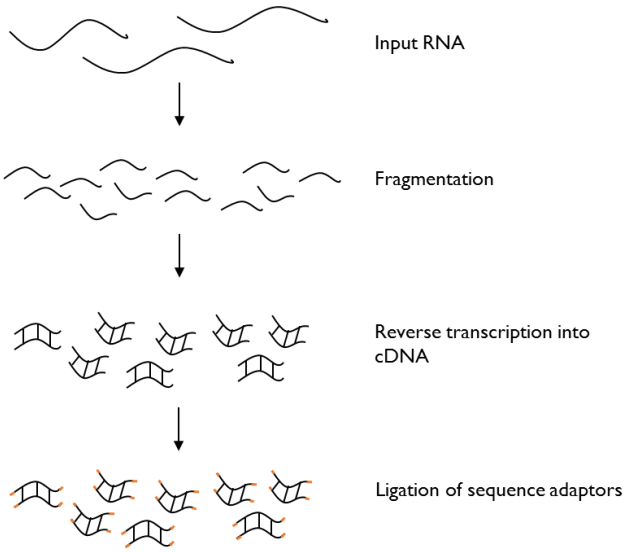

Figure 7: Preparation of a sequence library in RNA-seq

In (1), the isolated RNA is reverse transcribed to cDNA and fragmented, before sequencing adaptors are ligated to each end (Figure 7). In (2), the fragments are amplified and sequenced, before annotation to a reference genome (3), in our case the goat genome (Dong, Xie et al. 2013).
Prior to the process of obtaining material for library construction, a proper experiment design is important. One should be aware of biological variability, factors that can influence the results, as well as chance. Roughly, experiments can be either quantitative or qualitative, meaning that the method can be used to identify expressed transcripts in a tissue or measure differences in gene expression. Since our aim was the latter, and for statistical purposes, we decided to avoid pooling of samples.
As the Illumina sequencing platform is one of the most widely used platforms it will not be further presented in this section.

Obtaining good-quality starting material is essential to achieve a good result. The total RNA pool consists of approximately 90 % ribosomal RNA (rRNA). To select the mRNA, poly-A selection is commonly used since all eukaryotic mRNA is produced with a poly-A tail. An alternative is to use rRNA depletion kits; however, this is not recommended since rRNA depletion never is complete. For

the purpose of poly-A selection, magnetic beads with Oligo (dT) can be used to isolate mRNA. Paired-end sequencing means that both ends of a fragment is sequenced, a process that generates high-quality and alignable sequence data.

Data management is a large challenge in RNA-seq since the data collected can be up to several gigabytes in size. The data analysis is also demanding, and usually bioinformatics expertise is needed to process and analyze the enormous amount of data.

During analysis and prior to assembly, artefacts are removed from the total pool of read segments. Such artefacts can be adaptor sequences, low-complexity reads (short DNA sequences) and PCR duplicates. Sequencing errors are also removed, after which the reads are assembled into transcripts. The expression level of each transcript is estimated by counting the number of reads that align to each transcript. Whereas the reference-based method is very sensitive and can assemble low-expressed genes, the grade of success depends on the quality of the reference genome.

In order to interpret differences in read counts appropriately, one must also obtain information regarding the variances that are associated with these numbers. Several types of variances contribute to RNA-seq data, including sampling, technical and biological variance. When measuring differences between biological systems, one needs to be aware of the complexity and sensitivity of these systems. To achieve a list of differentially expressed genes (DEGs), a careful filtration of the gene list is required. The filtration standards are usually decided by the research group and depends on the aim of the experiment. In our data set, we used cut offs for fold change (log2 fold change +/- 0.5), and mean number of reads (> 100 reads in one of the groups) as inclusion criteria. However, a risk with these cut offs is that important genes are lost/are missed during analysis. Analysis of several data sets after the setting of different cut-offs may therefore be valuable.

5. Results and general discussion

The work included in this thesis originated from the serendipitous discovery of Norwegian Dairy goats with a mutation in *PRNP*, rendering animals devoid of PrP^C. The existence of these goats is a continuous confirmation that PrP^C deficiency is consistent with life, at least as a production animal in an ordinary Norwegian animal husbandry context, as indicated by seemingly normal health, resistance towards disease and reproduction. Within the prion research field, the model is highly valuable, especially since it is fundamentally different from previous artificial *PRNP* knockout animals.

Throughout the work in this thesis, some fundamental aspects of the physiology, immunity, stress response and reproduction in this unique PrP^C-deficient goat model has been addressed to gain deeper insight into the biology of the animals. Interesting and original data concerning possible functions of PrP^C have been retrieved, and should be further investigated in this and other models. The studies have formed a basis for further assessments, which could prove important for several reasons. Knowledge concerning PrP^C's normal physiological function is not only valuable for potential insight into prion disease pathogenesis, but could also provide valuable information concerning the consequences of silencing of PrP^C, the latter being discussed as a possible treatment option for prion disease (Kong 2006, White and Mallucci 2009). In addition, since the goats are completely resistant to prion disease, they could be valuable not only in breeding strategies in areas where scrapie is endemic, but also as producers of diverse biological reagents. A prerequisite for such use is normal health and reproduction.

In paper I, general aspects of blood parameters including cell counts and clinical chemistry, bone marrow composition and immune cell functions were assessed from $PRNP^{Ter/Ter}$ goats and compared with $PRNP^{+/+}$ goats. Paper II addressed the influence of lack of PrP^C on the transcriptome of isolated resting-state PBMCs, whereas in paper III, goat PBMCs and spermatozoa were utilized to investigate how PrP^C-deficient cells handled oxidative and genotoxic stress. Data from goat cells were supplemented with similar studies in human neuroblastoma SH-SY5Y cells expressing high or low levels of PrP^C. In the following sections, results are discussed in a broader manner than in the separate papers.

5.1. Hematological shift is a phenotype of *PRNP*$^{Ter/Ter}$ goats

Hematological analysis of 3-4-week old goat kids revealed increased number of RBCs in *PRNP*$^{Ter/Ter}$ animals compared with *PRNP*$^{+/Ter}$ and *PRNP*$^{+/+}$ genotypes (paper I). In addition, a tendency to higher numbers of neutrophils was observed. A closer analysis of the hematology data revealed that the RBCs' MCV was slightly lower (p-value=0.08) in the PrP^{C}-deficient animals. Whereas circulating reticulocytes are rarely seen in the blood of ruminants, even during anemias (Jones and Allison 2007), a complete lack of reticulocytes in blood smears suggested that the relatively smaller RBCs were mature and that the bone marrow activity was normal. The term "hematological shift" was chosen to describe the alteration, in line with other "shifts" in medicine used to describe an increase in a particular cell type. This shift was quite subtle and the values were within the normal reference range, suggesting that the effect of PrP^{C} loss is modest, which is consistent with clinical observations and overall health status of the goats.

Because animals of both genotypes displayed similar hematocrit levels, we wondered whether cell volume and number of RBCs in peripheral blood were reciprocally regulated to maintain stable hematocrit values, meaning that if one parameter increases, the other is reduced or vice versa. However, to my knowledge, this kind of regulation is not a commonly described phenomenon in hematological homeostasis.

As iron deficiency can affect erythropoiesis, the effects of PrP^{C} on RBC parameters could be explained by PrP^{C}´s role in iron homeostasis (Pushie, Pickering et al. 2011, Singh, Haldar et al. 2013, Haldar, Tripathi et al. 2015). Chronic iron deficiency was reported from studies of *Prnp* knockout mice, explained by reduced intestinal uptake and transport into parenchymal and hematopoietic cells (Singh, Kong et al. 2009). An interesting observation in these mice was that the iron deficiency seemed largely compensated, as the RBC counts and hematocrits were normal and hemoglobin only slightly lowered. The compensation was explained by changes in "iron management proteins" and a more labile iron storage. In the goat model, the *PRNP*$^{Ter/Ter}$ animals had normal mean corpuscular hemoglobin content (MCHC) and serum iron. The different effect of PrP^{C} loss on serum iron in mice and goats indicate that iron metabolism differ between species, and so far, the conclusion is that uptake and transfer of iron is likely not explaining why *PRNP*$^{Ter/Ter}$ goats differ from normal goats with respect to the number of RBCs and MCV values.

The number of studies in *Prnp* knockout mice addressing basal hematology data are few. Because we are working with a ruminant model, it was highly interesting to compare our results with *PRNP* knockout cattle, where animals at 10 months of age demonstrated similar hematological differences as the goats (Richt, Kasinathan et al. 2007). More specifically, cattle lacking PrP^C had a significant decrease in MCV (below the reference range), an increase in neutrophils, as well as a reduced MCHC. Additionally, a tendency for an increased number of RBCs (p-value=0.11) was observed. The striking similarity between the two different ruminant models, representing two completely different genetic backgrounds, strengthens the results and reduces the likelihood that a confounding factor could have influenced these data. Although a thorough characterization of $PRNP^{+/Ter}$ (heterozygous) animals was not within the scope of this thesis, it is interesting to note that half the normal amount of PrP^C, as indicated by flow cytometry studies of PBMCs, is sufficient to "rescue" the RBC numbers and normalize the MCV values. Whether this also holds true for other phenotypes remains to be investigated.

To the best of our knowledge, no other studies apart from the one in cattle and the present in goats have reported lowered MCV associated with PrP^C loss. Obviously, this phenotype can develop irrespective of the very different morphology of normal RBCs between the two species (Figure 9).

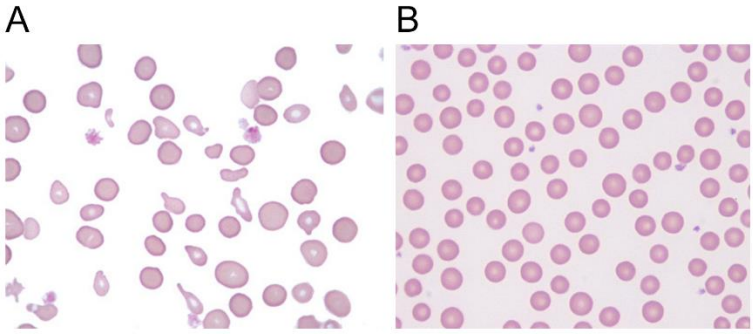

Figure 9: RBCs from goats (A) and cattle (B). Poikilocytosis is a normal feature of goat RBCs.
Image source: http://www.eclinpath.com/hematology/morphologic-features/red-blood-cells/normal-erythrocytes/

Since PrP^C has been suggested to be redundant during a normal physiological state, the effects of PrP^C loss is often investigated in situations where cells are undergoing stress or threats against homeostasis (Reviewed in Zeng, Zou et al. 2015). In paper I, we discussed if the hematological shift we observed in young goats could be related to an increased demand or physiological stress in the hematopoietic tissue during growth, and we referred to preliminary data indicating that the modest PrP^C effect was absent in adult goats. However, subsequent measurements of RBC parameters of older animals point

to a sustained effect with a higher number of RBCs with lower MCV in *PRNP*$^{Ter/Ter}$ goats (Cecilie Ersdal; personal communication), indicating that PrP^{C} influences basal mechanisms in erythropoiesis, a process that in all ages includes rapid cell division and moderate physiological stress. These data suggest that increased RBC production is a consistent trait of *PRNP*$^{Ter/Ter}$ animals, present regardless of physiological state.

What are the mechanisms behind an increased number of RBCs in *PRNP*$^{Ter/Ter}$ animals? The bone marrow might be the site where PrP^{C} influences the RBCs, although we did not detect morphological differences between *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ goats in this tissue. It is important to note that only one goat kid of each genotype was investigated and a more detailed study including a higher number of animals is needed to reach a conclusion. Similarly, no aberrations were detected in the bone marrow of *Prnp* knockout mice and there were no differences in the numbers of erythroid precursor cells or apoptotic cells during a normal physiological state (Zivny, Gelderman et al. 2008).
Studies of PrP^{C} expression in bone marrow of goats have not been performed; however, in mice, PrP^{C} is present on CD34^{+} HSCs (Zhang, Steele et al. 2006), and both human and mouse RBCs express PrP^{C} on their surface (Holada and Vostal 2000). To investigate PrP^{C} expression in caprine HSCs, an attempt was made at double-staining bone marrow cells for PrP^{C} and the stem cell markers c-kit and CD34. We found that PrP^{C} were expressed on several cell types in the bone marrow (Figure 8). However, due to technical difficulties, no clear conclusions regarding the co-localization of PrP^{C} with CD34 and c-kit on HSCs were drawn. PrP^{C} was clearly present on several blast cells, whereas the smaller and more differentiated erythroid precursors (normoblasts) seemed negative, an observation analogous to a finding from a study in mice (Panigaj, Brouckova et al. 2011). Similarly, granulocyte precursors were positively stained for PrP^{C}, in line with a previous report (Liu, Li et al. 2001). The strong staining of PrP^{C} on megakaryocytes was indisputable, consistent with studies of megakaryocytes and/or platelets in humans and some non-human primates (Starke, Harrison et al. 2005, Holada, Simak et al. 2007). It is interesting to note that the species differences are significant when it comes to PrP^{C} on hematopoietic and blood cells as demonstrated by lack of detectable PrP^{C} on platelets from sheep (Barclay, Hope et al. 1999, Herrmann, Davis et al. 2001), another small ruminant. Furthermore, several cells were weakly stained or negative for PrP^{C}. Further studies are needed to determine the lineage affiliation of these. *PRNP*$^{Ter/Ter}$ bone marrow was negative for PrP^{C}, as expected.

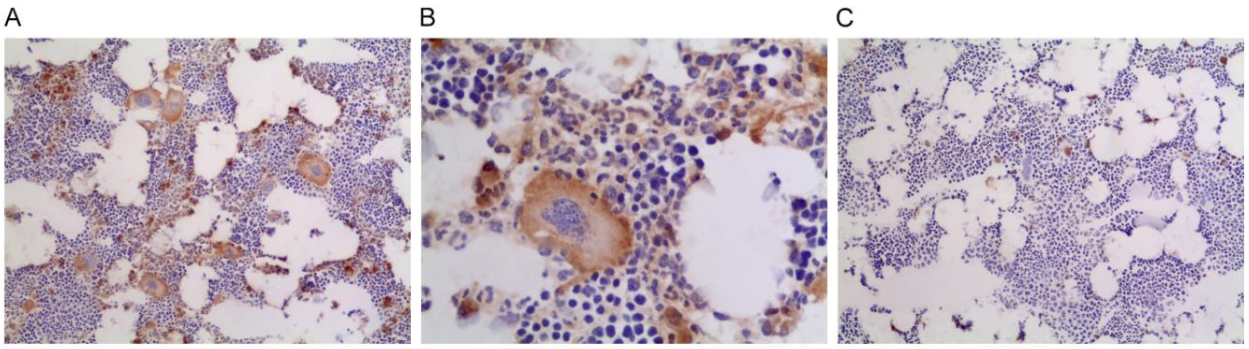

Figure 8: IHC staining of bone marrow against PrP^C (SAF32 antibody). A: $PRNP^{+/+}$ 10x, B: $PRNP^{+/+}$ 40x, C: $PRNP^{Ter/Ter}$ 10x

Several studies have investigated how PrP^C loss affects the stressed bone marrow. One study found that bone marrow transplants from *Prnp* knockout mice displayed impaired repopulation of bone marrow cells in irradiated mice after several rounds of serial transplantation (Zhang, Steele et al. 2006). In a study on induced hemolytic anemia, the response was less efficient in mice lacking PrP^C (Zivny, Gelderman et al. 2008). In the latter study, reduced erythropoietin production in the kidneys was suggested as the causative mechanism.

Experiments addressing severe stress hematopoiesis, such as hemolytic anemia, should be performed in goats to investigate if PrP^C-deficient goats mount a normal hematopoietic response in a stress situation, and whether the MCV remains reduced in such a situation.

Of some relevance for the present discussion is also a report on survival of RBCs in circulation. Transfusion experiments found that the amount of PrP^C on RBCs affected their survival time. RBCs without PrP^C disappeared more quickly from the circulation compared to normal RBCs, a finding that was confirmed in more than one *Prnp* knockout-mice model (Glier, Simak et al. 2015). The authors suggested that the antioxidative and antiapoptotic effects of PrP^C (Westergard, Christensen et al. 2007) might protect PrP^C-expressing RBCs against senescence and premature clearance by splenic macrophages. If similar mechanisms operate in $PRNP^{Ter/Ter}$ goats, a reduced circulating pool of RBCs would have been expected, which our observations directly contradict. This is also in agreement with the studies in paper III, pointing to a normal antioxidative ability of PrP^C-deficient goat cells and other studies in *Prnp* knockout mice showing normal half-life of RBCs in these mice (Singh, Kong et al. 2009).

In light of our findings presented in paper II, we question whether alterations in type I IFN signaling can affect bone marrow homeostasis. Within the HSC niche, type I IFNs is important for general maintenance (Pietras, Lakshminarasimhan et al. 2014), since interferon receptor I-deficient

(*ifnarI*$^{-/-}$) mice failed to produce hematopoietic cells (Essers, Offner et al. 2009, Sato, Onai et al. 2009). Furthermore, human patients treated with type I IFNs presented with hematological defects beyond the RBC lineage displaying cytopenias, anemia and thrombocytopenia (Gokce, Bilginer et al. 2012), and chronic IFN production caused HSC exhaustion and HSC depletion (Sato, Onai et al. 2009). Clearly, the effects of IFNs on the HSC niche are very complex and an effect of low to moderately elevated type I IFN-responsive genes on certain hematopoietic stem cell niches cannot be ruled out.

5.2. PrP^{C} loss and the influence on blood leukocyte subpopulations and functions

The blood leukocytes constitute the polymorphnuclear granulocytes, mainly neutrophils, and PBMCs. Several factors regulate the production of these cells in the bone marrow or primary lymphatic organs, including cytokines and growth factors.

In addition, the blood leukocytes have a certain turn-over rate regulated by clearance of senescent cells by the mononuclear-phagocyte system or they leave the blood either in connection to immune surveillance and homing or when called upon to produce a local or systemic inflammation.

A prerequisite for further investigations of PrP^{C} physiology was that the tissues we had chosen to investigate expressed PrP^{C}. In paper I, we confirmed the expression of PrP^{C} on PBMCs, whereas the expression in granulocytes was below the threshold of detection (paper I). The results are in accordance with the literature (Dodelet and Cashman 1998, Antoine, Cesbron et al. 2000).

In heterozygous *PRNP*$^{+/Ter}$ goats, PBMCs expressed both *PRNP* mRNA and PrP^{C} levels of around 50 % compared to the normal *PRNP*$^{+/+}$ PBMCs, showing that the cells don't compensate with increased transcriptional and/or translational activity from their single normal *PRNP* allele to obtain normal levels of PrP^{C}. It is interesting to speculate on whether this means that one allele is sufficient to produce the amount of PrP^{C} that the cells need during a normal physiological state. However, further studies are warranted to assess if *PRNP*$^{+/Ter}$ PBMCs respond differently from *PRNP*$^{Ter/Ter}$ and *PRNP*$^{+/+}$ cells to changes in cellular states, or if changes in other cellular pathways than those arising from studies in *PRNP*$^{Ter/Ter}$ cells can be revealed.

A brief comment was made in paper I regarding the tendency for an increased amount of neutrophils in *PRNP*$^{Ter/Ter}$ goats that was observed in the blood count, similar to the observation in *PRNP* knockout

cattle (Richt, Kasinathan et al. 2007). Interestingly, besides being important first-line defenders against microbes within the innate immune system, neutrophils and monocytes descend from the same precursor cell in the bone marrow. However, we did not detect changes in the levels of circulating monocytes. Rather than being a primary consequence of bone marrow alterations, it could be speculated that the increased neutrophil number is related to our finding in paper II, revealing an increased expression of type I IFN-responsive genes. Whether this is connected to the type I IFNs' effect on the bone marrow (Pietras, Lakshminarasimhan et al. 2014), thus leading to increased neutrophil production and release is unknown. Indeed, type I IFNs influence neutrophil survival and lifespan (Pylaeva, Lang et al. 2016). After type I IFN stimulation, increased production of IL-6 is detected (Zimmermann, Arruda-Silva et al. 2016). Furthermore, it was shown that mature neutrophils express higher levels of IFN-responsive genes than immature neutrophils, indicating that they are very sensitive to IFNs (Martinelli, Urosevic et al. 2004), and thus could be influenced by the modulating role of PrP^C on type I IFN signaling.

It is also of interest to note that goats without PrP^C display longer duration of sickness behavior after LPS stimulation, although no differences were detected in the number of circulating neutrophils or other immune cells throughout the study (Salvesen, Reiten et al. 2017). This could indicate that the finding is indeed related to an enhanced production of type I IFNs or other pro-inflammatory cytokines in *PRNP*$^{Ter/Ter}$ goats. One possible mechanism could be, as mentioned, that type I IFN stimulation increase neutrophil IL-6 secretion, which again could lead to sickness behavior. Alternatively, lack of PrP^C cause sickness behavior by increasing TNF-α activity (Ezpeleta, Boudet-Devaud et al. 2017). Whether these findings are connected remains to be investigated.

PrP^C has been linked to a diverse array of immune cell functions and is often reported upregulated during activation (Cashman, Loertscher et al. 1990, Mabbott, Brown et al. 1997, Mariante, Nobrega et al. 2012). A proper study of subpopulation sizes and putative functional aberrations due to PrP^C loss in the goat model was therefore of interest. Immunophenotyping of PBMCs revealed a similar composition of $CD3^+$, $CD8^+$ and γδ T cells, B cells, $CD14^+$ monocytes (paper I) and NK cells (not published due to incomplete characterization of this cell subset in the goat) in both genotypes, suggesting that PrP^C deficiency does not affect the relative levels of circulating mononuclear cells. Again, this is similar to *PRNP* knockout cattle although a tendency for a higher number of γδ T cells was found in knockout cattle compared to normal animals (Richt, Kasinathan et al. 2007).

A dynamic PrP^C expression in regulatory and memory T cells (Li, Liu et al. 2001) with increased PrP^C levels during T-cell proliferation (Cashman, Loertscher et al. 1990, Mabbott, Brown et al. 1997) suggests a role for PrP^C during T-cell activation and/or proliferation. However, when PBMCs from goats were stimulated with concanavalin A, IL-2 or IL-15 to induce proliferation of T and NK cells, none of the proliferation rates were significantly different between the two genotypes.

Our findings are in accordance with similar T-cell proliferation experiments in *PRNP* knockout cattle (Richt, Kasinathan et al. 2007). The authors of the cattle study also found that the humoral response after ovalbumin immunization was similar, indicating that activated T cells are able to cooperate with B cells in mounting a proper immune response despite PrP^C ablation. Although we did not specifically look at immune responses, all health-related data retrieved from $PRNP^{Ter/Ter}$ goats suggest that animals are able to mount sufficient and protective immune responses against microbes in their environment.

Phagocytosis is a complex process involving recognition of foreign material, gathering of multiple receptors, internalization, digestion and production of inflammatory signals. We sought to assess if loss of PrP^C influences this ability in $CD14^+$ monocytes activated with granulocyte macrophage colony-stimulating factor. The uptake of bacteria, yeast and latex was similar among the genotypes, indicating no effects on PrP^C deficiency on this specific cellular process. The result is in contrast to another *in vitro* study where macrophages from *Prnp* knockout mice displayed increased phagocytosis of *Escherichia coli* (Wang, Zhao et al. 2014). In another study, phagocytosis of latex beads by macrophages isolated from the *Prnp*-knockout Zürich I mouse line occurred at a decreased rate (Uraki, Sakudo et al. 2010), demonstrating that it is difficult to establish a clear connection between lack of PrP^C and phagocytic ability.

An issue with *PRNP*-flanking genes influencing phagocytosis was later detected (outlined in the *Introduction*), more specifically polymorphisms in the gene encoding the SIRP-α protein, *SIRPA* (de Almeida, Chiarini et al. 2005, Nuvolone, Kana et al. 2013), a well-known inhibitor of phagocytosis. Whether polymorphisms in this protein can affect other *PRNP*-related phenotypes is unclear, but based on the present finding we have no data to support an impact of SIRP-α polymorphisms on phagocytosis by PrP^C-deficient goat cells. The flanking gene problem is also addressed in section 4.1 Animals.

Increased levels of type I IFN-responsive genes at rest as detected in PBMCs (paper II) could likely have influenced basal immune functions of the cells in a paracrine or autocrine manner, but this does not seem to happen. This is interesting, since several mononuclear cell types are influenced by

constitutive type I IFN-signaling, and in macrophages, constitutive type I IFN signaling is involved in functional maintenance, particularly phagocytosis (Vogel and Fertsch 1984).

The effect of type I IFNs on T cells is complex, since IFN-α/-β are potent suppressors of proliferation in most cell types, but enhanced the survival and proliferation of $CD8^+$ blasts. Constitutive type I IFN-signaling is also involved in the maintenance of NK-cell biology at several levels (Muller, Aigner et al. 2017). The primary cytokine necessary for NK-cell proliferation is IL-15, which is a type I IFN-regulated gene. Mice deficient in *ifnar* or signal transducer and activator of transcription 1 (STAT1) have lower numbers of NK cells and display diminished NK-cell protection against virus infection due to reduced NK-cell proliferation (Madera, Rapp et al. 2016). Thus, in relation to the findings in paper II, it was interesting to find that isolated cells, including NK cells without PrP^C, did not proliferate at a different rate after stimulation with IL-15, compared to normal cells. It would be of interest to study T- and NK-cell functions, including proliferation rates after isolation of purer cell populations, particularly since these cells have significant levels of PrP^C (Durig, Giese et al. 2000).

5.3. Transcriptome studies

5.3.1. Type I IFN signaling is influenced by PrP^C

In paper II, recent advances in transcriptomic techniques were applied to search for manifestations of novel phenotypes in cells without PrP^C. The RNA-seq method is a powerful tool, which allows precise quantification of thousands of mRNA species from a cell or tissue sample. Today, RNA-seq has replaced microarray hybridization methods, commonly used in earlier investigations. The aim of the study in paper II was to retrieve data that could aid in the understanding of how lack of PrP^C influence gene regulation in isolated PBMCs in a basal, non-stimulated state and thus obtain clues to the normal function of PrP^C in immune cells. Several previous studies of gene expression related to loss of PrP^C in different animal and cell models have used either proteomic analysis looking at changes in protein levels or alterations in mRNA profiles. Generally, limited effects of PrP^C loss have been reported, e.g. in studies of mice brain where proteome (Crecelius, Helmstetter et al. 2008)- and microarray (Chadi, Young et al. 2010)-based studies revealed none or very few differentially expressed proteins or mRNAs. Other array studies of brain hippocampus (Benvegnu, Roncaglia et al. 2011) or cultured fibroblasts (Satoh, Kuroda et al. 2000) found only moderate changes in mRNA levels of genes related to a broad range of different cellular functions including cell signaling and communication, survival,

differentiation, calcium homeostasis and synaptic transmission. In an RNA-seq study of *Prnp* knockout mice, whole embryos at E6.5 and E7.5 revealed 73 and 263 DEGs, respectively. The DEGs were mostly related to cellular adhesion and proliferation, but also including genes coding for, or involved in, matrix metalloproteases, apoptosis, inflammatory response and response to oxidative stress networks (Khalife, Young et al. 2011). Interestingly, the study indicated a principal role for PrP^C during embryogenesis. In *Prnp* knockout embryos, PrP^C appeared to be compensated for by the PrP^C-related protein Shadoo (Young, Passet et al. 2009). The somewhat divergent results from various mouse studies on how PrP^C influences the transcription profile in cells inspired us to do a new transcriptome study utilizing the non-transgenic, non-conventional goat model. Because we were interested in the immunoregulatory role of PrP^C, isolated PBMCs were analyzed.

In accordance with the studies mentioned above, it was not surprising that only 0.7 % of the genes were differentially expressed between the *PRNP* genotypes in the PBMCs, a percentage very similar to what was found in the RNA-seq study on mouse embryos (Khalife, Young et al. 2011). Analysis of the data from the goat PBMCs using the Interferome database defined as many as 60 of the 86 functionally annotated genes to be so-called "interferon responsive" and the majority of the interferon-responsive genes fell into the type I IFN category. They were modestly upregulated in the goats without PrP^C, with fold changes ranging from 1.4 to 3.7. Among these were *ISG15*, *DDX58* (RIG-1), *MX1*, *MX2*, *OAS1*, *OAS2* and *DRAM1*, which have important roles in pathogen defense, cell proliferation, apoptosis, immunomodulation and DNA damage response. None of the abovementioned studies described findings similar to the present study in goat PBMCs where such a large fraction of DEGs was involved in one particular signaling pathway. In the mouse embryo study (Khalife, Young et al. 2011), immune-related genes such as TLR4, were regulated in the opposite direction as compared with the goat model. Differences in results could be related to species, or, even more likely, to the cell type, with immune cells probably being more sensitive to the lack of molecules modulating immune signaling pathways.

There are a number of possible mechanisms to explain how PrP^C might modulate type I IFN signaling in PBMCs. Lack of PrP^C might lead to increased levels of IFN-α/β in the blood and thus enhanced signaling through IFNAR, initiating phosphorylation of a cascade of downstream transcription factors, ultimately leading to transcription of interferon-responsive genes (summarized in Figure 10). The type I IFN-mediated immune response launched by IFN-α and –β production from several cellular sources,

particularly plasmacytoid DCs, provides a first line of defense against viral pathogens (Asselin-Paturel and Trinchieri 2005). Whereas the transcription of type I IFNs normally is low, it can be rapidly induced by viral and bacterial products via Toll-like receptors, located both extra- and intracellularly in mammalian cells (Kawai and Akira 2011). We were concerned whether minor differences in microbial burden between the two genotypes could have produced a low-grade inflammation in the *PRNP*$^{Ter/Ter}$ goats, and, subsequently, activation of type I IFN signaling. However, all animals were healthy at the point of blood sampling (paper I), with no signs of inflammation, neither clinically nor based on blood parameters.

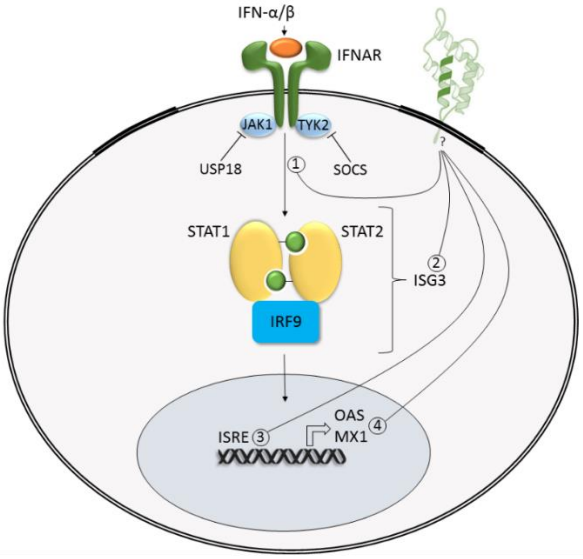

Figure10. Simplified model of the type I IFN pathway including a few theories on how and where PrP^C could modulate the type I IFN pathway (1-4). Areas with thick lines on the cell membrane represent lipid rafts. Cell signaling by type I IFNs occurs upon binding to the IFNAR receptor, a heterodimer consisting of the IFNAR1 and IFNAR2 chains. Binding to IFNAR2 recruits IFNAR, and a signal is initiated by phosphorylation of tyrosine kinase 2 (Tyk2) and janus kinase 1 (Jak1), further activating the STAT1-STAT2 heterodimer. It is not clear whether PrP^C is able to interact with IFNAR (1). Several binding partners for PrP^C have been described, although the functional importance of these have been heavily discussed. USP18 and SOCS are known negative regulators of the type I IFN pathway; perhaps could PrP^C be involved in negative regulation of the receptor together with these. The STAT1-STAT2 complex transmigrates into the nucleus and interacts with the transcription factor IFN regulatory factor (IRF) 9. Together, they form IFN-stimulated gene factor 3 (ISGF3), which binds to upstream sequence elements and activates the transcription of IFN-responsive genes, here exemplified by OAS and MX1. Other explanations of PrP^C interaction involve modulation of the activity and/or assembly of ISG3 (3), or PrP^C could possibly be able to initiate transcription of IFN-responsive genes or contribute to longer half-life of these (4). The location of PrP^C implies that the protein is dependent on intramembrane and intracellular signaling molecules.

Priming of immune responses with basal-level expression of signaling molecules is a well-known biological phenomenon and is important for sustaining many different cellular processes. This has

evolved to ensure a rapid protective response to microbes (Reviewed in Gough, Messina et al. 2012). Such a primed systemic immune state is stimulated by the content and the commensal bacteria in the intestine leading to IFN-β production by DCs (Kawashima, Kosaka et al. 2013). In this respect, enterocytes of PrP^C-deficient goats might be equipped with tight junctions impairing intestinal barrier functions as found in mice (Petit, Besnier et al. 2013) resulting in increased systemic type I IFN levels. We were unable to detect any alteration in expression of IFN-α and -β or other cytokines in the PBMCs, and this indicates that systemic immune-system activation by microbial products from gut or sub-clinical infections that could have led to increased levels of type I IFN-responsive genes, is not likely. Local IFN expression in immune cells within the lamina propria of the gut could be a source, although this would probably have given a somewhat higher expression of important components in the type I IFN-signaling pathway. However, we were unable to detect changes in mRNA the levels encoding neither activating nor inhibiting molecules in either genotypes (Paper II, Table 1), making such influence also unlikely. Importantly, *in vitro* experiments with SH-SY5Y displaying high and low PrP^C expression showed that cells with low PrP^C responded with stronger *MX2* expression to exposure with the same concentration of IFN-α, indicating that the modulatory role of PrP^C affects the PBMCs directly at the cellular level.

In biological systems, a certain degree of immune activation is generally advantageous, whereas activation above a certain threshold may lead to harmful consequences (Essers, Offner et al. 2009, Nallar and Kalvakolanu 2014, Porritt and Hertzog 2015). Thus, any activity needs to be balanced by activating and dampening substances on different levels. Considering the immune-dampening effects of PrP^C, it is plausible that PrP^C is a negative modulator of one or several of the steps in the type I IFN pathway. An excellent review on negative regulators of type I IFN signaling can be found elsewhere (Richards and Macdonald 2011). However, deeper knowledge concerning the ability of negative regulators to fine-tune basal activity, or anti-viral and other responses is needed.
The present work does not give the answer to whether PrP^C modulates the type I IFN response directly or indirectly, but provides basis for some considerations for future work.

Based on the cell biology of PrP^C, it may be speculated where interactions with type I IFN-signaling may occur. PrP^C is tethered to the outside of the cell membrane via its GPI-anchor, located in lipid rafts and has been suggested to have many binding partners (Gauczynski, Peyrin et al. 2001, Lopes,

Hajj et al. 2005, Linden, Cordeiro et al. 2012) and pleiotropic properties making it a cell surface scaffold protein (Linden 2017). These are proteins with the ability to bind several ligands (Santos, Beraldo et al. 2013), mediate crosstalk between signaling cascades (Dard and Peter 2006), and modulate signaling molecular complexes in a cell type specific manner. PrP^C is known to modulate several signaling pathways, including the PI3K/Akt pathway (Vassallo, Herms et al. 2005, Roffe, Beraldo et al. 2010, Llorens, Carulla et al. 2013) and it remains to be investigated if PrP^C also can influence type I IFN signaling in a similar manner.

Based on the subtle, but distinct inflammatory profile in resting *PRNP*$^{Ter/Ter}$ PBMCs, we wondered whether cells without PrP^C were in a primed state and able to react stronger to type I IFN stimuli compared with normal cells. To test this, we measured the cellular response in SH-SY5Y cells with high and low PrP^C levels after exposure to IFN-α. Interestingly, non-transfected SH-SY5Y cells with very low PrP^C levels responded with a significantly increased *MX2* transcription compared with PrP^C-containing SH-SY5Y cells, confirming that cells without PrP^C respond stronger when activated by type I IFNs.

Furthermore, data from an independent experiment from an *in vivo* LPS challenge of *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ goats (Salvesen, Reiten et al. 2016, Salvesen, Reiten et al. 2017), not included in this thesis, supported that the basal gene expression of interferon-responsive genes in circulating blood leukocytes was slightly higher in the *PRNP*$^{Ter/Ter}$ cells compared with the *PRNP*$^{+/+}$ cells. After LPS administration, the difference between the two genotypes was slightly more pronounced, illustrating that this primed phenotype also exists in an *in vivo* setting. Again, when assessing these results, we should keep in mind that the goats do not seem to be overrepresented in disease statistics, suggesting that the phenotype does not lead to overall aberrant immune responses. Additionally, no effects of PrP^C loss was found when cells were subjected to normal immune cell duties such as proliferation of T/NK cells and phagocytosis (paper I). These are essential processes influenced by type I IFNs (Vogel and Fertsch 1984, Reiter 1993, Welsh, Bahl et al. 2012), but effects on the IFN response caused by lack of PrP^C were too subtle to influence the measured phenotypes.

In the study mentioned above, additional transcriptomic analysis of choroid plexus and hippocampus in *PRNP*$^{Ter/Ter}$ goats after LPS-induced neuroinflammation showed an activation of cytokine-responsive genes that was skewed towards a more profound type I IFN response (Salvesen, Reiten et al. 2017).

Considering that only minor differences could be detected in the choroid plexus and the hippocampus prior to LPS challenge, it was intriguing to identify a more prolonged sickness behavior in *PRNP*$^{Ter/Ter}$ animals compared with *PRNP*$^{+/+}$ goats, a phenotype that might be related to an increased type I IFN response. The study corroborates the finding that PrP^C is involved in innate immunity signaling and that a type I IFN-phenotype can be found also in non-immunological tissues.

Altogether, our findings strengthen the view that PrP^C is involved in modulation of immune responses, more specifically in certain pathways of innate immunity, a continuation of a view recently presented in a review by several members of our research group (Bakkebo, Mouillet-Richard et al. 2015).

5.3.2. A comment on the similarities between the expression profile early in prion disease and the profile of cells lacking PrP^C

The increased levels of type I IFN-responsive genes is a previously unrecognized phenotype in cells without PrP^C. Could the expression profile of goat PBMCs without PrP^C be relevant for prion disease pathogenesis and the debate concerning whether loss-of-function or gain-of-function of PrP^C is involved in initiation and progression of neurodegeneration? Keeping our results in mind, it was incredibly fascinating to recognize that a similar expression profile was detected during early stages of prion disease (Baker, Lu et al. 2004). Increased expression of IFN-responsive genes was observed early in the disease course, about 50 days before detectable amount of PrP^{Sc} and presence of neuropathology. Similar to our findings in paper II, increased type I IFN expression was not detected, which could have aided in explaining the results. However, increased phosphorylation of STAT1 was found (Baker, Lu et al. 2004). Whether downstream signaling components are constantly slightly more activated in cells from PrP^C-deficient goats remains to be investigated. Similarly, another study found increased levels of IFN-responsive genes in three rodent models at the onset of clinical symptoms (Stobart, Parchaliuk et al. 2007).

Whether sampling time is important to detect this effect is unclear, as some studies have failed to detect a clear IFN signature during early stages of prion disease (Booth, Bowman et al. 2004, Skinner, Abbassi et al. 2006). Several IFN-responsive genes were detected in scrapie-infected brain tissue from mice at end-stage (Riemer, Queck et al. 2000), indicating that IFNs could contribute to pathology also during later stages of prion disease. Indeed, it was shown that type I IFNs can be directly toxic to neuronal cells and induce apoptosis through activation of JAK-STAT and PKR signaling and downregulation of the P13K/Akt pathway (Dedoni, Olianas et al. 2010). However, gene expression

studies during later stages of prion disease may not be relevant for the initial phenotype of disease due to the overwhelming activation of genes resulting from cell death and the M1 pro-inflammatory phenotype of microglia in the later stages (Aguzzi, Nuvolone et al. 2013). The impact of enhanced levels of type I IFN-responsive genes on brain tissue, induced by PrP^C loss-of-function, during prion disease, remains to be investigated. In the alternative gain-of-function hypothesis, aggregates of PrP^{Sc} could interact with PrP^C and induce subversion of PrP^C function, as outlined in section 1.2.7, followed by some form of toxic signaling associated with a change in the expression of IFN-responsive genes.

5.4. Stress-protective properties of PrP^C

5.4.1. PrP^C expression in male gonads

As a background for studies of PrP^C in male reproduction, an examination of PrP^C's expression pattern in reproductive tissue was necessary. In paper III, WB confirmed the presence of PrP^C in the testicle and the spermatozoa. By IHC and IF we were able to visualize the expression pattern in the testicle and epididymis. Our findings are in line with several previous studies (Shaked, Rosenmann et al. 1999, Gatti, Metayer et al. 2002, Peoc'h, Serres et al. 2002, Ecroyd, Sarradin et al. 2004).

Diglycosylated, full-length PrP^C was clearly present in both the testicle and spermatozoa, although in the testicle, the molecular weight of PrP^C was slightly higher than in spermatozoa. After deglycosylation, full-length PrP^C with a molecular weight of 27 kDa, in addition to an 18 kDa band, were detected in both the testicle and spermatozoa, the latter likely corresponding to a C_2 cleavage fragment (see Figure 1) known to occur after oxidative stress (McMahon, Mange et al. 2001). The fragment is also detected during a normal physiological state in brain tissue; however, at low levels (Campbell, Gill et al. 2013). Whether this cleavage is more common in the testicle and spermatozoa than other tissues such as the brain is unknown. A similar study of ram spermatozoa detected a 24-25 kDa band, which, after deglycosylation, was reduced to 17 kDa (Ecroyd, Sarradin et al. 2004). Two additional bands in spermatozoa with molecular weights of 22-24 kDa were detected in spermatozoa, which could possibly derive from a cleavage of PrP^C close to the C-terminus. Notably, the P4 antibody used in paper III would not detect any C_1 fragment resulting from α cleavage and in order to achieve a more complete analysis of the different PrP^C fragments, an extensive epitope mapping with a range of antibodies is necessary.

PrP^C expression during spermatogenesis has been investigated previously (Ford, Burton et al. 2002, Peoc'h, Serres et al. 2002, Fujisawa, Kanai et al. 2004), and analogously to Ford et al. (Ford, Burton et al. 2002), we found a vivid staining of Sertoli cells. In the Sertoli cells, a strong PrP^C staining of ORO-positive lipid vacuoles within the basal parts of the cells was detected and the origin of these vacuoles was discussed. Sertoli cells are nursing cells that facilitates the maturation of spermatogenic cells from the stem cell stage to the release of spermatozoa into the lumen of the seminiferous tubuli. In the final maturation stage, the elongated spermatids shed a cytoplasmic droplet that are taken up by the Sertoli cells at the luminal side of the tubules and become residual bodies, structures that in paper III were found positive for both ORO lipid stain and PrP^C. The residual bodies are processed in the Sertoli cells and may end up as larger ORO positive vacuoles at the basal side. Another source for these vacuoles is apoptotic spermatogonia (Wang, Wang et al. 2006). Whether the source of the lipid vacuoles in Sertoli cells is residual bodies and/or degradation products from apoptotic spermatogenic cells remains to be investigated; however, the strong staining on residual bodies suggest that co-transport of PrP^C and lipids from the residual body stage to the larger vacuoles seems possible. Interestingly, as much as half of the developing spermatogonia can go into apoptosis in the testicle, although some variation between species exists (Allan, Harmon et al. 1992), this is a phenomenon required for adequate and efficient production of spermatozoa (Nakanishi and Shiratsuchi 2004). In this regard, with PrP^C's proposed anti-apoptotic trait in mind (Park, Jeong et al. 2015), it is worth mentioning that we observed no differences between the genotypes concerning degree of apoptosis or number of ORO-positive vacuoles, indicating that PrP^C does not have any negative impact on the spermatogenic development. However, few animals were investigated, and a higher number is necessary to reach a conclusion.

The Sertoli cell is responsible for the blood-testis-barrier via intercellular tight junctions and these structures are shown in enterocytes to be influenced morphologically and functionally by lack of PrP^C (Morel, Fouquet et al. 2008). During the spermatogenic cycle, Sertoli cells furnish the primary spermatocytes with iron, which is needed for mitosis, meiosis and mitochondriogenesis and the iron, potentially toxic to the mature spermatids, is recycled back to the Sertoli cells through the residual bodies (Leichtmann-Bardoogo, Cohen et al. 2012). As PrP^C is connected to the transport of iron (Haldar, Tripathi et al. 2015), a potential function could be as transporter of iron between germ cells and Sertoli cells.

IHC showed weak staining of PrP^C in developing spermatozoa precursor cells, in contrast to the human testicles (Peoc'h, Serres et al. 2002), and we wondered if the source of PrP^C in spermatozoa may be from outside the spermatogenic cell line. The intricate physical association between Sertoli cells and the different germ cell stages facilitates transfer of proteins between the cell types and in the case of PrP^C, this could occur from the Sertoli cell to the spermatids. This form of protein transfer is known to occur from epididymal epithelium to spermatozoa (Sullivan, Saez et al. 2005, Martin-DeLeon 2015); however, the finding of different isoforms of PrP^C in ram fluid and spermatozoa indicates that the PrP^C on spermatozoa is not derived from the seminal fluid (Gatti, Metayer et al. 2002, Ecroyd, Sarradin et al. 2004). Furthermore, the columnar epithelium in epididymis appeared negative in our sections when visualized by IHC and IF, questioning the occurrence of transport of PrP^C in epididysomes shed from the epithelium. The interstitial tissue was strongly positive for PrP^C, however, and a transport of PrP^C from epididymis to spermatozoa cannot be ruled out based on the IHC and IF alone. Whether epididymal fluid from goat bucks contains PrP^C is unknown and the precise mechanism for loading of PrP^C on spermatozoa would be interesting to investigate further.

5.4.2. Spermatozoa lacking PrP^C shows normal resistance against oxidative stress

PrP^C displayed abundant expression in the testicles and in the interstitial tissue of epididymis, as shown by IF and IHC, and by WB we were able to detect PrP^C on spermatozoa, however PrP^C's precise role in these organs is unknown (Allais-Bonnet and Pailhoux 2014). In view of data from mice demonstrating that PrP^C significantly protected spermatozoa against Cu^{2+}-induced stress (Shaked, Rosenmann et al. 1999), a surprising finding was that i*n vitro* Cu^{2+}exposure of spermatozoa revealed no differences between the genotypes in motility, acrosome intactness or ATP levels. Notably, the same Cu^{2+} concentrations were used in both studies. However, in the study by (Shaked, Rosenmann et al. 1999), spermatozoa isolated from epididymis were used, whereas we used ejaculated spermatozoa in our studies. The spermatozoa are under continuous maturation via proteins and other molecules secreted from the epithelium during the passage through the epididymal segments (Domeniconi, Souza et al. 2016) and it may be that this process can influence the spermatozoa's susceptibility to ROS. Whether the differences in results could be due to species differences or the use of different methods is not clear. Generally, PrP^C has been reported to bind Cu (Stockel, Safar et al. 1998, Aronoff-Spencer, Burns et al. 2000), and PrP^C-deficient cells suffer more from Cu treatment

than cells with PrP^C (Canello, Friedman-Levi et al. 2012). Our results do not confirm any protection of PrP^C against Cu exposure *in vitro* when investigating a series of important spermatozoa parameters. A larger fraction of the PrP^C-containing cells fell into the "high ROS category" after incubation with H_2O_2 and $FeSO_4$, indicating that spermatozoa from *PRNP*$^{+/+}$ animals are able to accumulate and tolerate larger levels of ROS, which is the opposite of what would be expected if PrP^C encompassed ROS-protective properties. The findings are not in accordance with previous studies showing increased levels of ROS in cells without PrP^C (Choi, Anantharam et al. 2007, Aude-Garcia, Villiers et al. 2011, Bertuchi, Bourgeon et al. 2012, Zanetti, Carpi et al. 2014).

An explanation to this finding would be that the high ROS generation is beneficial to the cell, at least as long as the antioxidant system, perhaps including PrP^C, can protect the cell from damage. In line with this, we did not detect changes in viability between spermatozoa from the two genotypes. Spermatozoa produce ROS themselves for functions such as capacitation and hyperactivation, allowing rapid movement along the female reproductive tract (Wright, Milne et al. 2014), and perhaps is PrP^C somehow necessary in this process. However, more detailed studies are needed to reveal the consequences of lower ROS levels in PrP^C-deficient spermatozoa, although no data support decreased fertility in *PRNP*$^{Ter/Ter}$ goats.

Altogether, our findings suggest that PrP^C loss does not obliterate the normal physiology of spermatozoa after thawing, which is an important finding also in the context of breeding.

The apparent normal fertility and spermatozoa parameters in goats without PrP^C is a paradox in relation to the high PrP^C levels in the gonads of normal bucks. Probably, redundant and compensatory mechanisms secure the important reproductive functions. The prion-like protein Dpl is highly expressed in the testicles (Rondena, Ceciliani et al. 2005, Espenes, Harbitz et al. 2006, Kocer, Gallozzi et al. 2007) and previous research has speculated if Dpl is compensatory upregulated after PrP^C-ablation or is able to take over PrP^C's function in PrP^C-deficient cells or tissues (Allais-Bonnet and Pailhoux 2014). While no expression studies or Dpl staining was performed in this work, this could be a subject to follow up.

Excess type I IFN signaling appears to cause infertility in mice overexpressing IFN-β by inducing apoptosis of germ cells (Satie, Mazaud-Guittot et al. 2011). While we detected no morphological differences in testicular tissue in the goat model, it is interesting to note that the interferon-responsive

genes *OAS*, *MX* and *PKR* are also constitutively expressed in the testicles, particularly in Sertoli cells (Dejucq, Chousterman et al. 1997, Melaine, Lienard et al. 2003, Starace, Galli et al. 2008).
In this context, it could be interesting to investigate whether the testicles from PrP^C-deficient goats also express a moderately higher level of type I IFN-responsive genes, similar to the PBMCs (paper II), and if this can alter the immune response and pathologies observed during infections. If lack of PrP^C should influence IFN-signaling I in the testicles, the modulation is obviously below the threshold that may cause infertility.

5.4.3. Responses of PBMCs and SH-SY5Y cells to oxidative and genotoxic stress are not influenced by PrP^C

No apparent cytoprotection was awarded by the presence of PrP^C upon exposure to reagents inducing oxidative and genotoxic stress in PBMCs and SH-SY5Y cells, as reflected in similar viability and the accumulated levels of 7-m(dG) regardless of *PRNP* genotype. If PrP^C was stress-protective in circumstances where oxidative stress was generated, an increased viability would be expected in PrP^C-expressing cells. Rather, we observed the opposite, indicating no protection from PrP^C expression *in vitro*. This is surprising, since increased survival was demonstrated in PrP^C-expressing cells exposed to oxidative stress induced by xanthine oxidase (Brown, Schulz-Schaeffer et al. 1997, Brown, Nicholas et al. 2002), H_2O_2 (White, Collins et al. 1999, Oh, Choi et al. 2012) and paraquat (Senator, Rachidi et al. 2004, Dupiereux, Falisse-Poirrier et al. 2008). We noted an increase in *PRNP* transcription, most distinctly in SH-SY5Y cells, in line other studies (overview given in Haigh and Brown 2006). An attempt was also made at measuring ROS levels in PBMCs and SH-SY5Y cells, however due to technical difficulties and limitations in time we were unable to finish these studies. Preliminary results, however, did not indicate any major alterations on ROS levels in PrP^C-deficient cells. Further studies are needed to fully elucidate if PrP^C contributes to intracellular levels of ROS.
Interestingly, several of the studies looking at cellular homeostasis after PrP^C ablation report a decreased activity of antioxidant enzymes (Brown, Schulz-Schaeffer et al. 1997, White, Collins et al. 1999, Klamt, Dal-Pizzol et al. 2001), although with exceptions (Brown, Nicholas et al. 2002), a finding which could explain the reportedly increased ROS levels. To provide an image of the antioxidant status of PBMCs, we were able to include data from the RNA-seq study (paper II). The basal levels of gene transcripts in PBMCs of selected enzymes involved in the antioxidant system were not significantly different between the genotypes, validating that PBMCs without PrP^C do not suffer from increased

levels of ROS during a normal physiological state. The finding is in contrast to increased levels of oxidation and lipoperoxidation found in PrP^C-ablated cells (Klamt, Dal-Pizzol et al. 2001, Wong, Liu et al. 2001, Senator, Rachidi et al. 2004), demonstrating increased ROS levels during steady-state.
An issue of debate has been whether PrP^C is involved in DNA repair (Bravard, Auvre et al. 2015). While we did not look at the base-excision repair pathway directly, RNA-seq results allowed us to include DNA repair enzyme transcripts from various pathways, however no effects of PrP^C deficiency could be found on the transcription of these during a normal physiological state.
Whereas our studies were not supportive of a cytoprotective role of PrP^C *in vitro*, our findings do not rule out a role for PrP^C in the *in vivo* resistance towards oxidative and genotoxic stress.

5.5. Main conclusions

Throughout the work in this thesis, the goat model has proved to be a unique and valuable tool in the search for PrP^C functions. By utilizing and comparing *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ goats we have set up a new framework in which future research can and should be continued.

Goats without PrP^C display few disturbances in circulating blood cells, although a hematological shift appeared in 3-4 week-old goat kids characterized by increased numbers of RBCs with a reduced MCV, and a tendency for an increased number of neutrophils. Basal immune cell functions appeared to be unaffected by PrP^C loss, a finding that calls previous studies on T-cell proliferation and phagocytosis into question.

Transcriptomic studies of circulating PBMCs revealed a subtle immunological signature in *PRNP*$^{Ter/Ter}$ cells, suggesting that PrP^C is involved in immunomodulation.
The shift in transcriptome was characterized by an increased level of type I IFN-responsive genes.
From our analyses, *PRNP*$^{Ter/Ter}$ PBMCs appeared to be in a primed state, which was confirmed by direct and indirect stimulation of the type I IFN pathway:

- SH-SY5Y cells displayed an enhanced transcriptional response of the type I IFN-responsive gene *MX2* when IFN-α was applied *in vitro*.
- Leukocytes isolated from the blood of *PRNP*$^{Ter/Ter}$ goats during a normal physiological state and after an LPS challenge *in vivo* showed increased levels of IFN-responsive genes

Although no mechanistic insight was given based on these findings, a thorough background was *given* for further studies.

No protective role of PrP^C could be found *in vitro* after induction of oxidative and genotoxic stress in PBMCs and SH-SY5Y cells.
PrP^C is widely expressed in the testicles, particularly in Sertoli cells, indicating that PrP^C could exhibit an important role during spermatogenesis. PrP^C is also expressed in spermatozoa, but no protective effect could be detected when oxidative stress was induced *in vitro*.

The possible link between increased levels of type I IFN-responsive genes and the other findings in this work is unclear.

5.6. Future perspectives

It will be crucial to continue the research concerning on PrP^C's immunomodulatory role, which can have far-reaching consequences if confirmed in other experimental models. In particular, mechanistic studies will be important to be able to fully dissect the pathways where PrP^C may be involved. One such approach could be to investigate the systemic effects of type I IFNs either *in vivo* or *in vitro*.
Salvesen et al. induced neuroinflammation with LPS and transcriptome changes in the choroid plexus and the hippocampus showed a similar profile with enhanced levels of type I IFN-responsive genes as in our investigated PBMCs (Salvesen, Reiten et al. 2017). This indicates that the findings are generalizable, however further gene expression studies in other cells and tissues are needed to fully understand the whole picture. This project is already started and ongoing.
Continued search for these pathways is also achievable *in vitro* by utilizing SH-SY cells or other cell lines.
Perhaps primary cells isolated from the goats could be used instead of PBMCs. In this regard, we have isolated gut epithelial and fibroblast cell cultures from goats with and without PrP^C, and studies of these are ongoing. Further studies of neutrophils or T cells could also be interesting study objects as they have shown dynamic regulation of PrP^C expression after activation.
Another option would be to investigate if the phenotype could be reproduced in other models, e.g. the newly introduced *Prnp* knockout mouse (Nuvolone, Hermann et al. 2016).

The transcriptome data would be interesting to follow up with studies of protein levels and activities. As the present studies have shown that PrP^C modulates the level of IFN responsive genes, a deeper understanding of these processes could be gained by investigating the level of activation of the proteins involved in the IFN I signaling pathway, often done by looking at the ratio between phosphorylated end dephosphorylated forms.

The finding of high levels of PrP^C in testicular tissue and spermatozoa, another immune-privileged site, calls for a deeper understanding of PrP^C's function and its possible immunomodulatory role in this tissue. Induction of inflammation and investigation of pathological alterations and changes in gene expression could be one way of pursuing this issue.

An interesting genotype for further studies are *PRNP*$^{+/Ter}$ goats. It would be interesting to continue investigating these animals as we showed that PBMCs from these animals express half the amount of cell-surface PrP^C compared with *PRNP*$^{+/+}$ cells. The amount of expressed PrP^C needed to "rescue" a phenotype is unclear and requires further investigation.

Last, but not least, further studies of RBCs would be profitable. The link between PrP^C and HIF-αis poorly understood and could yield valuable information on the cross-talk between sensing of oxidative stress and inflammation. Studies of bone marrow development and fetal blood could be one approach.

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Appendix: enclosed papers I-III

Paper I

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
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Hematological shift in goat kids naturally devoid of prion protein

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The physiological role of the cellular prion protein (PrP^C) is incompletely understood. The expression of PrP^C in hematopoietic stem cells and immune cells suggests a role in the development of these cells, and in PrP^C knockout animals altered immune cell proliferation and phagocytic function have been observed. Recently, a spontaneous nonsense mutation at codon 32 in the *PRNP* gene in goats of the Norwegian Dairy breed was discovered, rendering homozygous animals devoid of PrP^C. Here we report hematological and immunological analyses of homozygous goat kids lacking PrP^C ($PRNP^{Ter/Ter}$) compared to heterozygous ($PRNP^{+/Ter}$) and normal ($PRNP^{+/+}$) kids. Levels of cell surface PrP^C and *PRNP* mRNA in peripheral blood mononuclear cells (PBMCs) correlated well and were very low in $PRNP^{Ter/Ter}$, intermediate in $PRNP^{+/Ter}$ and high in $PRNP^{+/+}$ kids. The $PRNP^{Ter/Ter}$ animals had a shift in blood cell composition with an elevated number of red blood cells (RBCs) and a tendency toward a smaller mean RBC volume ($P = 0.08$) and an increased number of neutrophils ($P = 0.068$), all values within the reference ranges. Morphological investigations of blood smears and bone marrow imprints did not reveal irregularities. Studies of relative composition of PBMCs, phagocytic ability of monocytes and T-cell proliferation revealed no significant differences between the genotypes. Our data suggest that PrP^C has a role in bone marrow physiology and warrant further studies of PrP^C in erythroid and immune cell progenitors as well as differentiated effector cells also under stressful conditions. Altogether, this genetically unmanipulated PrP^C-free animal model represents a unique opportunity to unveil the enigmatic physiology and function of PrP^C.

Keywords: cellular prion protein, PrP^C, hematology, hematopoiesis, phagocytosis, T-cell proliferation

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Introduction

The cellular prion protein (PrP^C) was first described as the substrate for PrP scrapie (PrP^{Sc}) (Prusiner, 1982; Brandner et al., 1996a,b), a misfolded and aggregation-prone form of the protein detected in brain tissue of animals diagnosed with transmissible spongiform encephalopathies, now often called prion diseases. These are fatal neurodegenerative diseases occurring naturally in humans and ruminants and include Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, scrapie, and chronic wasting disease. The process of template-directed self-replication of PrP^{Sc} constitutes the core of the "protein only" hypothesis (Prusiner et al., 1998), stating that the prion agent consists solely of misfolded PrP^C conformers, and in accordance with this, PrP^C knock-out (KO) mice do not replicate prions, nor do they develop prion disease (Bueler et al., 1992).

PrP^C is a highly conserved GPI-anchored protein (Steele et al., 2007) expressed abundantly in the central nervous system (CNS) (Kretzschmar et al., 1986), but also at lower levels in many other cells like hematopoietic (Zhang et al., 2006) and embryonic stem cells (Miranda et al., 2011), immune cells (Isaacs et al., 2006) and various epithelial cell types (Horiuchi et al., 1995; Ford et al., 2002), suggesting that PrP^C might have important functions within these cell types (Bendheim et al., 1992). In view of this, it was puzzling that PrP^C KO mice developed normally without overt phenotypic abnormalities (Bueler et al., 1992; Manson et al., 1994). Subsequent analyses have; however, revealed various phenotypes such as altered circadian rhythms (Tobler et al., 1996), behavior abnormalities (Roesler et al., 1999; Massimino et al., 2013), increased susceptibility to oxidative stress (Wong et al., 2001), and increased excitability of neurons (Khosravani et al., 2008).

In bone marrow, PrP^C is expressed in long-term hematopoietic stem cells (Dodelet and Cashman, 1998; Zhang et al., 2006) and may contribute to maintenance of stem cell properties, since bone marrow stem cells derived from PrP^C KO mice, contrary to similar cells from wild-type mice, fail to repopulate the bone marrow of irradiated recipient mice, especially after serial transplantations (Zhang et al., 2006). Interestingly, the PrP^C expression in immune cells is regulated according to their lineage fate. The observation that PrP^C expression is maintained at a high level in mononuclear cell precursors and downregulated in granulocytic and erythroid cells during their maturation in the bone marrow suggests that PrP^C plays a role in the dynamic development of these cells (Dodelet and Cashman, 1998). PrP^C is upregulated in activated T lymphocytes (Mabbott et al., 1997) and neutrophils (Mariante et al., 2012) and has been suggested to act as a signaling molecule in the activation of immune cells (Mattei et al., 2004; Krebs et al., 2006), but the definite role of PrP^C in these processes remains unclear.

Whether PrP^C modulates macrophage functions, in particular phagocytic capacity is controversial. Peritoneal macrophages devoid of PrP^C displayed increased phagocytosis toward apoptotic cells in comparison with wild-type macrophages (De Almeida et al., 2005), suggesting that PrP^C acts as a negative modulator of phagocytosis. Similar results were recently reported in a study using primary cell culture of bone marrow-derived macrophages from ZrchI type PrP^C KO mice in comparison with similar cells derived from C57BL/6 mice (Wang et al., 2014), where the PrP^C-depleted macrophages displayed increased phagocytic capacity toward fluorescently labeled *E. coli*, enhanced phagosome maturation and cytokine expression. Uraki and co-workers; however, reached the opposite conclusion when using immortalized bone marrow-derived macrophages from ZrchI type PrP^C KO mice, observing that loss of PrP^C reduced phagocytic capacity toward fluorescent latex beads (Uraki et al., 2010).

Likewise, the proliferative response of T cells *in vitro* upon cytokine stimulation has been studied in cells from transgenic mice and cattle with and without PrP^C. Murine studies demonstrated a reduced proliferative response (Mabbott et al., 1997) and altered cytokine profile (Bainbridge and Walker, 2005) in Concanavalin A (Con A)-stimulated T cells from PrP^C KO mice indicating a role for PrP^C in T-cell pathways leading to proliferation. Data from transgenic PrP^C KO cattle (Richt et al., 2007) revealed no differences in the proliferative response of T cells when stimulated with anti-CD3 antibody, Con A and phytohemagglutinin. A further clarification of PrP^C's roles in stem cell and immune cell maturation and function is desirable and will be helpful in understanding PrP^C's functions in general.

Recently, a unique line of otherwise healthy Norwegian dairy goats carrying a nonsense mutation at codon 32 (premature termination codon, Ter) in the *PRNP* reading frame was discovered (Benestad et al., 2012). The mutation results in an early termination of PrP^C synthesis and consequently animals homozygous for this mutation are devoid of PrP^C. This is, to our knowledge, the first species identified that is naturally devoid of PrP^C. These animals are genetically unmanipulated and therefore represent a unique spontaneous animal model to complement the transgenic models available for studies of PrP^C functions.

Here, we report results of hematological and immunological analyses of goat kids without PrP^C expression ($PRNP^{Ter/Ter}$) compared to heterozygotes ($PRNP^{+/Ter}$) and normal ($PRNP^{+/+}$) goats.

Methods

Animals and Sampling

The animals included in this study were of the Norwegian Dairy Goat Breed obtained from a research herd of around 100 animals at the Norwegian University of Life Sciences. Based on daily monitoring and enhanced health surveillance through a national goat health monitoring service, the health status of this flock is considered to be very good. Scrapie outbreaks have never occurred and there have been no cases of caprine arthritis encephalitis, Johne's disease or caseous lymphadenitis during the last 5 years (i.e., diseases subject to surveillance and control program in Norway) (Nagel-Alne et al., 2014). The entire flock was previously analyzed for *PRNP* genotypes (Benestad et al., 2012) and through selective breeding goat kids with the desired $PRNP^{Ter/Ter}$ genotypes were retrieved. No animals died of disease during the observation time and all $PRNP^{Ter/Ter}$ offspring developed normally during their first 6 months with no signs of behavioral or health problems. The Norwegian Animal Research Authority approved the protocol with reference to the Norwegian regulation on animal experimentation (FOR-1996-01-15-23, § 2) which is based upon the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Blood was drawn from the jugular vein into heparinized tubes and tubes without anticoagulant from age matched goat kids at 3–4 weeks of age. For hematological and clinical chemistry analyses, goat kids carrying the genotypes $PRNP^{Ter/Ter}$ ($n = 8$), $PRNP^{+/Ter}$ ($n = 16$), and $PRNP^{+/+}$ ($n = 24$) were included.

Hematological analyses were performed with an Advia® 2120 Hematology System using Advia 2120 MultiSpecies System Software and clinical chemistry analyses were performed with Advia 1800 Chemistry System (both from Siemens AG Healthcare Sector).

Peripheral blood mononuclear cells (PBMCs) were isolated from $PRNP^{Ter/Ter}$ ($n = 8$) and $PRNP^{+/+}$ ($n = 8$) goat kids by gradient centrifugation (Lymphoprep, Axis-Shield) at 1760 × g. Red blood cells (RBC) were lysed by brief exposure to sterile water prior to counting and trypan blue viability assessment using a Countess Automated Cell Counter (Life Technologies).

Morphology

For morphological studies, two age-matched male kids, one $PRNP^{Ter/Ter}$ and one $PRNP^{+/+}$, were necropsied at 3 months of age. Macroscopic examinations were performed routinely. Fresh bone marrow imprints and smears were made and evaluated after staining with May Grünewald Giemsa. Histological slides were prepared from formalin fixed and paraffin-embedded tissues and stained with hematoxylin and eosin (HE) prior to examination by light microscopy.

Real-time RT-PCR Analysis

Total RNA was isolated from PBMCs using the Qiagen RNeasy mini plus kit (Qiagen) following the manufacturer's instructions. RNA concentration and purity was analyzed using NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific), and quality was assessed using RNA Nano Chips on an Agilent 2100 Bioanalyzer (both from Agilent Technologies). RNA was stored at −80°C. cDNA was synthesized using the SuperScript III Reverse Transcriptase, RNase Out, dNTP mix and Random Primers (all from Invitrogen, Life Technologies) at the following conditions: 5 min at 65°C, >1 min on ice, 5 min at 25°C, 1 h at 50°C, and 15 min at 70°C.

Quantitative-PCR was conducted with LightCycler 480 Sybr Green I Master mix (Roche), with *PRNP* as target gene (F: GTG GCT ACA TGC TGG GAA GT; R: AGC CTG GGA TTC TCT CTG GT) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as reference gene (F: GGT TGT CTC CTG CGA CTT CA; R: TGG AAA TGT GTG GAG GTC GG). cDNA corresponding to 1 ng RNA was used per reaction. The samples had a total volume of 20 µl, and were run on a LightCycler 480 System (Roche). Conditions: 5 min at 95°C; 40 cycles of 10 s at 95°C, 10 s at 60°C, and 10 s at 72°C; and melting curve with 5 s at 95°C, 1 min at 65 and 97°C. Relative expression levels were calculated using an externally run standard curve for the PBMCs generated from one $PRNP^{+/+}$ animal, run in duplicates, with one randomly selected $PRNP^{+/+}$ as positive control (in-run).

Flow Cytometry for Cell Surface PrP and Immune Cell Markers

Immunophenotyping in flow cytometry was performed as previously described (Olsen et al., 2013). Briefly, isolated PBMCs, or whole blood if indicated, were incubated with Fixable Yellow Dead Cell Stain Kit (Life Technologies, Thermo Fisher Scientific Inc.) followed by primary monoclonal antibodies (mAbs), brief incubation with 30% normal goat serum to block Fc-receptors, and finally fluorescence-labeled goat-anti-mouse secondary antibodies (see Supplementary Table 1). To detect the intracellular CD3 epitope, surface-labeled cells were permeabilized with Intracellular Fixation and Permeabilization Buffer Set (eBioscience, Affymetrix Inc.) according to the manufacturer's instructions. Labeled cells were analyzed in a Gallios flow cytometer and data were processed using Kaluza 1.2 software (both Beckman Coulter, Inc.). Cell gates were designed to select for single and viable mononuclear cells.

Lymphocyte Proliferation Test

PBMCs were incubated in a flat-bottom 96 well plate for 72 h in complete medium with a density of 2×10^5 cells per well. Cells were stimulated with Concanavalin A (ConA; Sigma-Aldrich) (200 µg/ml), recombinant ovine interleukin (IL)-2 (10,000 U/ml) (Connelley et al., 2011) or recombinant human (rh)IL-15 (Affymetrix/eBioscience) (25,000 U/ml). Unstimulated cells were used as controls. Each treatment was run in three to six parallels. Proliferation was measured as 24 h uptake of 3H-thymidine (Perkin-Elmer, Waltham, USA) as previously described (Storset et al., 2001) in counts per minute (CPM). Parallels outside ±50% of the median were excluded, and net proliferation (net CPM) was calculated as mean of stimulated cells minus mean of unstimulated cells.

Isolation of Monocytes and Phagocytosis Assays

$CD14^+$ monocytes were positively selected from isolated PBMCs by anti-human CD14 MACS MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) using 10 µl beads per 10^7 cells. The purity was consistently measured between 90 and 95% by flow cytometry using anti-CD14 mAbs (TUK4; IgG2a; AbD Serotec). $CD14^+$ monocytes were seeded in a 96 well non-adherent plate (Corning Costar Ultra-Low Attachment multiwell plates) in complete medium [RPMI + 10% fetal calf serum (FCS) + 1% Penicillin/Streptomycin; Gibco] at 2×10^5 cells per well and incubated for 24 h at 37°C and 5% CO_2 in the presence of 5000 U/ml recombinant bovine granulocyte macrophage colony stimulating factor (rbGM-CSF) that had been expressed in 293T cells as described (Lund et al., 2012) based on a btGM-CSF plasmid (kindly provided by D. Werling, RVC, UK). The resulting activated monocytes were incubated with latex beads (FluoroSpheres 430/465, 20 µl corresponding to 4×10^6 beads, 20 beads/cell, Life Technologies), pHrodo red *E. coli* or pHrodo red Zymosan (both from Life Technologies) in separate wells. Phagocytosis was terminated after 30 min of incubation (37°C, 5% CO_2) by placing the plate on ice. The particle uptake was measured in a Gallios flow cytometer. The monocyte gate was adjusted to exclude free particles using cell-free particle-only samples as control.

To confirm that the particles had been phagocytized, monocytes were centrifuged onto slides at 1000 rpm for 5 min. The cells were fixed in acetone, blocked with goat serum and immunolabeled with mAb against human CD68 (Dako). The cytospots were incubated with secondary antibodies, Alexa 488 goat-anti-mouse IgG1 or Alexa 594 goat-anti-mouse IgG1, mounted in Prolong Gold Antifade reagent with DAPI (all from Life Technologies) and analyzed by standard fluorescence and confocal microscopy.

Statistical Analysis

All data was analyzed for statistical significance with the Mann-Whitney test and data reported with medians. A level of $P < 0.05$ was considered statistically significant and shown as * and **

in figures. GraphPad prism version 6.04 software was used for statistical analyses.

Results

Expression of PrP^C in Leukocytes Correlates with Genotype

The absence of PrP^C on PBMCs from *PRNP*$^{Ter/Ter}$ goats was confirmed by three different anti-PrP^C mAbs (**Figure 1A** and data not shown). The fluorescence intensity of anti-PrP^C-stained PBMCs showed that cells from *PRNP*$^{+/Ter}$ goats expressed approximately half the density of surface PrP^C as compared to those from *PRNP*$^{+/+}$ goats. When co-staining for immune cell subpopulations, this pattern of PrP^C expression was similar in monocytes (CD14$^+$), B cells (B-B2$^+$), and T cells (CD3$^+$) (data not shown). Anti-PrP^C mAbs staining of whole blood revealed that PrP^C was not expressed on the surface of granulocytes in goats of either genotype, while its presence was confirmed in the lymphocyte gate in *PRNP*$^{+/+}$ goats (**Figure 1B**).

To investigate whether reduced PrP^C levels in *PRNP*$^{+/Ter}$ and *PRNP*$^{Ter/Ter}$ animals would lead to compensatory upregulation of *PRNP* mRNA, relative mRNA expression levels in PBMCs and bone marrow were analyzed. As shown in **Figures 1C,D**, the *PRNP* mRNA expression level in PBMCs from *PRNP*$^{Ter/Ter}$ animals was 16.8% of the expression in *PRNP*$^{+/+}$ animals, whereas the expression level in *PRNP*$^{+/Ter}$ PBMCs was 42.4% of the *PRNP*$^{+/+}$ animals. In the bone marrow, the relative *PRNP* mRNA expression in the *PRNP*$^{Ter/Ter}$ goat was 34% of the *PRNP*$^{+/+}$ goat investigated (**Figure 1D**). Collectively, these data indicate that no compensatory mechanism is counteracting loss of PrP^C at *PRNP* mRNA expression level and that *PRNP*$^{Ter/Ter}$-encoding mRNA is degraded.

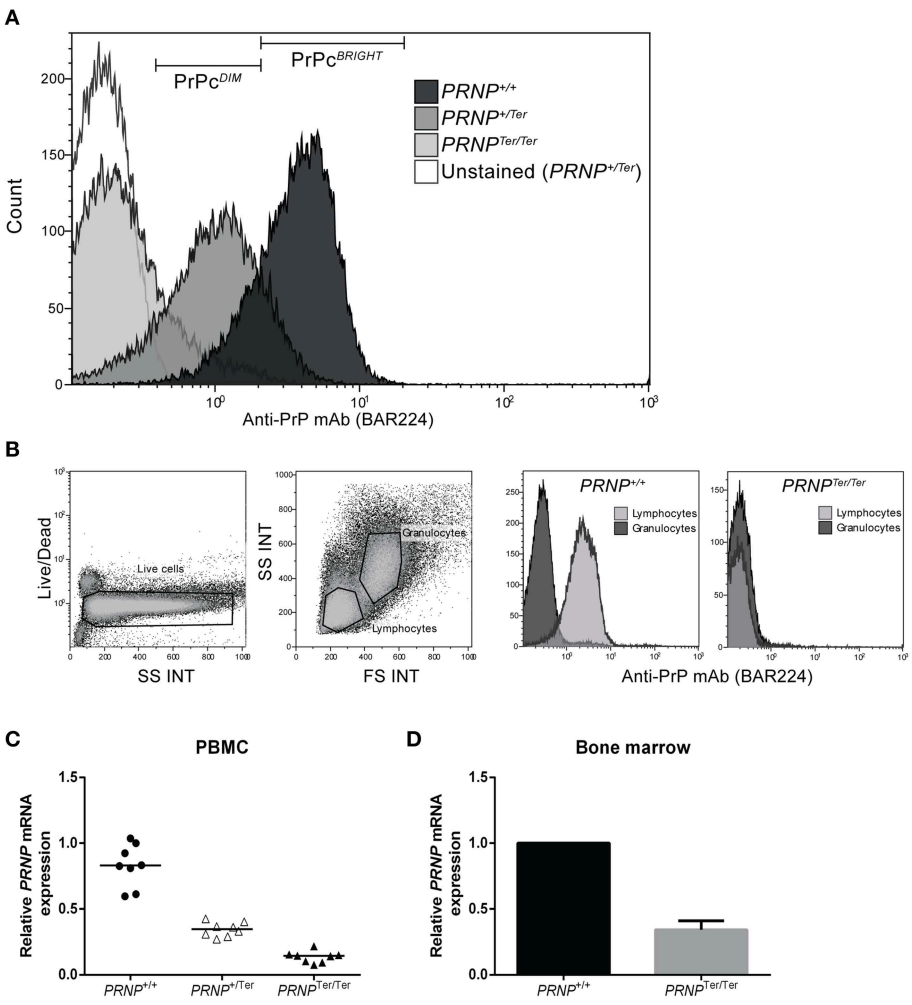

FIGURE 1 | (A–D) Relative PrP^C expression and *PRNP* mRNA expression in PBMCs. **(A)** Representative plot of PrP^C expression on PBMCs is shown using anti-PrP^C mAb BAR224. **(B)** Whole blood analysis showing gating of live cells into granulocyte and lymphocyte populations based on FS and SS characteristics. The two rightmost plots show representative PrP^C expression on lymphocytes and granulocytes in *PRNP*$^{+/+}$ ($n = 6$) and *PRNP*$^{Ter/Ter}$ ($n = 2$) animals. **(C)** Relative *PRNP* mRNA expression in PBMCs (each genotype $n = 8$). **(D)** Relative *PRNP* mRNA expression in bone marrow (each genotype, $n = 1$).

Red Blood Cell Numbers are Elevated in *PRNP*$^{Ter/Ter}$ Animals

To analyze whether reduced levels or complete lack of PrP^C influences the cellular or chemical composition of the blood, we performed hematological and clinical chemistry analyses of *PRNP*$^{Ter/Ter}$, *PRNP*$^{+/Ter}$ and *PRNP*$^{+/+}$ goat kids between 3 and 4 weeks of age.

Clinical chemistry revealed a slightly, but significantly, lower magnesium level and an increased creatine level in *PRNP*$^{Ter/Ter}$ animals compared to *PRNP*$^{+/+}$, both well within the normal reference range (see Supplementary Table 2).

The number of RBCs was increased in *PRNP*$^{Ter/Ter}$ goat kids (**Table 1** and **Figure 2A**) as compared to the *PRNP*$^{+/+}$ and *PRNP*$^{+/Ter}$ groups. Mean cell volume of the RBCs (MCV) was not significantly different between the groups, although the distribution of values suggested a clear tendency of lower MCV in the *PRNP*$^{Ter/Ter}$ animals compared to the *PRNP*$^{+/+}$ and *PRNP*$^{+/Ter}$ groups (**Table 1** and **Figure 2B**).

The groups did not differ in hematocrit (HCT) values (**Table 1**), suggesting that the reduced RBC volume was compensated by an increased number of RBCs or vice versa. To investigate if differences in iron uptake and metabolism could have any influence on RBC number and MCV we investigated the iron levels in blood serum. However, iron levels did not differ between the genotypes (**Figure 2C**).

TABLE 1 | Hematology results.

	Reference range	Median			*P*-values		
		A: *PRNP*$^{+/+}$	B: *PRNP*$^{+/Ter}$	C: *PRNP*$^{Ter/Ter}$	A vs. B	B vs. C	A vs. C
White blood cells ($\times 10^9$/l)	4–16	7.5	7.4	8.65	0.75	0.73	0.34
Red blood cells ($\times 10^{12}$/l)	8–18	10.61	10.58	11.78	0.8	**0.006**	**0.007**
Hemoglobin (g/l)	75–125	66	67	67	0.51	0.87	0.82
Hematocrit (l/l)	0.22–0.38	0.25	0.26	0.25	0.51	0.8	0.92
Mean corpuscular volume (fl)	16–25	23.65	24.3	21.6	0.67	0.051	0.08
Mean corpuscular hemoglobin content (g/l)	320–370	267.5	262	270	0.51	0.11	0.058
Rdw (%)	23–35	53.8	53.1	52.2	0.99	0.62	0.76
Neutrophils ($\times 10^9$/l)	1.5–8	2.25	2.35	3.75	0.35	0.33	0.068
Lymphocytes ($\times 10^9$/l)	2–9	4.8	4.35	4.35	0.28	0.75	0.6
Monocytes ($\times 10^9$/l)	0–0.5	0.35	0.45	0.45	0.028	0.91	0.08

P-values < 0.05 are shown in bold.

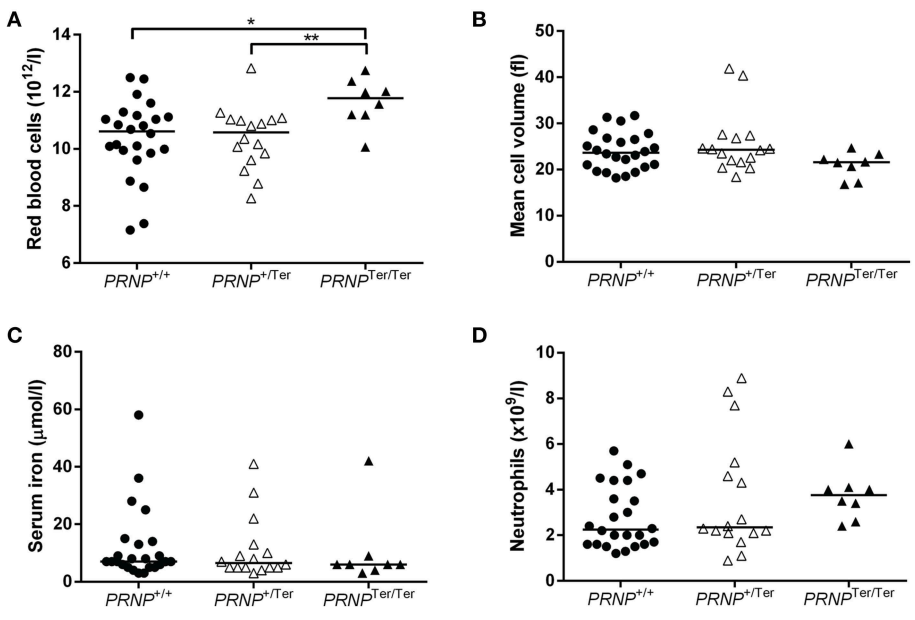

FIGURE 2 | Selected hematology parameters and serum iron values from 3 to 4 weeks old goat kids. Dot plots showing **(A)** the number of RBCs, **(B)** the RBC mean cell volume, **(C)** the serum iron levels, and **(D)** absolute number of neutrophils in *PRNP*$^{Ter/Ter}$ ($n = 8$), *PRNP*$^{+/Ter}$ ($n = 16$), and *PNRP*$^{+/+}$ ($n = 24$) animals. * and ** indicate $P < 0.05$.

In blood smears from $PRNP^{Ter/Ter}$ ($n = 8$) and $PRNP^{+/+}$ ($n = 24$) animals a marked poikilocytosis was observed, as expected in young kids. The neutrophil granulocytes were mature and showed no signs of left shift in any of the genotypes. No reticulocytes were present in any of the smears (data not shown).

Within the leukocyte populations, there was a tendency of a higher neutrophil count in the $PRNP^{Ter/Ter}$ animals as well as an increase in the monocyte number in $PRNP^{Ter/Ter}$ and $PRNP^{+/Ter}$ animals compared to the $PRNP^{+/+}$ group (**Table 1** and **Figure 2D**). The majority of the hematology values were within the general reference ranges for goats, except mean cell hemoglobin content (MCHC), red cell distribution width (RDW), and hemoglobin, where values fell below the presented reference range in all genotypes. However, breed-specific reference ranges were not available, and since these low values were present in all groups they would most likely be within normal ranges for rapidly growing goat kids of this breed.

Observing that PrP^C was highly expressed in a range of immune cells in normal goats we investigated whether lack of PrP^C expression influenced the numbers of these cells in peripheral blood. The relative numbers of monocytes ($CD14^+$), B cells ($B\text{-}B2^+$), T cells ($CD3^+$) as well as the gamma-delta ($WC1^+$) and $CD8^+$ subsets of T cells were quantified by flow cytometry (**Figure 3**). $CD4^+$ T-cell labeling was excluded from the study due to methodological problems. No differences in numbers were revealed for any of these leukocyte subsets between the $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ genotypes.

Morphological Analysis of Bone Marrow

To investigate whether the observed difference in hematological profile could be reflected by morphological changes in bone marrow, smears, and imprints of bone marrow from 3 months old $PRNP^{+/+}$ ($n = 1$) and $PRNP^{Ter/Ter}$ ($n = 1$) goat kids were analyzed. However, there were no differences in myeloid/erythroid ratio between the two genotypes, and the precursor cells had a normal appearance. In addition, there was no observable difference in degree of apoptosis (data not shown). Altogether, no evident morphological changes

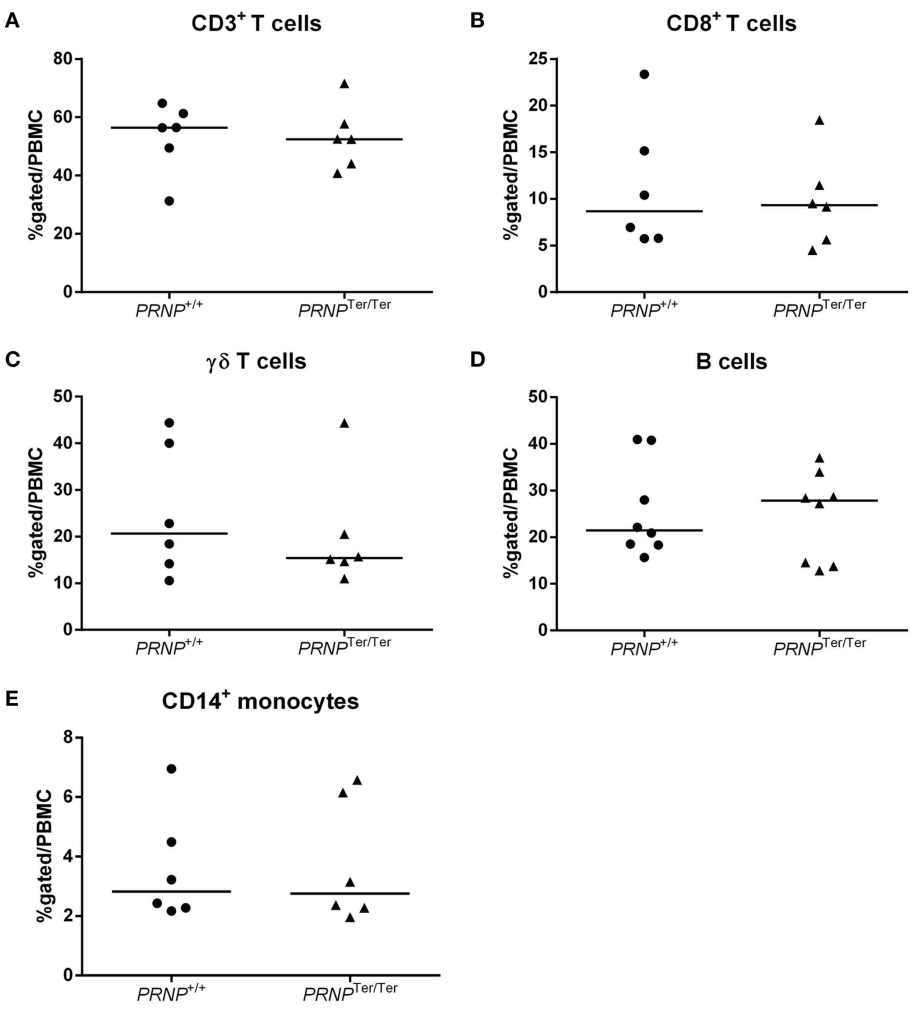

FIGURE 3 | Relative immune cell population sizes in 3–4 weeks old goat kids. Flow cytometric analysis of **(A)** $CD3^+$ T cells, **(B)** $CD8^+$ T cells, **(C)** γδ T cells, **(D)** B cells, and **(E)** $CD14^+$ monocytes out of the total PBMC population.

were found within the bone marrow of the *PRNP*$^{Ter/Ter}$ goat kid.

Monocyte Phagocytosis and T-cell Proliferation Appear Unaltered in *PRNP*$^{Ter/Ter}$ Animals

To determine whether PrP^{C} might have a functional impact on white blood cells, we performed phagocytosis and proliferation studies to assess two major functional properties of leukocytes. Positively selected CD14^{+} monocytes from peripheral blood were cultured for 24 h to stabilize cells following isolation, and supplemented with GM-CSF to activate the cells and prevent apoptosis (Bratton et al., 1995). The resulting short-term activated monocytes were incubated with latex beads, bacteria (*Escherichia coli*), or zymosan-covered yeast cells (*Saccharomyces cerevisiae*) for 30 min. Fluorescence and confocal microscopy of cytospots confirmed the cellular uptake of particles (**Figures 4A–C** and data not shown). A majority of the monocytes were CD68^{+}, consistent with monocytes or macrophages as previously described (Fadini et al., 2013). These cells had numerous vacuoles in the cytoplasm and a round to bean-shaped nucleus. All particle types were efficiently phagocytized by activated monocytes (**Figures 4D,E**). When comparing activated monocytes from *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ goats by flow cytometry, we detected no significant differences between the genotypes in the proportions of cells that had taken up fluorescent particles, for none of the particle types (**Figures 4 F–H**). There was also no significant difference in the numbers of particles per cell measured as median fluorescent intensity of positive cells; or in the case of latex beads, the number of cells that had engulfed 2 beads or more (**Figure 4E** and data not shown).

To investigate if PrP^{C} expression could be involved in cellular proliferation, we stimulated PBMCs *in vitro* using the mitogen Con A or the cytokines IL-2 or IL-15 to cover proliferation of T cells and NK cells/innate lymphocytes (**Figure 5**). The cell cultures proliferated well in response to these stimuli, but no significant differences between the groups were observed, although a slightly higher median response of cells from the *PRNP*$^{Ter/Ter}$ group was noted for all stimulations.

Collectively, these results suggest that loss of PrP^{C} does not have any major influence on the phagocytic ability of activated monocytes or the proliferative capacity of T or NK cells *in vitro*.

Discussion

The PrP^{C} is phylogenetically conserved and widely present in vertebrates (Harrison et al., 2010), pointing to an essential role for the organism. Non-transgenic animals naturally devoid of PrP^{C}, which has not been reported until recently (Benestad et al., 2012), could provide essential information regarding PrP^{C} physiology and function. In the present study we have observed that such *PRNP*$^{Ter/Ter}$ goats present with hematological changes, although without morphological changes in the bone marrow or alterations in major immune parameters.

By investigating complete blood counts of 3–4 weeks old goat kids we demonstrated that *PRNP*$^{Ter/Ter}$ animals had a significantly higher number of RBC compared to matched *PRNP*$^{+/+}$ controls. Tendencies of changes in other hematological values were also observed, such as lowered MCV and increased amounts of neutrophil granulocytes. These results are strikingly similar to the hematological observations in 10 months old PrP^{C} KO cattle (Richt et al., 2007), which had increased numbers of RBC, WBC, and neutrophil granulocytes, and lowered MCV and MCH. The authors of the study questioned if the gene cassette used in the transgenic procedure rather than the absence of PrP^{C} *per se* could have caused the observed differences. Our findings in naturally mutated animals strengthen the likelihood that PrP^{C} loss may physiologically affect RBC parameters and neutrophil numbers in young ruminants. Preliminary hematology results from adult goats have revealed no differences between the genotypes in any of the hematological values (unpublished results).

Several experimental approaches have shown that phenotypes related to loss of PrP^{C} are only clearly evident under various stressful conditions, such as tissue damage, infection, or anemia (Zivny et al., 2008; Gourdain et al., 2012). Likewise, it could be speculated that the increased demand for cell proliferation in the bone marrow during growth represents a physiological stress which reveals an otherwise cryptic phenotype caused by loss of PrP^{C}. Accordingly; a relatively subtle impact would not be observable in adult goats with lower bone marrow activity.

Mice lacking PrP^{C} have been reported to be in a chronic state of systemic iron deficiency, ascribed to inefficient uptake and transport into the blood stream, as well as inefficient uptake in various recipient cells, which could be rescued by the reintroduction of PrP^{C} (Singh et al., 2009). To rule out any impact of iron deficiency on the erythroid lineage we analyzed iron levels in all investigated animals. Although a smaller cell volume could be a result of an iron deficiency, we did not detect any difference in circulating iron levels that would support a state of systemic iron deficiency in *PRNP*$^{Ter/Ter}$ animals.

Studies in PrP^{C} KO mice have suggested a possible influence of PrP^{C} on long-term hematopoietic stem cells (LT-HSCs), as these cells showed reduced regenerative capacity when undergoing serial transplantations (Zhang et al., 2006). However, before the interventions, mice were normal with respect to blood cell levels and presence of cell lineage profiles in the bone marrow. In the present study, we did not observe morphological evidence of bone marrow impairment, as apoptosis rate and myeloid/erythroid ratio was found similar in a *PRNP*$^{Ter/Ter}$ animal compared to a normal control. More comprehensive and cell-specific investigations are however needed to clarify whether there are changes in bone marrow cellular composition in growing *PRNP*$^{Ter/Ter}$ kids, and further studies are needed to reach a final conclusion regarding possible differences between genotypes with respect to stem cell phenotypes in animals, both under normal and stressful conditions.

We detected high PrP^{C} expression in peripheral blood mononuclear leukocytes but not granulocytes of *PRNP*$^{+/+}$ animals, similar to previous reports that have led to a particular interest for PrP^{C} functions in immune cells, several of which have suggested PrP^{C} involvement in immune cell functions (Dodelet and Cashman, 1998; Barclay et al., 1999; Durig et al., 2000; Holada and Vostal, 2000; Herrmann et al., 2001; Halliday

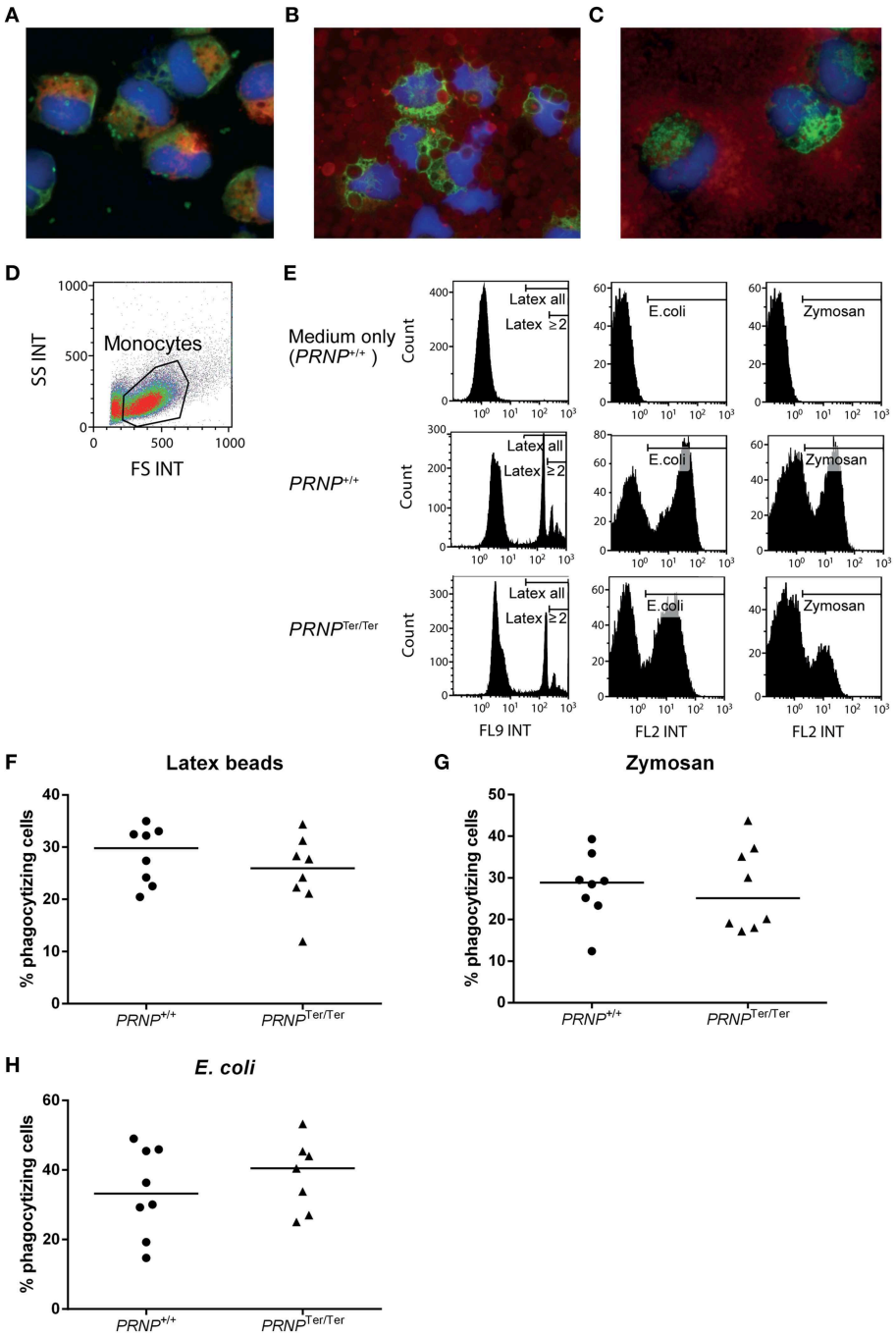

FIGURE 4 | Phagocytosis assays. Cytospots confirmed the cellular uptake of **(A)** latex beads (green fluorescence), **(B)** *E. coli* (red fluorescence), and **(C)** Zymosan (red fluorescence) in activated monocytes. Additional staining for nuclei (blue) and CD68 (**A** red, **B** and **C** green). **(D)** Gating of live cells based on FS and SS characteristics in flow cytometry. **(E)** Particle uptake in $PRNP^{Ter/Ter}$ and $PRNP^{+/+}$ cells based on the results from two representative animals. Medium only was used as control. Gates indicate particle-containing cells, and in the case of latex beads, also gates for cells that had engulfed 2 particles or more **(F–H)** Compiled results of all animals showing percentage of monocytes containing **(F)** latex beads, **(G)** Zymosan and **(H)** *E. coli*, as measured by flow cytometry. For each assay, $n = 8$, except $PRNP^{Ter/Ter}$ *E. coli* where $n = 7$.

et al., 2005; Dassanayake et al., 2012). Hematological analysis suggested a higher count of neutrophil granulocytes and possibly monocytes in $PRNP^{Ter/Ter}$ kids, while flow cytometric analyses did not confirm any difference in $CD14^+$ monocyte numbers, and relative numbers of other major circulating mononuclear cell subsets also appeared unaffected by PrP^C loss. Similar results,

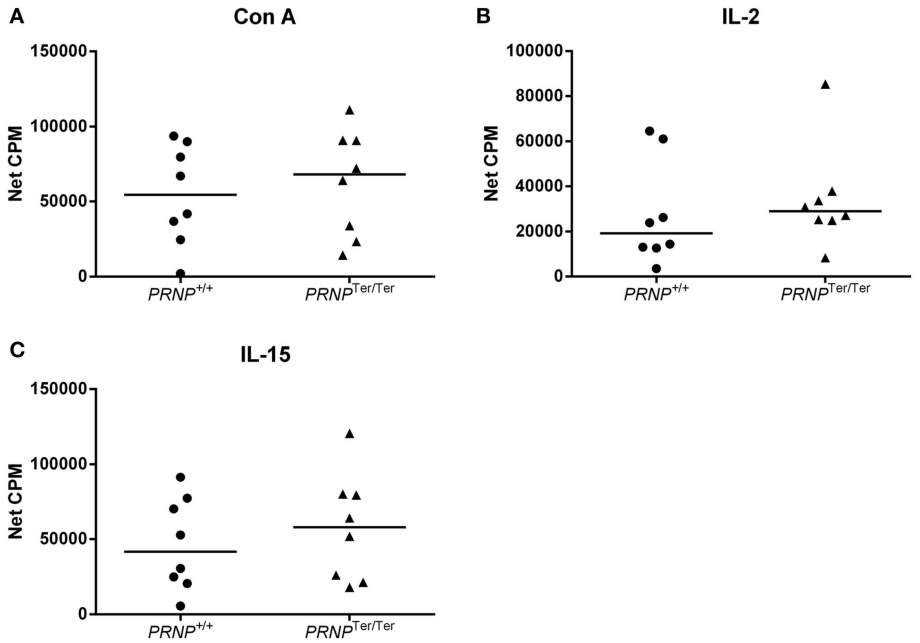

FIGURE 5 | Lymphocyte proliferation test. Proliferation of T cells after 72 h stimulation with **(A)** Con A, **(B)** IL-2, and **(C)** IL-15, measured as beta emission following DNA incorporation of tritiated thymidine (net CPM = sample CPM − control CPM). In all cases $n = 8$.

including increased neutrophil counts, have been found in KO cattle, although a tendency of more numerous γδ T cells in a group of four PrP^C cattle compared to four WT cattle, was found (Richt et al., 2007). Few similar studies have been done in murine PrP^C KO models but in a double KO, namely PrP^C and Doppel KO mice, no alterations in the immune cell populations were noted (Genoud et al., 2004). Seen together these results indicate that mechanisms that regulate the levels of immune cells in the blood are largely independent of PrP^C.

It has been shown that PrP^C is expressed in regulatory and memory T cells (Li et al., 2001), and that levels of PrP^C increase during T-cell proliferation (Cashman et al., 1990; Mabbott et al., 1997). Furthermore, T cells lacking PrP^C display reduced proliferation rates (Mabbott et al., 1997). Here, we stimulated PBMCs *in vitro* with mitogen or cytokines to assess alternative proliferation pathways but detected no difference in the proliferation rates between the genotypes. This correlates with the findings in transgenic cattle (Richt et al., 2007). Nevertheless, a slightly higher mean response in the $PRNP^{Ter/Ter}$ group consistent through all stimulations could suggest that proliferative differences may be present in more restricted lymphocyte subsets. Furthermore, we quantified the uptake of bacteria, yeast and latex particles by activated blood monocytes in order to approach different activation pathways of phagocytosis (Flannagan et al., 2012). We did not find that PrP^C deficiency had any influence on the proportion or efficiency of monocytes to phagocytize any of these particle types. Earlier studies on phagocytosis have been performed with cells from PrP^C KO mice and have given conflicting results. In an *in vitro* study of phagocytosis of *E. coli* by bone marrow-derived macrophages, the activity was enhanced in cells from PrP^C KO mice (Wang et al., 2014). Conversely, macrophages from PrP^C KO mice were shown to phagocytize latex beads at a lower rate than macrophages from WT mice (Uraki et al., 2010). In another study, De Almeida et al. (2005) found that PrP^C is a negative modulator of phagocytosis, as peritoneal macrophages from PrP^C KO mice phagocytized apoptotic cells at a higher rate than cells from WT mice *in vitro* and *in vivo*, a result confirmed by two different mouse strains. The results from the latter study have later been linked to polymorphisms in PrP^C flanking genes involved in phagocytosis, rather than the loss of PrP^C in itself (Nuvolone et al., 2013), and similar concerns might be raised regarding other reports on PrP^C functions from PrP^C KO models. Our results from naturally PrP^C-devoid animals are not in support of a principal mechanistic role of the prion protein neither in lymphocyte proliferation nor in phagocytosis.

In summary, this study showed that naturally occurring PrP^C-deficient goat kids displayed a generally healthy constitution, moderate shifts in red blood cell and possibly granulocytic cell lineages but not in other major circulating hematopoietic cells. No impairment of major immune cell functions was detected. These results point toward a role for PrP^C related to maturation and release of selected cell lineages from the bone marrow, and underscore previous observations that PrP^C loss appears to have limited physiological or pathological consequences for the animal, at least under conventional living conditions and in the absence of significant stressors. The present natural goat model offers a unique opportunity to study the function of PrP^C *in* and *ex vivo* without the confounding factors of genetic engineering, which may hopefully help to

shed light on the elusive nature of this protein and its related diseases.

Author Contributions

PB, AE, MT, MB, MR designed the study. MR, PB, MB performed the experiments and MR, PB, MT, AE, MB analyzed the data. AL assisted with the monocyte isolation protocol and experiments and HB contributed to the analysis of hematology data, blood smears and bone marrow imprints, and smears. MR, MB, HB, AL, IO, MT, AE, PB wrote or critically reviewed the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2015.00044

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table 1: Primary and secondary antibodies

Primary antibodies

Molecule recognized	Monoclonal /Polyclonal	Clone	Isotype	Typical expression pattern	Manufacturer	Raised against	Used in (FCM = flow cytometry, CS = cytospots)
B-B2	M	BAQ44a	mouse IgM	B-cells	VMRD / Kingfisher Biotech	Bovine	FCM
CD14	M	VPM65	mouse IgG1	Monocytes	Serotec	Ovine	FCM
CD14	M	Tük4	mouse IgG2a	Monocytes	AbD Serotec	Human	FCM
CD14	M	CAM36a	mouse IgG1	Monocytes	VMRD / Kingfisher Biotech	Bovine	FCM
CD3	M	CD3-12	ratIgG1	T cells	AbD Serotec, Ltd., Oxford, UK	Human	FCM
CD4	M	GC50A1	mouse IgM	T cell subset	VMRD / Kingfisher Biotech	Bovine	FCM
CD68	M	EBM11	mouse IgG1	Macrophages, monocytes	Dako	Human	CS
CD8a	M	BAQ111a	mouse IgM	T cell subset	VMRD / Kingfisher Biotech	Bovine	FCM
CD8b	M	BAT82a	mouse IgG1	T cell subset	VMRD / Kingfisher Biotech	Bovine	FCM
FITC isotype control	M	eBRG1	rat IgG1	Unknown	Affymetrix / eBioscience		FCM
Pacific blue isotype control	M	MOPC-173	rat IgG2a	Unknown	BioLegend		FCM
PrP^C	M	BAR224	mouse IgG2a	Various	Bartin Pharma	Ovine	FCM
PrP^C	M	SAF32	mouse IgG2b	Various	Bartin Pharma	Hamster	FCM
PrP^C	M	6H4	mouse IgG1	Various	Prionics	Human	FCM
TCR1	M	GB21a	mouse IgG2b	Gamma-delta T-cells	VMRD / Kingfisher Biotech	Bovine	FCM

Secondary antibodies

Fluorochrome	Monoclonal /Polyclonal	Clone	Isotype recognized	Manufacturer	Used in
Alexa 488	P	(N/A)	Goat anti-mouse IgG1	Life Technologies / Molecular Probes	FCM, CS

Alexa 488	P	(N/A)	Goat anti-mouse IgG2a	Life Technologies / Molecular Probes	FCM
Alexa 488	P	(N/A)	Goat anti-mouse IgG2b	Life Technologies / Molecular Probes	FCM
Alexa 488	P	(N/A)	Goat anti-mouse IgG3	Life Technologies / Molecular Probes	FCM
Alexa 488	P	(N/A)	Goat anti-mouse IgM	Life Technologies / Molecular Probes	FCM
Alexa 594	P	(N/A)	Goat anti-mouse IgG1	Life Technologies / Molecular Probes	CS
PE	P	(N/A)	Goat anti-mouse IgG1	Southern Biotechnologies	FCM
PE	P	(N/A)	Goat anti-mouse IgG2b	Southern Biotechnologies	FCM
PE	P	(N/A)	Goat anti-mouse IgG3	Southern Biotechnologies	FCM
PerCp-efluor710	M	R2a-21B2	Rat anti-mouse IgG2a	Affymetrix / eBioscience	FCM
PerCp-efluor710	M	M1-14D12	Rat anti-mouse IgG1	Affymetrix / eBioscience	FCM
Alexa 647	P	(N/A)	Goat anti-mouse IgG2a	Life Technologies / Molecular Probes	FCM
Alexa 647	P	(N/A)	Goat anti-mouse IgG1	Life Technologies / Molecular Probes	FCM
Alexa 647	P	(N/A)	Goat anti-mouse IgG2b	Life Technologies / Molecular Probes	FCM
APC-efluor780	M	II/41	Goat anti-mouse IgM	Affymetrix / eBioscience	FCM

Supplementary Table 2

	Reference range	**Median**			**P-values**		
		A: *PRNP*$^{+/+}$	B: *PRNP*$^{+/Ter}$	C: *PRNP*$^{Ter/Ter}$	A vs. B	B vs. C	A vs. C
Aspartat aminotransferase (U/l)	70-188	65	64	64	0,84	0,87	0,68
Gamma-glutamyl transeferase (U/l)	33-159	35	39	43,5	0,2	0,54	0,12
Glutamate dehydrogenase (U/l)		5,5	7	8	0,58	0,34	0,39
Creatinin kinase (U/l)	138-721	147,5	218,5	213,5	0,06	0,78	0,076
Total protein (g/l)	64-91	59	60	59	0,24	0,56	0,98
Urea (mmol/l)	2,7-11,9	3,5	3,4	2,95	0,56	0,5	0,43
Creatine (µmol/l)	61-120	60	61,5	64,5	0,6	0,23	0,049
Total bilirubin (µmol/l)							
Hydroxybutyric acid (mmol/l)	0,2-0,8	0,1	0,1	0,1	0,08	0,16	0,62
Glucose (mmol/l)	2,7-4	6,2	6,05	6,25	0,81	0,46	0,35
Inorganic phosphate (mmol/l)	1,1-3	3,45	3,4	3,7	0,58	0,15	0,08
Calsium (mmol/l)	2,2-2,8	2,7	2,7	2,75	0,85	0,48	0,53
Magnesium (mmol/l)	0,79-1,22	1,085	1,045	0,985	0,15	0,27	0,005
Albumin (g/l)	31,8-44,5	32,4	32,85	33,65	0,25	0,92	0,62
Alpha 1 (g/l)	6-10,3	7,95	6,95	7,65	0,05	0,12	0,63
Alpha 2 (g/l)	5,2-8,9	8,25	8,55	8,5	0,11	0,94	0,09
Beta 1 (g/l)	0,8-4	0,9	0,9	1	0,78	0,22	0,25
Beta 2 (g/l)	2,6-5	2,65	2,65	2,35	0,81	0,37	0,29
Gamma (g/l)	12,5-31,3	6,3	7,75	5,95	0,14	0,22	0,42
A/G	0,59-1,28	1,25	1,22	1,25	0,77	0,78	0,91
Fe (µmol/l)	16-40	7	6,5	6	0,61	0,75	0,27

Paper II

PLOS ONE

RESEARCH ARTICLE

Loss of prion protein induces a primed state of type I interferon-responsive genes

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Abstract

The cellular prion protein (PrP^C) has been extensively studied because of its pivotal role in prion diseases; however, its functions remain incompletely understood. A unique line of goats has been identified that carries a nonsense mutation that abolishes synthesis of PrP^C. In these animals, the PrP-encoding mRNA is rapidly degraded. Goats without PrP^C are valuable in re-addressing loss-of-function phenotypes observed in *Prnp* knockout mice. As PrP^C has been ascribed various roles in immune cells, we analyzed transcriptomic responses to loss of PrP^C in peripheral blood mononuclear cells (PBMCs) from normal goat kids ($n = 8$, $PRNP^{+/+}$) and goat kids without PrP^C ($n = 8$, $PRNP^{Ter/Ter}$) by mRNA sequencing. PBMCs normally express moderate levels of PrP^C. The vast majority of genes were similarly expressed in the two groups. However, a curated list of 86 differentially expressed genes delineated the two genotypes. About 70% of these were classified as interferon-responsive genes. In goats without PrP^C, the majority of type I interferon-responsive genes were in a primed, modestly upregulated state, with fold changes ranging from 1.4 to 3.7. Among these were *ISG15*, *DDX58* (RIG-1), *MX1*, *MX2*, *OAS1*, *OAS2 and DRAM1*, all of which have important roles in pathogen defense, cell proliferation, apoptosis, immunomodulation and DNA damage response. Our data suggest that PrP^C contributes to the fine-tuning of resting state PBMCs expression level of type I interferon-responsive genes. The molecular mechanism by which this is achieved will be an important topic for further research into PrP^C physiology.

Introduction

The cellular prion protein (PrP^C) can misfold into disease-provoking conformers (PrP scrapie; PrP^{Sc}) that give rise to several neurodegenerative prion diseases, such as Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in

cattle [1]. The seeding of PrP^{Sc} in brain tissue acts as a template for further misfolding of PrP^{C}, ultimately leading to severe neurodegeneration and neuronal death [1].

PrP^{C} is abundant throughout the nervous system, and, albeit at lower levels, in most other tissues of the body [2]. The protein is conserved in mammalian species [3, 4] and expressed already during early embryonal stages [5]. It was therefore surprising that $Prnp^{0/0}$ mice developed normally and revealed no major phenotypes besides being prion-disease resistant [6–8]. Interestingly, in four $Prnp^{0/0}$ mouse models (*Ngsk*, *Rcm0*, *ZrchII*, and *Rikn*), ablation of the *Prnp* gene induced severe degeneration of cerebellar Purkinje neurons [9–12]. This was, however, subsequently shown to be caused by ectopic expression of the prion-like protein Doppel (*Dpl*) in the brain, as a side-effect of the transgenic protocols [10]. Two additional *Prnp*-ablated mouse lines (*ZrchI* and *Npu*) displayed no neurodegeneration [7, 8]. Furthermore, other experiments have shown that a polymorphism in another *Prnp* flanking gene, *Sirp-alpha*, could significantly influence the interpretation of data that concerns the roles for PrP^{C} in phagocytosis [13]. Despite these inherent challenges with *Prnp*-null models [14], collectively known as the flanking-gene problem, the $Prnp^{0/0}$ lines have proven extremely valuable in exploring PrP^{C} physiology. They have provided clues regarding maintenance of axonal myelin [15–17], modulation of circadian rhythms [18], and neuronal excitability [19], in addition to protective roles in severe stress such as ischemia [20] and hypoxic brain damage [21].

A more general problem is the gap between mice and human physiologies [22–24]. The two species diverged about 65 million years ago, and differ substantially in both size and life span. Mice have evolved into short-lived animals relying on massive reproductive capacity, whereas humans reside at the other end of the spectrum, with low reproduction rates and life spans of approximately 80 years. This is of particular significance in modeling chronic human diseases that take decades to develop, and often involve subtle immunological imbalances [22]. In addition, translation to human medicine has proven challenging.

Recently, we identified what seems to be a unique line of dairy goats carrying a nonsense mutation that completely abolishes synthesis of PrP^{C} [25]. This spontaneous, non-transgenic model, is referred to as $PRNP^{Ter/Ter}$. Approximately 10 percent of the Norwegian dairy goat population carries the mutated allele. These animals appear to have normal fertility and behavior in all aspects of standard husbandry. We have no data to suggest that they are over-represented in disease statistics or otherwise failing in production performance. Careful analysis of hematological and blood biochemical parameters, as well as basic immunological features, did not reveal any abnormalities [26]. It was, however, noted that goats without PrP^{C} had slightly elevated numbers of red blood cells, identical to an observation in transgenic cattle without PrP^{C} [27], suggesting that this is a true biological loss-of-function phenotype, at least in ruminants.

Peripheral blood mononuclear cells (PBMCs) express moderate, but dynamic, levels of PrP^{C} [28]. We observed that goats heterozygous for the mutation ($PRNP^{+/Ter}$) express half the amount of cell surface PrP^{C} on PBMCs [26]; however, a 50 percent reduction in levels compared to PBMCs from $PRNP^{+/+}$ goats did not stimulate compensatory expression from the normal allele. Intrigued by this, and the fact that many reports have pointed to putative functions for PrP^{C} in immune cells (reviewed in [29], [30, 31]), mRNA sequencing of PBMCs derived from normal goats and goats without PrP^{C} was performed. The main goal of this study was to evaluate whether the loss of PrP^{C} elicits a transcriptional response in PBMCs that could reveal biological processes involving PrP^{C}. Our findings show that in the absence of PrP^{C}, a subtle, but highly significant change in the transcriptional profile of PBMCs is seen, dominated by upregulation in the expression of type I interferon-responsive genes.

Results

RNA-seq data quality control

High quality RNA sequencing data (FASTQ) were derived from Beijing Genome Institute (BGI), with an average total reads of 58,806,319 per sample, average total mapped reads of 42,168,758, and average uniquely mapped reads of 38,253,898 per sample (S1 Fig). To validate the sequencing data, primers (S1 Table) were designed for 12 randomly selected differentially expressed genes (DEGs), using reverse transcription (RT) quantitative real-time PCR (qPCR) on the original RNA. As shown in Fig 1, qPCR analysis of mRNA levels correlated well with the RNA-seq analysis ($r = 0.9616$, $p < 0.0001$, Pearson correlation). Minor discrepancies could be due to sample variations, as RNA from only six goats per group were used for qPCR validation, compared with eight goats per group for RNA-seq analysis.

Lack of PrP^C subtly alters the transcriptome in immune cells

A high correlation was observed between averaged $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ normalized gene expression data ($r = 0.99$, Pearson correlation). However, we found that not all $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ goats could be clearly separated from each other, probably reflecting the phenotypic diversity of the goats (S2 Fig). Despite this, using edgeR [32] and a p-value cut-off < 0.05, 735 genes were differentially expressed between the two genotypes (S1 File). Further filtration of the gene list using cut offs for fold change (log2 FC ± 0.5) and mean number of reads (> 100 reads in one of the groups) generated a high-confidence gene list of 127 DEGs, of which 67 were upregulated and 60 were downregulated in the $PRNP^{Ter/Ter}$ genotype (S2 Table). Of note, as we have previously shown that the PBMC cell populations, mainly T cells, B cells and monocytes, are stable between the two genotypes compared in our study [26], the DEGs result from real genotype-associated shifts in gene expression, not shifts in the cell populations. Reassuringly, the *PRNP* gene was among the DEGs, with very few reads mapping to this locus in the mutant. The chromosomal distribution of the DEGs is found in S3 Fig. The *PRNP* gene is located on chromosome 13 in goats. Only 1 (*SIGLEC1*) of the 86 annotated DEGs also maps to

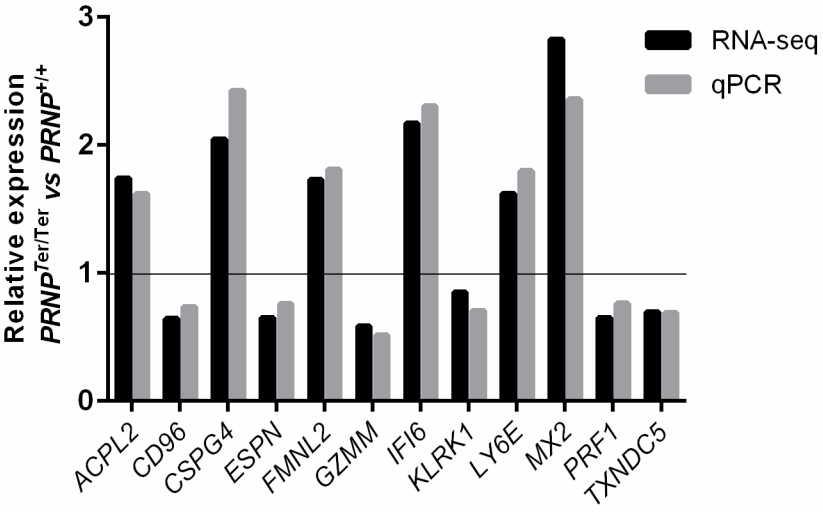

Fig 1. Validation of RNA sequencing data with quantitative PCR. Validation of 12 randomly chosen, differentially expressed genes was performed with qPCR using the original RNA. Expression data from the two methods are presented as relative expression between $PRNP^{Ter/Ter}$ and $PRNP^{+/+}$ animals (RNA-seq data $n = 8$, qPCR $n = 6$; $r = 0.9616$, $p < 0.0001$, Pearson correlation).

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chromosome 13. This gene is expressed at a low level and is irrelevant for the findings in our study.

Of the average total number of genes expressed in PBMCs from both genotypes, only 0.7 percent of the genes were altered upon loss of PrP^C (Fig 2A). Using Ingenuity Pathway Analysis (IPA), of the 127 high-confidence DEGs, 86 genes were functionally annotated. Interestingly, 22 of these genes were categorized as "Viral infection" (p-value = $3.27x10^{-5}$), and additional genes were related to other anti-virus-associated terms. The majority of these genes were upregulated in the $PRNP^{Ter/Ter}$ genotype compared with the $PRNP^{+/+}$ genotype. Of the top canonical pathways, "Interferon signaling" was by far the most affected (p-value = $8.92x10^{-6}$). Due to these findings, we performed further analyses of the annotated DEGs using the Interferome database [33]. Strikingly, 60 of the 86 annotated DEGs were interferon-responsive genes (Fig 2B). Of these, 42 were upregulated (red bar) and 18 downregulated (blue bar) in the $PRNP^{Ter/Ter}$ genotype. Fig 2C shows the inter-individual variation in gene expression of all samples represented in a heatmap, and hierarchical clustering analysis of the 60 interferon-responsive genes revealed a clustering of downregulated and upregulated genes between the $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ genotypes.

Since the observed data could be due to altered expression levels of interferons or components in type I interferon signaling, we analyzed expression levels of a number of genes that could affect the expression of interferon-responsive genes. However, differences between the genotypes were not detected (Table 1), except for *IFNB2-like*, which was slightly downregulated in the $PRNP^{Ter/Ter}$ genotype (p-value = 0.025).

Introduction of *PRNP* inhibited *MX2* gene expression in SH-SY5Y cells

To test whether PrP^C could influence IFN-α responsiveness in a cell culture system with a different genetic makeup, we used human neuroblastoma SH-SY5Y cells, which normally express extremely low levels of PrP^C. SH-SY5Y clones stably expressing human PrP^C were generated (SH-SY5Y PrP^{high}) and assessed with regard to glycosylation and proteolytic processing to ensure physiological post-translational modification and trafficking of PrP^C (S4 Fig). Eight clones stably expressing PrP^C as well as untransfected SH-SY5Y cells were exposed to 3 U/ml IFN-α for 3h. One of the transfected clones showed aberrantly high *MX2* gene expression levels and was excluded from the analysis. Of the seven clones included in the experiment, six displayed a significantly reduced response to IFN- α, as assessed by the interferon-responsive gene *MX2* expression levels, compared with the untransfected SH-SY5Y cells, using Dunnett's post hoc test for multiple comparisons (Fig 3) (n = 4, mean ± SEM). The levels of PrP^C expression did not directly correlate with the degree of *MX2* expression-level inhibition; however, this was not expected due to the complexity of the interferon signaling pathway, and the possible distance between PrP^C interference and *MX2* gene expression. On average, the clones showed a significantly inhibited response to IFN- α (p-value = 0.0001) compared with the untransfected SH-SY5Y cells, using a two-way ANOVA.

Increased interferon-responsive gene expression in blood leukocytes devoid of PrP^C after LPS challenge

In an independent, parallel study [34, 35], goats were challenged intravenously with lipopolysaccharide (LPS), thereby indirectly stimulating interferon pathways. RNA was extracted from circulating blood leukocytes, and gene expression of interferon-responsive genes was assessed by FLUIDIGM qPCR. As shown in Fig 4A, basal level expression (0h) of several interferon-responsive genes was slightly higher in the $PRNP^{Ter/Ter}$ (n = 13) genotype than in the $PRNP^{+/+}$ (n = 12) genotype, albeit being significantly different for only *IFI6* (p-value = 0.037). Moreover,

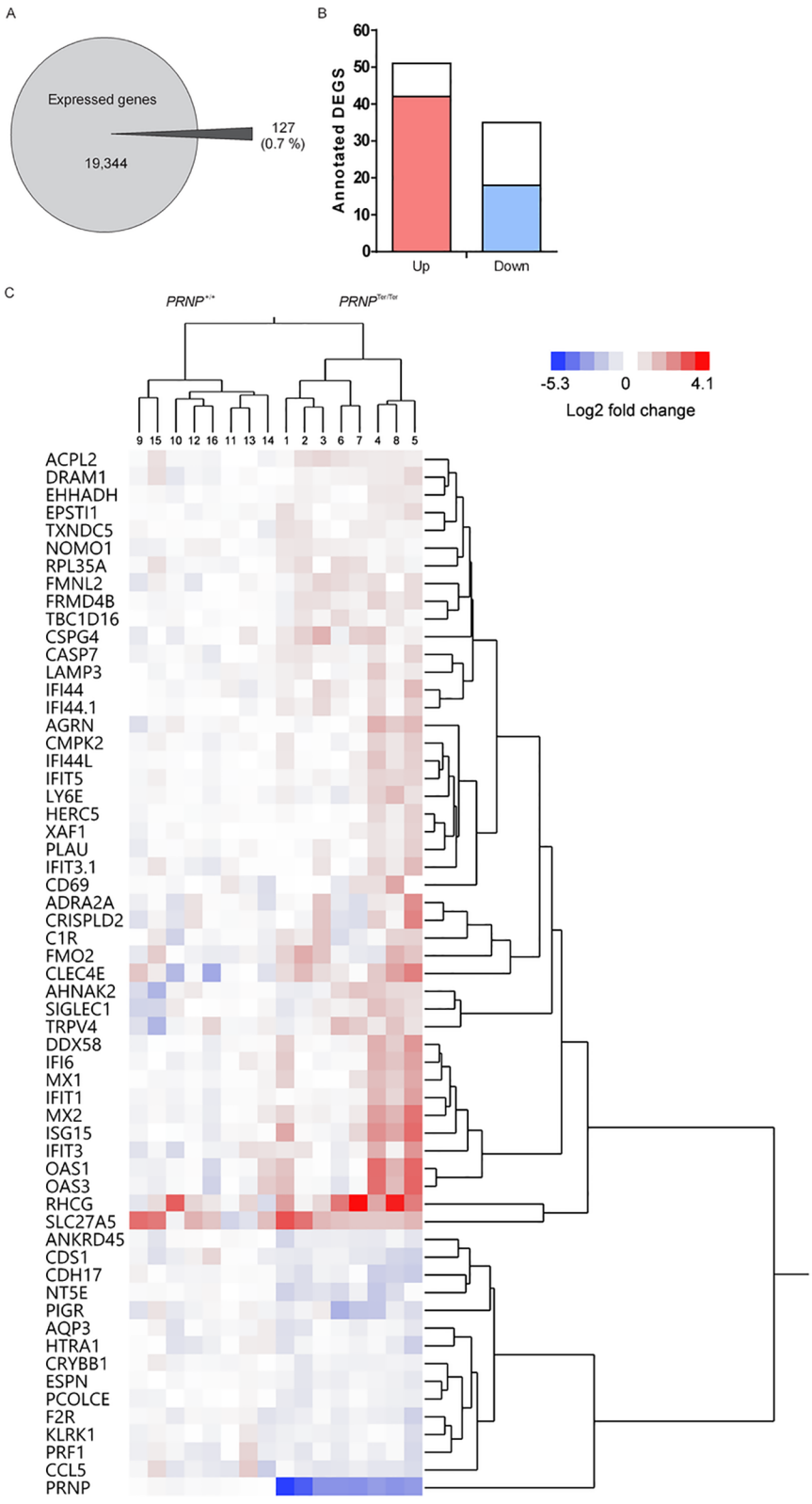

Fig 2. Interferon-responsive genes dominate among the differentially expressed genes in goats lacking PrP^{C}. Graphical presentation of (A) the total number and percentage of differentially expressed genes (DEGs) between the two genotypes, compared to the average total number of genes expressed in peripheral blood mononuclear cells from both genotypes, and (B) the total number of upregulated and

downregulated annotated DEGs. The fraction of upregulated (red) and downregulated (blue) interferon-responsive genes among the DEGs are also shown. (C) Hierarchical clustering of the interferon-responsive genes among the DEGs and expression data from all individual goats of both genotypes. Hierarchical clustering was performed using the ward algorithm on log2-normalized fold changes.

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STAT1 mRNA expression levels did not differ between the genotypes. One hour after LPS challenge, the mRNA expression level of interferon-responsive genes increased slightly and the difference between the two genotypes was more pronounced (Fig 4B), with three genes showing a statistically significant difference in expression level (*ISG15* (p-value = 0.049), *IFIT1* (p-value = 0.02), and *MX1* (p-value = 0.019), assessed by multiple t-tests).

Discussion

Similar to observations in transgenic mice [6], goats [36], and cattle [27] with knockout (KO) of *PRNP*, the $PRNP^{Ter/Ter}$ goats display no obvious loss-of-function phenotype [25, 26]. Consequently, only subtle transcriptomic alterations were expected, corroborating data from KO mouse models [37–41]. Accordingly, this study revealed subtle expression differences affecting less than a percent of the expressed genes. However, analysis of the annotated DEGs using the Interferome database [33], identified a distinct expression profile, with 70 percent of the DEGs being classified as interferon responsive, of which several were among the top upregulated genes. Importantly, animals were age-matched and derived from the same research flock. The health status of this herd is frequently monitored and considered excellent. Prior to sampling, animals were assessed clinically by a veterinarian and found healthy, which was also confirmed by hematological analysis in an accompanying study [26]. Furthermore, we were unable to detect any differences in gene expression levels of neither interferons nor IFN signaling components. A flanking gene problem will also be present in the $PRNP^{Ter/Ter}$ goats; however, preliminary data indicate that this is very limited compared to inbred knockout mouse models. In the absence of alternative explanations, we consider the observed gene expression profile to be a true signature of PrP^{C} loss-of-function. It is likely that this profile, which is evident at rest in the outbred and immunocompetent goats, might be even weaker or absent in inbred transgenic mice, housed in pathogen-depleted environments. It is, however, interesting to note that studies of prion disease in mice have revealed a gene expression profile similar to that observed in PrP^{C}-deficient goats. Analysis of transcripts from mouse whole brain throughout the course of experimental CJD revealed an upregulation of several interferon-responsive genes, e.g. *OAS*, *ISG15*, and *IRF*-family members. Importantly, the upregulation of these genes occurred very early in the course of the disease, approximately 50 days before the onset of neuropathological signs and detection of PrP^{Sc} [42]. Similar findings were recently reported in another study of prion-infected mice [43]. In a hamster model of scrapie, several interferon-responsive genes, including those encoding OAS and Mx protein, were upregulated during development of scrapie [44]. In addition, three interferon-responsive genes, assessed by qPCR studies, were moderately upregulated in a hamster model and different mouse models inoculated with scrapie strains [45]. Recently, transcriptomic data from cerebellar organotypic cultured slices infected with prions showed that a slight upregulation of several interferon-responsive genes was evident at 38 and 45 days post infection [46]. It is tempting to speculate that some of the observed gene expression alterations at very early stages of prion disease could, at least partly, reflect induced loss-of-PrP^{C} function, and, thus, explain the similarity with the expression profile reported here. Further investigations are clearly needed to test this hypothesis.

Studies of human SH-SY5Y neuroblastoma cells transfected with human *PRNP* displayed a significantly dampened response (MX2 expression) to a low-level IFN-α stimulation,

Table 1. Mean unique reads of genes related to Interferon signaling from *PRNP*$^{+/+}$ (*n* = 8, ± SEM) and *PRNP*$^{Ter/Ter}$ (*n* = 8, ± SEM) goats.

Gene symbol	Transcript ID	*PRNP*$^{+/+}$	*PRNP*$^{Ter/Ter}$
Interferons			
IFNA-H-like	XM_005683618.1	0.3 ± 0.3	0.0 ± 0.0
IFNB2-like	XM_005702021.1	63.0 ± 5.3	43.4 ± 4.2 *
IFNK	XM_005683589.1	0.1 ± 0.1	0.0 ± 0.0
IFNO1-like	XM_005683620.1	26.5 ± 6.8	19.1 ± 4.9
IFNT2A	XM_005683606.1	0.9 ± 0.4	0.9 ± 0.5
IFNG	XM_005680208.1	38.4 ± 10.9	27.8 ± 4.5
IFNL3	XM_005692539.1	0.1 ± 0.1	0.0 ± 0.0
IFNL4-like	XM_005692540.1	0.5 ± 0.3	0.3 ± 0.2
Interferon receptors			
IFNAR1	XM_005674742.1	11565.5 ± 613.5	11818.3 ± 683.0
IFNAR2	XM_005674684.1	3484.1 ± 245.4	3664.9 ± 188.7
IFNGR1	XM_005684807.1	3056.4 ± 268.9	3772.4 ± 252.6
IFNGR2	XM_005674741.1	7492.8 ± 179.1	8209.5 ± 408.9
IFNLR1	XM_005677011.1	95.5 ± 10.8	122.4 ± 22.4
Interferon signaling components			
JAK1	XM_005678310.1	31579.9 ± 920.9	31909.0 ± 908.7
JAK2	XM_005683698.1	2399.1 ± 109.3	2587.5 ± 84.7
JAK3	XM_005682189.1	11636.9 ± 600.5	9816.8 ± 603.9
TYK2	XM_005682457.1	4528.3 ± 205.6	4775.3 ± 328.4
STAT1	XM_005676277.1	26477.4 ± 2414.9	28314.6 ± 1119.4
STAT2	XM_005680347.1	5548.9 ± 332.1	6363.6 ± 408.4
STAT3	XM_005693850.1	98.5 ± 10.5	92.5 ± 8.2
STAT4	XM_005676278.1	2101.9 ± 158.6	1949.5 ± 120.6
STAT5A	XM_005693847.1	5250.6 ± 172.7	5365.3 ± 194.9
STAT5B	XM_005693846.1	4604.1 ± 137.3	4511.0 ± 155.2
STAT6	XM_005680308.1	15197.3 ± 704.8	15596.6 ± 692.7
IRF1	XM_005682621.1	12308.6 ± 1155.2	10936.5 ± 1329.5
IRF2	XM_005698710.1	624.5 ± 26.6	663.4 ± 17.1
IRF3	XM_005692726.1	1073.9 ± 74.3	1169.1 ± 60.4
IRF4	XM_005696935.1	1482.5 ± 157.3	1379.5 ± 140.9
IRF5	XM_005679456.1	764.9 ± 61.4	811.8 ± 65.6
IRF6	XM_005691036.1	7.3 ± 2.2	5.4 ± 1.9
IRF8	XM_005691907.1	3565.8 ± 219.0	3824.8 ± 210.8
IRF9	XM_005685224.1	205.8 ± 16.7	234.5 ± 28.2
Inhibitors and enhancers			
IRF2BP-like	XM_005686182.1	2686.3 ± 135.5	2794.5 ± 123.7
IRF2BP1	XM_005692789.1	1265.3 ± 33.0	1232.4 ± 32.3
IRF2BP2	XM_005699013.1	8090.8 ± 600.5	8588.9 ± 793.4
PIAS1	XM_005685148.1	1266.8 ± 66.9	1320.0 ± 86.4
PIAS2	XM_005697179.1	390.6 ± 16.9	405.0 ± 19.2
PIAS3	XM_005677741.1	99.1 ± 7.8	99.5 ± 8.1
PIAS4	XM_005682570.1	39.4 ± 2.7	40.0 ± 4.8
SOCS2	XM_005679820.1	0.8 ± 0.4	1.4 ± 0.6
SOCS3	XM_005694412.1	372.0 ± 48.8	346.4 ± 47.5
SOCS4	XM_005685884.1	845.4 ± 24.5	823.3 ± 28.0
SOCS5	XM_005686570.1	1748.9 ± 82.0	1737.6 ± 75.8

(*Continued*)

Table 1. (*Continued*)

Gene symbol	Transcript ID	*PRNP*$^{+/+}$	*PRNP*$^{Ter/Ter}$
SOCS6	XM_005709580.1	137.5 ± 14.4	144.6 ± 14.0
SOCS7	XM_005709575.1	2286.3 ± 193.8	2144.5 ± 198.7
IL18	XM_005689450.1	21.3 ± 4.5	18.9 ± 3.6
PTK2	XM_005688815.1	82.4 ± 11.4	92.1 ± 7.4
PTK2B	XM_005684041.1	99.3 ± 11.6	114.5 ± 17.5

*: p = 0.025

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compared with untransfected cells that are virtually devoid of PrP^{C}. Furthermore, in an independent, parallel study involving older goat kids than those recruited for the RNA seq study, animals were challenged with LPS, which is a potent pro-inflammatory compound. In contrast to mice, which are relatively tolerant towards LPS, goats have a similar sensitivity as humans [34, 35]. In line with data from the present RNA sequencing study, resting state expression levels of interferon-responsive genes in leukocytes were slightly elevated in the *PRNP*$^{Ter/Ter}$ genotype. Interestingly, the expression differences between the genotypes were increased one hour after LPS injection. Apparently, leukocytes without the expression of PrP^{C} upregulated interferon-responsive genes more rapidly than their PrP^{C}-expressing counterparts. The regulation of interferon-responsive genes expression level is multifaceted and tightly controlled at several levels [47, 48], involving receptor downregulation, upregulation of a plethora of inhibitors as well as epigenetic modifications.

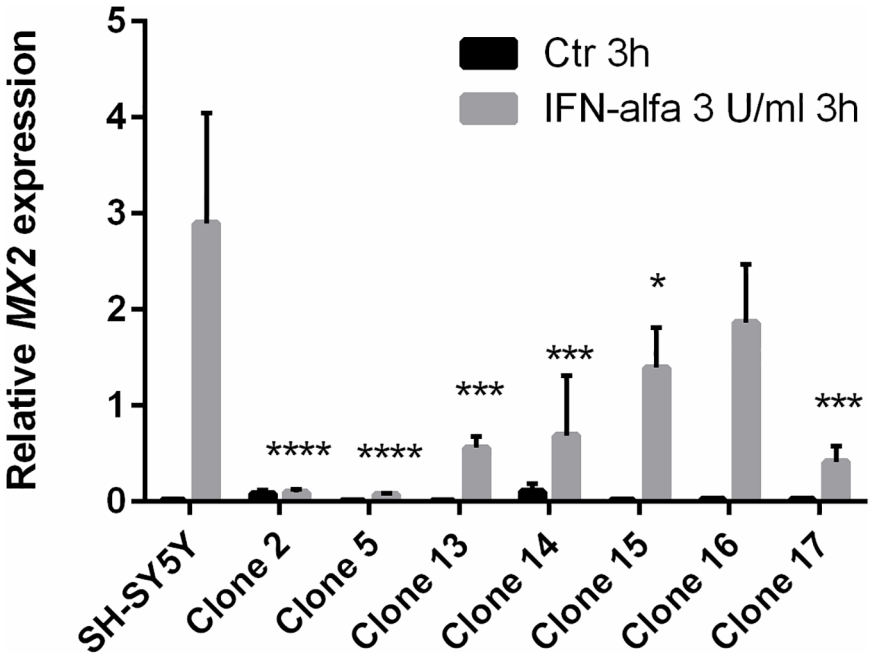

Fig 3. PrP^{C} suppresses upregulation of *MX2* gene expression upon INF-α stimulation in SH-SY5Y cells. Untransfected human neuroblastoma SH-SY5Y cells and seven different clones transfected with a plasmid containing human *PRNP* to produce SH-SY5Y clones expressing human PrP^{C}, were stimulated for 3h with IFN-α (3 U/ml) (mean ± SEM, *n* = 4), and *MX2* gene expression was assessed. Six out of seven clones displayed a significantly lower response to IFN-α compared with the untransfected SH-SY5Y cells, using Dunnett's post hoc test for multiple comparisons.

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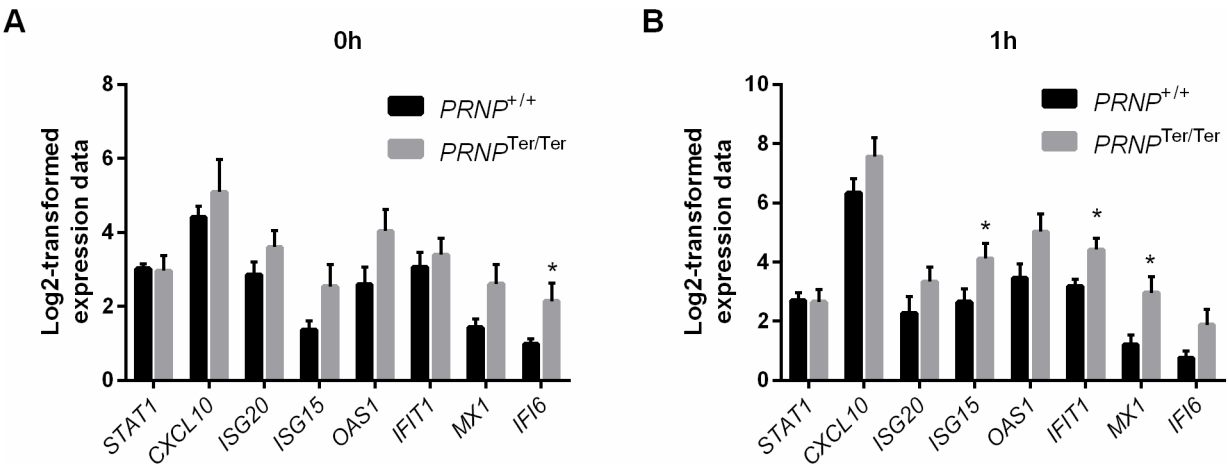

Fig 4. Expression of interferon-responsive genes in blood leukocytes after *in vivo* lipopolysaccharide (LPS) challenge in goats without PrP^C. RNA was extracted from circulating blood leukocytes from both genotypes, and gene expression was analyzed by FLUIDIGM qPCR. (A) Basal expression level (0 h) of selected interferon-responsive genes and *STAT1* in $PRNP^{+/+}$ ($n = 12$) and $PRNP^{Ter/Ter}$ ($n = 13$) animals. (B) Gene expression of interferon-responsive genes and *STAT1* after *in vivo* LPS challenge (1 h) from $PRNP^{+/+}$ ($n = 7$) and $PRNP^{Ter/Ter}$ ($n = 8$) animals. Values are mean ± SEM. Statistical significance is indicated by *, p-value < 0.05, as assessed by multiple t-tests.

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Taken together, our data suggest that PrP^C contributes to dampening of type I interferon signaling at rest and that loss of PrP^C induces a primed state of interferon-responsive genes. Accordingly, direct or indirect stimulation of type I IFN signaling, elicits a somewhat stronger immediate response when PrP^C is absent. These data do not conflict with roles acclaimed to the prion protein. Indeed, they might strengthen previous observations and provide mechanistic hints of PrP^C physiology.

Material and methods

Animals

The animals (FOTS approval number ID 8058) included in the study were of the Norwegian Dairy Goat Breed obtained from a research herd of approximately 100 winter-fed goats at the Norwegian University of Life Sciences. Based on health surveillance through membership in the Goat health monitoring service and The Norwegian Association of Sheep and Goat Farmers and daily monitoring, the general health status of the herd is considered to be good. The entire flock was previously genotyped [25] concerning *PRNP* genotypes, and through selective breeding, goat kids with the two genotypes $PRNP^{+/+}$ ($n = 8$; 4 female and 4 male) and $PRNP^{Ter/Ter}$ ($n = 8$; 4 female and 4 male) were retrieved. Prior to inclusion in the experiment, all goat kids were examined clinically and found to be healthy.

Isolation of peripheral blood mononuclear cells

Blood was sampled from the jugular vein into EDTA tubes at 2–3 months of age. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (Lymphoprep®, Axis-Shield, Dundee, Scotland) at 1760 x *g* without brake, and washed with PBS supplemented with EDTA (2 mM). Red blood cells were lysed by brief exposure to sterile water, and washed with PBS supplemented with EDTA (2 mM) prior to counting and trypan blue viability assessment using a Countess® Automated Cell Counter (Life Technologies, Thermo Fisher Scientific, Waltham, MA).

Cell culture studies

Human neuroblastoma SH-SY5Y cells (Sigma-Aldrich, Merck, Kenilworth, NJ) were cultured in Eagle's Minimum Essential Medium and Ham's F12 (1:1) (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine and antibiotics (1% streptomycin and penicillin) (all from Gibco, Thermo Fisher Scientific), and cultivated in T25 flasks at 37˚C with 5% (v/v) CO_2 at saturated humidity. SH-SY5Y cells were stably transfected with a plasmid construct, pCI-neo (Promega, Madison, WI) encoding human *PRNP*, using jetPRIME (Polyplus, Illkirch, France) according to the manufacturer's instructions. Transfected cells were grown under selection pressure of Geneticin (Thermo Fisher Scientific), and nine different single clones with variable levels of PrP^{C} (SH-SY5Y PrP^{high}) were isolated (S4 Fig). Clone no. 8 showed an abnormal phenotype, and was excluded from the studies.

Western blotting

Untransfected SH-SY5Y cells and transfected SH-SY5Y PrP^{high} clones were lysed in homogenizer buffer (Tris HCl 50 uM, NaCl 150 mM, EDTA 1 mM, DOC 0.25%, NP40 1%) supplemented with protease inhibitor cocktail (Roche complete, Roche Holding AG, Basel, Switzerland). Protein concentrations were measured using Protein assay (Bio-Rad, Hercules, CA). To obtain deglycosylated protein, 20 μg of total protein were incubated overnight with PNGase-F (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions.

Fifty μg of protein or the deglycosylated samples were separated on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12% Criterion™ XT Bis-Tris, Bio-Rad), and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Little Chalfont, United Kingdom). After incubation with blocking buffer (5% non-fat milk in TBS-Tween) for 90 minutes at room temperature, samples were incubated in 1% non-fat milk in TBS-Tween containing mouse anti-PrP^{C} primary antibody diluted 1:4000 (6H4, Prionics, Thermo Fischer Scientific) over-night at 4˚C. Subsequently, the membrane was washed and incubated for 90 minutes in 1% non-fat milk containing Alkaline Phosphatase (AP)-conjugated anti-mouse IgG diluted 1:4000 (Novex, Life Technologies, Thermo Fischer Scientific). Membrane was developed using EFC™ substrate (GE Healthcare) and visualized with Typhoon 9200 (Amersham Bioscience, GE Healthcare).

Isolation and sequencing of RNA

Total RNA was extracted using the Qiagen RNeasy mini plus kit (Qiagen, Germantown, MD) following the manufacturer's instructions. RNA concentration and purity was analyzed using NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific) or Epoch Microplate Spectrophotometer (BioTek Instruments Inc, Winooski, VT), and quality was assessed before RNA sequencing using RNA Nano Chips on an Agilent 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA). RNA was stored at -80˚C. Individual RNA samples of high quality (RIN $\geq$ 9.8) were sequenced by mRNA poly-A-tail, paired-end sequencing (Illumina HiSeq 2000) with 91 bp read-lengths (Beijing Genomics Institute (BGI), Hong Kong), retrieving a minimum depth of 5G clean data per sample. In detail, after the total RNA extraction and DNase I treatment, magnetic beads with Oligo (dT) were used to isolate mRNA. Mixed with the fragmentation buffer, the mRNA was fragmented into short fragments, and cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. The short fragments were connected with adapters. After agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification as templates. During the QC steps, Agilent

2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample library.

For the IFN-studies, RNA quality was assessed by TAE/formamide RNA gel electrophoresis. RNA samples were mixed with formamide (50% v/v, Sigma) and orange loading dye (New England Biolabs), denatured by heating for 5 min at 65˚C, put on ice, and loaded on 1% agarose gel containing 1xTAE buffer (0.04 M Tris-acetate, 1 mM EDTA) and visualized with SYBR™ Safe (Invitrogen, Thermo Fisher Scientific).

Analysis of RNA sequencing data

Reads were mapped to the goat genome assembly (CHIR_1.0) using SOAP2 [49]. Reads per gene were obtained using SOAP2 and the goat genome annotation (RefSeq, CHIR_1.0). Read counts were normalized to reads per kilobase per million mapped reads (RPKM) [50]. Testing for differentially expressed genes was performed using the function exactTest in edgeR [32].

Expression analysis by reverse transcription (RT) quantitative real-time PCR (qPCR) analysis

cDNA was synthesized using SuperScript III Reverse Transcriptase, RNase Out, dNTP mix and Random Primers (all from Invitrogen, Thermo Fisher Scientific) at the following conditions: 5 min at 65˚C, >1 min on ice, 5 min at 25˚C, 1 h at 50˚C and 15 min at 70˚C.

For the RNA sequencing validation study, qPCR was conducted with LightCycler 480 Sybr Green I Master mix (Roche). cDNA corresponding to 2.5 ng RNA was used per reaction. The samples were run in duplicates in a total volume of 20 μl on a LightCycler 96 System (Roche). Conditions: 5 min at 95˚C; 40 cycles of 10 sec at 95˚C, 10 sec at 60˚C and 10 sec at 72˚C; and melting curve with 5 sec at 95˚C, 1 min at 65˚C and 97˚C. Relative expression levels were calculated using a standard curve generated from one randomly selected animal, run in triplicate, with GAPDH as a reference gene, and one randomly selected animal as a positive control. The average of six $PRNP^{Ter/Ter}$ animals was divided by the average of six $PRNP^{+/+}$ animals, and compared relative to RNA sequencing data.

For the interferon-treatment studies using SH-SY5Y cells, qPCR was conducted with LightCycler 480 Sybr Green I Master mix (Roche). cDNA corresponding to 10 ng RNA was used per reaction. The samples were run in triplicate in a total volume of 10 μl on a LightCycler 96 System (Roche). Conditions: 5 min at 95˚C; 40 cycles of 10 sec at 95˚C, 10 sec at 60˚C and 10 sec at 72˚C; and melting curve with 5 sec at 95˚C, 1 min at 65˚C and 97˚C. Relative expression levels were calculated using the ΔΔCt method. ActB was used as a reference gene. An inter-run calibrator was included on every plate as a positive control. The qPCR-amplified sample was run on a 1% agarose gel, and visualized using SYBR™ Safe (Thermo Fisher Scientific).

LPS challenge and FLUIDIGM qPCR of whole blood leukocyte interferon-responsive genes

An intravenous LPS challenge was performed (0.1 μg/kg, *Escherichia coli* O26:B6) in 16 Norwegian dairy goats age 6–7 months (*8* $PRNP^{+/+}$ (female) and 8 $PRNP^{Ter/Ter}$ (7 female, 1 castrated male)) (FOTS approval number IDs 5827, 6903, and 7881), and 10 controls were included (5 of each genotype). In brief, blood samples were collected in PAX-gene blood RNA tubes before (0 h) and after LPS challenge (1 h). High quality RNA (RIN 9.0 ± 0.34) was extracted using the PAXgene Blood miRNA kit, and cDNA synthesis was performed in two replicates (QuantiTect Reverse Transcription Kit). The relative expression of ISGs in

circulating leukocytes was assessed after qPCR on the Fluidigm Biomark HD platform and data analysis using GenEx5 software (MultiD, Sweden). The full study protocol, method description, and primer sequences can be found in [34, 35].

Statistical analysis

Multiple *t*-tests or two-way ANOVA followed by Dunnett's post hoc test for multiple comparisons were used for statistical analysis of the data using Graph Pad Prism v. 6.07 (Graphpad, La Jolla, CA). For correlation analysis, the Pearson correlation coefficient was calculated. Mean values are presented ± SEM.

Ethics statement

The animal experiments were performed in compliance with ethical guidelines, and approved by the Norwegian Animal Research Authority (FOTS approval number IDs 8058, 5827, 6903, and 7881) with reference to the Norwegian regulation on animal experimentation (FOR-2015-06-18-761).

Supporting information

S1 Fig. Individual number of reads obtained from RNA sequencing. Total reads, total mapped reads and uniquely mapped reads across all samples, $n = 16$, 8 of each genotype.
(TIF)

S2 Fig. Hierarchical clustering dendrogram. Hierarchical clustering dendrogram of all genes after normalization of expression data (RPKM) using Euclidean distance and complete linkage.
(TIF)

S3 Fig. Chromosomal distribution of differentially expressed genes. (A) Frequency of differentially expressed genes (735 genes) per chromosome. Total number of genes per chromosome were obtained from National Center for Biotechnology Information (NCBI), based on the Capra hircus CHIR_1.0-Primary Assembly. (B) Chromosomal distribution of annotated differentially expressed genes (86 genes).
(TIF)

S4 Fig. Clones of human neuroblastoma SH-SY5Y cells expressing human *PRNP*. Protein expression of PrP^C for untreated and PNGase-F-treated untransfected human neuroblastoma SH-SY5Y cells and SH-SY5Y clones transfected with human *PRNP* ($n = 8$), determined by Western Blot analysis using 6H4 mouse anti-PrP^C as the primary antibody. Protein bands correspond to glycosylated PrP^C, deglycosylated PrP^C and PrP^C C1 fragment as indicated.
(TIF)

S1 Table. Forward and reverse primers used for qPCR.
(DOCX)

S2 Table. Differentially expressed genes between $PRNP^{Ter/Ter}$ ($n = 8$) and $PRNP^{+/+}$ ($n = 8$) goats (127 genes).
(DOCX)

S1 File. Differentially expressed genes (735 genes).
(XLSX)

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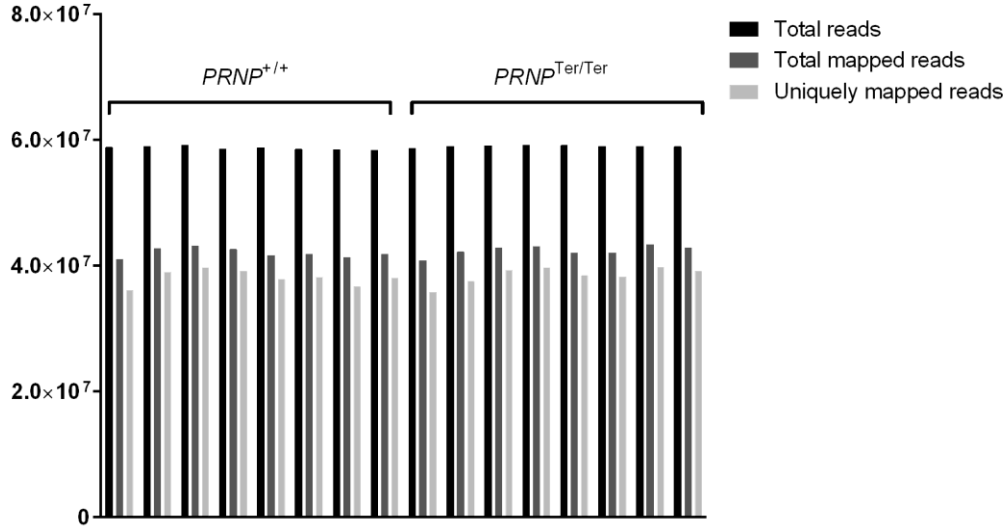
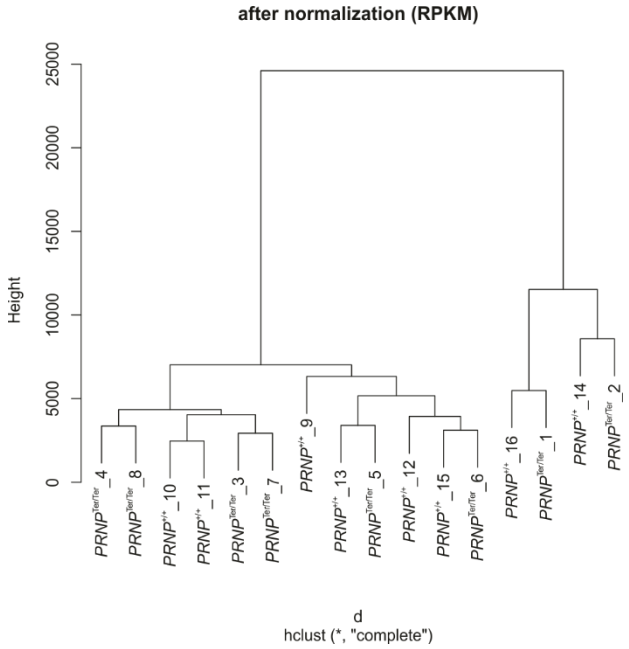
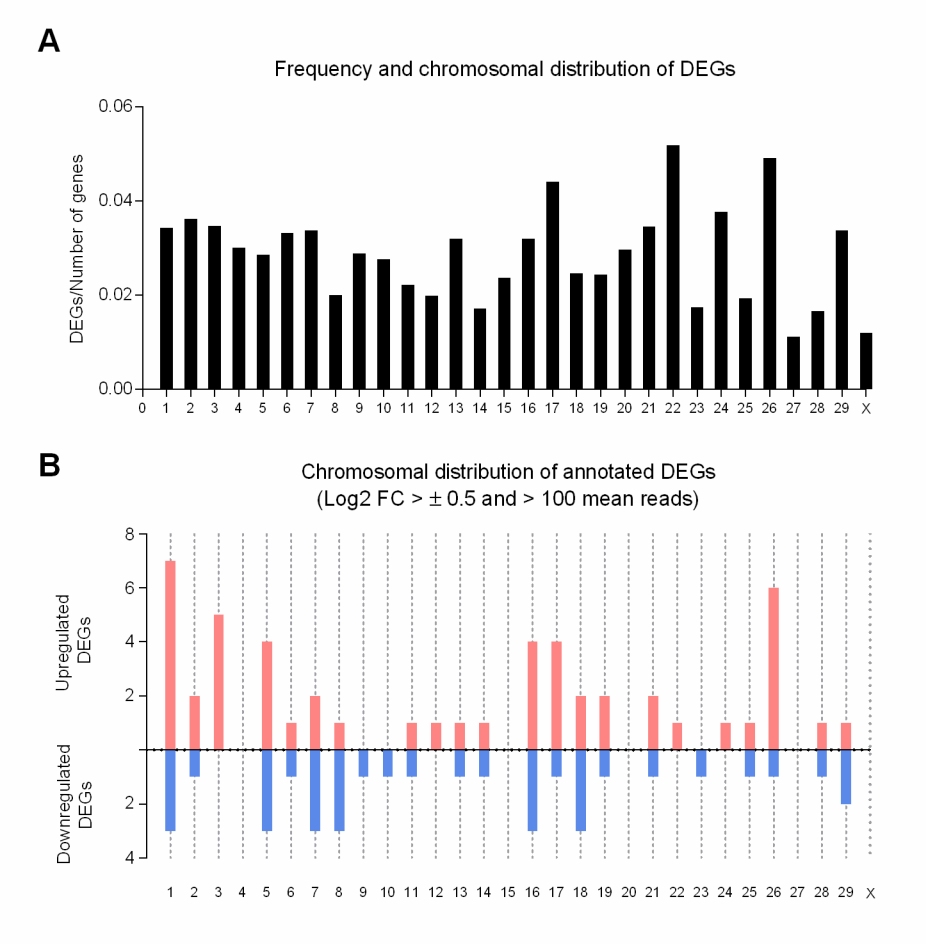
Supplementary figure 1



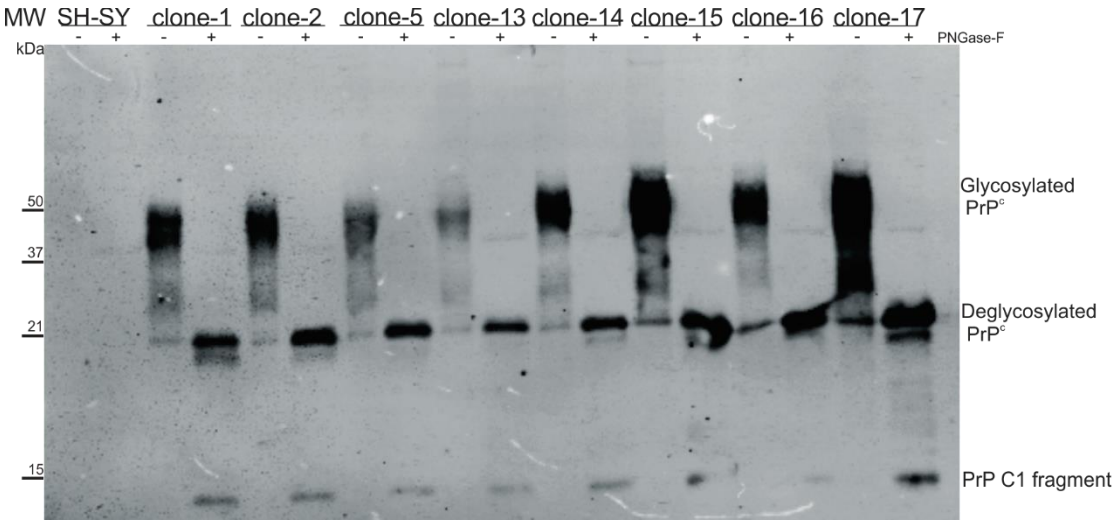
Supplementary figure 2



Supplementary figure 3



Supplementary figure 4



S1 Table: Forward and reverse primers used for qPCR

Gene symbol	Species	Forward 5' to 3'	Reverse 5' to 3'
ACPL2	Goat	TTCTAAAAGGCGCACGATGC	TTCTGGCCTTTGCAAACCAC
ActB	Human	CCGCGAGAAGATGACCCAGAT	GGATAGCACAGCCTGGATAGCA
CD96	Goat	ATGCATTTGGTCAGGGGAGTAG	AGCCAGGCAAAGCGTAAATG
CSPG4	Goat	ATGTTCAGCGTCATCATCCC	TGCCTGTCTTGTTGCGTTTG
ESPN	Goat	AAAGTGCCCAAGCTGTTGAC	AGAGCTCTGCACACTTTGAC
FMNL2	Goat	TGCCGAAACCAAGAATGCAG	TGTGTCCTGCAGTTTTTCCG
GAPDH	Goat	GGTTGTCTCCTGCGACTTCA	TGGAAATGTGTGGAGGTCGG
GZMM	Goat	TGCACCGACATCTTCAAACC	GCTGCACAATGCTCCTTAGAAC
IFI6	Goat	TATCGCTGTTCCTGTGCTACC	AAGCTCGAGTCGCTGTTTTC
KLRK1	Goat	ATGGAACCTGTGCAGTCTATGG	TGGCGAATGGCTTTTGAGTC
LY6E	Goat	AAGCAAAGCAACTGGGACTG	CAAGTTCACCACGTTCTTGAGG
MX2	Goat	TTCACGGAAACCAGCAAACG	TGCATCATGGCTTTCTGCAC
MX2	Human	AGAGGCAGCGGAATCGTAAC	GGTGTTCCGGTAGCTGATCC
PRF1	Goat	ACCATCGTTCAAGGCATGTG	ACCATCGACATTGGAATGGC
PRNP	Goat	GTGGCTACATGCTGGGAAGT	AGCCTGGGATTCTCTCTGGT
PRNP	Human	CTGCTGGATGCTGGTTCTCT	GTGTTCCATCCTCCAGGCTT
TXNDC5	Goat	AAGTTTTACGCGCCATGGTG	AGTACTTGCTGCAGAGGTTCC

S2 Table: Differentially expressed genes between *PRNP*$^{Ter/Ter}$ (*n* = 8) and *PRNP*$^{+/+}$ (*n* = 8) goats

Gene symbol*	Transcript ID	Log2FC	Ratio	Description
ACPL2	**XM_005675502.1**	**0,80**	**1,74**	**acid phosphatase-like 2**
ADAMDEC1	**XM_005684016.1**	**-1,32**	**0,40**	**ADAM-like, decysin 1**
ADRA2A	**XM_005698608.1**	**0,87**	**1,82**	**adrenoceptor alpha 2A**
AGRN	**XM_005690869.1**	**0,88**	**1,84**	**agrin**
AHNAK2	**XM_005695474.1**	**0,77**	**1,71**	**AHNAK nucleoprotein 2**
ANKRD45	**XM_005690886.1**	**-0,82**	**0,57**	**ankyrin repeat domain 45**
ANO9	**XM_005700107.1**	**-0,61**	**0,66**	**anoctamin 9**
AQP3	**XM_005684063.1**	**-0,59**	**0,67**	**quaporin 3 (Gill blood group)**
ATP8B3	**XM_005682922.1**	**0,73**	**1,66**	**ATPase, aminophospholipid transporter, class I, type 8B, member 3**
BAC7.5	XM_005696009.1	-2,01	0,25	Bac7.5 protein
C1R	**XM_005680921.1**	**0,73**	**1,66**	**complement component 1, r subcomponent, transcript variant X2**
C3H1orf162	XM_005677896.1	-0,61	0,66	chromosome 3 open reading frame, human C1orf162
CASP7	**XM_005698497.1**	**0,65**	**1,57**	**caspase 7, apoptosis-related cysteine peptidase, transcript variant X1**
CCDC14	**XM_005675042.1**	**0,54**	**1,45**	**coiled-coil domain containing 14**
CCDC67	**XM_005699410.1**	**-0,69**	**0,62**	**coiled-coil domain containing 67**
CCDC8	**XM_005709596.1**	**-1,47**	**0,36**	**coiled-coil domain containing 8**
CCL5	**XM_005693201.1**	**-0,86**	**0,55**	**chemokine (C-C motif) ligand 5**
CD69	**XM_005680868.1**	**0,69**	**1,61**	**CD69 molecule**
CD96	**XM_005674903.1**	**-0,65**	**0,64**	**CD96 molecule**
CDH17	**XM_005689238.1**	**-0,96**	**0,51**	**cadherin 17, LI cadherin (liver-intestine)**
CDHR5	**XM_005700099.1**	**0,59**	**1,51**	**cadherin-related family member 5**
CDS1	**XM_005681958.1**	**-0,96**	**0,51**	**CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1**
CLEC4E	**XM_005680910.1**	**1,54**	**2,90**	**C-type lectin domain family 4, member E**
CLU	**XM_005683560.1**	**-0,72**	**0,61**	**clusterin**
CMPK2	**XM_005687096.1**	**0,60**	**1,51**	**cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial**
CRISPLD2	**XM_005691843.1**	**0,93**	**1,91**	**cysteine-rich secretory protein LCCL domain containing 2**
CRYBB1	**XM_005691704.1**	**-0,54**	**0,69**	**crystallin, beta B1**
CSPG4	**XM_005695190.1**	**1,03**	**2,04**	**chondroitin sulfate proteoglycan 4**
DDX58	**XM_005683566.1**	**1,16**	**2,24**	**DEAD (Asp-Glu-Ala-Asp) box polypeptide 58**
DHDH	**XM_005692697.1**	**-0,77**	**0,59**	**dihydrodiol dehydrogenase (dimeric)**
DOCK6	**XM_005682467.1**	**0,83**	**1,77**	**dedicator of cytokinesis 6**
DRAM1	**XM_005680638.1**	**0,60**	**1,52**	**DNA-damage regulated autophagy modulator 1**
DTWD2	**XM_005682735.1**	**-0,85**	**0,56**	**DTW domain containing 2**
EHHADH	**XM_005675175.1**	**0,51**	**1,42**	**enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase**
EPB41L3	**XM_005697125.1**	**0,70**	**1,62**	**erythrocyte membrane protein band 4.1-like 3**
EPSTI1	**XM_005687584.1**	**0,64**	**1,56**	**epithelial stromal interaction 1 (breast)**
ESPN	**XM_005690834.1**	**-0,64**	**0,64**	**espin**
F2R	**XM_005685292.1**	**-0,74**	**0,60**	**coagulation factor II (thrombin) receptor**
FCRL2	**XM_005677296.1**	**0,63**	**1,54**	**Fc receptor-like 2**
FMNL2	**XM_005676239.1**	**0,78**	**1,72**	**formin-like 2**
FMO2	**XM_005690635.1**	**1,11**	**2,16**	**flavin containing monooxygenase 2 (non-functional)**
FRMD4B	**XM_005695748.1**	**0,76**	**1,69**	**FERM domain containing 4B**
GZMM	**XM_005682880.1**	**-0,78**	**0,58**	**granzyme M (lymphocyte met-ase 1)**
HERC5	**XM_005681669.1**	**0,54**	**1,46**	**HECT and RLD domain containing E3 ubiquitin protein ligase 5**
HTRA1	**XM_005698569.1**	**-0,57**	**0,67**	**HtrA serine peptidase 1**
IFI44	**XM_005678196.1**	**0,83**	**1,78**	**interferon-induced protein 44, transcript variant X2**
IFI44	**XM_005678197.1**	**0,73**	**1,66**	**interferon-induced protein 44, transcript variant X3**
IFI44L	**XM_005678249.1**	**0,75**	**1,68**	**interferon-induced protein 44-like**
IFI6	**XM_005676790.1**	**1,11**	**2,16**	**interferon, alpha-inducible protein 6**
IFIT1	**XM_005698194.1**	**0,99**	**1,99**	**interferon-induced protein with tetratricopeptide repeats 1**
IFIT3	**XM_005698195.1**	**1,06**	**2,09**	**interferon-induced protein with tetratricopeptide repeats 3, transcript variant X1**
IFIT3	**XM_005698196.1**	**0,68**	**1,60**	**interferon-induced protein with tetratricopeptide repeats 3, transcript variant X2**
IFIT5	**XM_005698239.1**	**0,73**	**1,66**	**interferon-induced protein with tetratricopeptide repeats 5**
IMPG2	**XM_005674860.1**	**-0,87**	**0,55**	**interphotoreceptor matrix proteoglycan 2**
ISG15	**XM_005690795.1**	**1,69**	**3,23**	**ISG15 ubiquitin-like modifier**
KIAA1324	**XM_005677961.1**	**0,51**	**1,43**	**KIAA1324 ortholog, transcript variant X1**

KLRF1	**XM_005680867.1**	**-1,05**	**0,48**	**killer cell lectin-like receptor subfamily F, member 1**
KLRK1	**XM_005680842.1**	**-0,50**	**0,71**	**killer cell lectin-like receptor subfamily K, member 1**
LAMP3	**XM_005675214.1**	**0,59**	**1,51**	**lysosomal-associated membrane protein 3, transcript variant X1**
HA25	XM_005701684.1	1,24	2,36	HA25 (LOC100860813)
GPR68	XM_005702029.1	-0,59	0,67	ovarian cancer G-protein coupled receptor 1-like (LOC102168821)
CCL5	XM_005699389.1	0,80	1,74	c-C motif chemokine 5-like (LOC102169556)
	XM_005675140.1	0,83	1,78	uncharacterized LOC102170912
FADS2	XM_005699818.1	-0,97	0,51	fatty acid desaturase 2-like (LOC102171133)
APOL3	XM_005701671.1	-0,63	0,65	apolipoprotein L3-like (LOC102171143)
	XR_311005.1	1,21	2,32	uncharacterized LOC102171392
TPCN1	XM_005686411.1	0,81	1,75	two pore calcium channel protein 1-like (LOC102171434)
LYZ1	XM_005680192.1	-0,71	0,61	lysozyme C-1-like (LOC102172037)
	XM_005701454.1	0,86	1,82	SLA class II histocompatibility antigen, DQ haplotype D alpha chain-like (LOC102172887)
	XM_005701898.1	-0,73	0,60	antigen WC1.1-like (LOC102174561)
ZNF347	XM_005692737.1	0,59	1,51	zinc finger protein 347-like (LOC102174966)
HBBC	XM_005689813.1	-1,60	0,33	hemoglobin subunit beta-C-like (LOC102175045)
	XM_005701422.1	-0,89	0,54	BOLA class I histocompatibility antigen, alpha chain BL3-6-like (LOC102176782)
	XM_005701685.1	0,86	1,81	boLa class II histocompatibility antigen, DQB*0101 beta chain-like (LOC102176786)
EMR3	XM_005682432.1	-0,93	0,52	EGF-like module-containing mucin-like hormone receptor-like 3-like (LOC102178529)
C4BPA	XM_005690428.1	0,68	1,60	C4b-binding protein alpha chain-like (LOC102179403)
PRSS2	XM_005679544.1	-0,74	0,60	anionic trypsin-like (LOC102179545)
MYADM	XM_005695019.1	-1,05	0,48	myeloid-associated differentiation marker-like (LOC102179985)
USP41	XM_005680768.1	0,70	1,63	putative ubiquitin carboxyl-terminal hydrolase 41-like (LOC102180290)
	XM_005691749.1	0,52	1,43	uncharacterized LOC102180790
MRP4	XM_005701314.1	-1,76	0,29	multidrug resistance-associated protein 4-like (LOC102181111)
	XM_005701539.1	-0,87	0,55	BOLA class I histocompatibility antigen, alpha chain BL3-7-like (LOC102182025)
	XM_005701270.1	-0,78	0,58	uncharacterized LOC102183501
	XM_005701814.1	-0,66	0,63	antigen WC1.1-like (LOC102183687)
	XM_005696678.1	-0,92	0,53	BOLA class I histocompatibility antigen, alpha chain BL3-7-like (LOC102184105)
KLRD1	XM_005680843.1	-0,66	0,63	natural killer cells antigen CD94-like (LOC102184229)
MRP4	XM_005701542.1	-1,16	0,45	multidrug resistance-associated protein 4-like (LOC102184240)
SERPINB3	XM_005697328.1	-0,81	0,57	serpin B3-like (LOC102184299)
OAS1	XM_005701870.1	1,70	3,24	2'-5'-oligoadenylate synthase 1-like (LOC102185558)
	XM_005701959.1	-1,46	0,36	BOLA class I histocompatibility antigen, alpha chain BL3-7-like (LOC102185917)
	XR_311067.1	-1,85	0,28	uncharacterized LOC102186545, transcript variant X1
LGALS9	XM_005701701.1	1,22	2,33	galectin-9-like (LOC102186681)
DD3	XM_005701156.1	-0,93	0,52	dihydrodiol dehydrogenase 3-like (LOC102187204), transcript variant X1
SIGLEC14	XM_005701965.1	1,06	2,09	sialic acid-binding Ig-like lectin 14-like (LOC102188938)
	XM_005701559.1	-0,95	0,52	antigen WC1.1-like (LOC102190214)
	XM_005696530.1	-0,64	0,64	DLA class II histocompatibility antigen, DR-1 beta chain-like (LOC102190745)
METAP2	XM_005700899.1	0,57	1,49	methionine aminopeptidase 2-like (LOC102190867)
OAS1	XM_005691488.1	1,42	2,68	2'-5'-oligoadenylate synthase 1-like (LOC102190983), transcript variant X2
LY6E	**XM_005688801.1**	**0,70**	**1,62**	**lymphocyte antigen 6 complex, locus E (LY6E)**
MARK1	**XM_005690579.1**	**0,58**	**1,50**	**MAP/microtubule affinity-regulating kinase 1 (MARK1)**
MX1	**XM_005675747.1**	**1,09**	**2,13**	**myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (MX1)**
MX2	**XM_005675746.1**	**1,50**	**2,82**	**myxovirus (influenza virus) resistance 2 (mouse) (MX2)**
NOMO1	**XM_005697968.1**	**0,54**	**1,45**	**NODAL modulator 1 (NOMO1)**
NT5E	**XM_005684823.1**	**-0,98**	**0,51**	**5'-nucleotidase, ecto (CD73) (NT5E)**
OAS1	**XM_005709622.1**	**1,68**	**3,20**	**2'-5'-oligoadenylate synthetase 1, 40/46kDa (OAS1)**
OAS3	**XM_005709604.1**	**1,65**	**3,13**	**2'-5'-oligoadenylate synthetase 3, 100kDa (OAS3)**
PARPBP	**XM_005680530.1**	**-0,62**	**0,65**	**PARP1 binding protein**
PCOLCE	**XM_005697822.1**	**-0,55**	**0,68**	**procollagen C-endopeptidase enhancer**
PIGR	**XM_005690418.1**	**-0,84**	**0,56**	**polymeric immunoglobulin receptor**
PLAU	**XM_005699221.1**	**0,57**	**1,48**	**plasminogen activator, urokinase**

PRF1	**XM_005699151.1**	**-0,61**	**0,65**	**perforin 1 (pore forming protein)**
PRNP	**XM_005688157.1**	**-3,68**	**0,08**	**prion protein**
RHCG	**XM_005694970.1**	**-1,95**	**0,26**	**Rh family, C glycoprotein**
RPL35A	**XM_005675050.1**	**0,65**	**1,57**	**ribosomal protein L35a, transcript variant X2**
SEZ6L	**XM_005691699.1**	**1,90**	**3,73**	**seizure related 6 homolog (mouse)-like (SEZ6L)**
SIGLEC1	**XM_005688201.1**	**0,74**	**1,67**	**sialic acid binding Ig-like lectin 1, sialoadhesin**
SIGLEC14	**XM_005692896.1**	**0,84**	**1,79**	**sialic acid binding Ig-like lectin 14**
SLC27A5	**XM_005693070.1**	**-1,81**	**0,29**	**solute carrier family 27 (fatty acid transporter) member 5**
STS	**XM_005701386.1**	**-0,77**	**0,59**	**steroid sulfatase (microsomal), isozyme S**
SULT1C4	**XM_005686652.1**	**-0,54**	**0,69**	**sulfotransferase family, cytosolic, 1C, member 4**
TBC1D16	**XM_005694405.1**	**0,55**	**1,47**	**TBC1 domain family, member 16**
TFCP2L1	**XM_005676324.1**	**-0,73**	**0,60**	**transcription factor CP2-like 1**
TIGIT	**XM_005674945.1**	**-0,59**	**0,67**	**T cell immunoreceptor with Ig and ITIM domains**
TJP3	**XM_005682657.1**	**-0,61**	**0,66**	**tight junction protein 3**
TRPV4	**XM_005691683.1**	**0,90**	**1,87**	**transient receptor potential cation channel, subfamily V, member 4**
TXNDC5	**XM_005696892.1**	**-0,53**	**0,69**	**thioredoxin domain containing 5 (endoplasmic reticulum)**
XAF1	**XM_005693425.1**	**0,53**	**1,45**	**XIAP associated factor 1, transcript variant X2**

*Annotated genes are marked with bold letters

Paper III

Stress resilience of spermatozoa and blood mononuclear cells without prion protein

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Abstract

The cellular prion protein PrP^C is highly expressed in neurons, but also present in non-neuronal tissues, including the testicles and spermatozoa. Most immune cells and their bone marrow precursors also express PrP^C. Clearly, this protein operates in highly diverse cellular contexts. Investigations into putative the stress-protective roles for PrP^C have resulted in an array of functions, such as inhibition of apoptosis, stimulation of anti-oxidant enzymes, scavenging roles and a role in nuclear DNA repair. We have studied stress resilience of spermatozoa and peripheral blood mononuclear cells (PBMCs) derived from non-transgenic goats that lack PrP^C (*PRNP*$^{Ter/Ter}$) compared with cells from normal (*PRNP*$^{+/+}$) goats. Spermatozoa were analyzed for freeze tolerance, DNA integrity, viability, motility, ATP levels and acrosome intactness at rest and after acute stress, induced by Cu^{2+} ions, as well as levels of reactive oxygen species (ROS) after exposure to $FeSO_4$ and H_2O_2. Surprisingly, PrP^C-negative spermatozoa reacted similarly to normal spermatozoa in all read-outs. Moreover, *in vitro* exposure of PBMCs to Doxorubicin, H_2O_2 and methyl methanesulfonate (MMS), revealed no effect of PrP^C on cellular survival or global accumulation of DNA damage. Similar results were obtained with human neuroblastoma (SH-SY5Y) cell lines stably expressing varying levels of PrP^C. RNA sequencing of PBMCs (n = 8 of *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$) showed that basal level expression of genes encoding DNA repair enzymes, ROS scavenging and antioxidant enzymes were unaffected by the absence of PrP^C. Data presented here questions the *in vitro* cytoprotective roles previously attributed to PrP^C, although not excluding such functions in other cell types or tissues during inflammatory stress.

Introduction

The cellular prion protein (PrP^{C}) is the substrate for prion propagation in which the protein is misfolded to the pathogenic scrapie conformer (PrP^{Sc}) (1). Neurons have limited capacity to degrade or otherwise dispose safely of PrP^{Sc}, which ultimately causes their demise. Aggregates of PrP^{Sc}, containing infectious prions, in the central nervous system (CNS) and to varying degrees in peripheral organs, are pathognomonic for incurable prion diseases such as Creutzfeldt Jakob disease in humans, scrapie in sheep and chronic wasting disease in deer (2). Understanding the physiological function of PrP^{C} is important for deciphering the pathogenesis of prion diseases and for development of prevention strategies.

During its synthesis, PrP^{C} is translocated into the endoplasmic reticulum and the secretory pathway. It undergoes several post-translational modifications, including attachment of two N-glycans and a C-terminal glycosylphosphatidylinositol (GPI) anchor that ultimately tethers the protein to the outer leaflet of the plasma membrane (3). Many aspects concerning PrP^{C}'s sub-cellular localization, post-translational modifications and participation in various cellular processes are still incompletely understood.

The protein is abundantly present in the central and peripheral nervous system, but also, at lower levels, in most other tissues. The widespread expression of the gene encoding PrP^{C} (*Prnp*) already during embryonal development (4, 5) and in adult animals (6), suggests that it functions in diverse physiological and cellular contexts. Initial analysis of mice with genetic knockout of PrP^{C} ($Prnp^{-/-}$) showed that they developed and remained healthy without displaying any aberrant phenotypes (7, 8), apart from being completely resistant to prion disease (9). Subsequently, a large catalogue of putative PrP^{C} functions has evolved, including that PrP^{C} is cytoprotective (10). Experiments involving hypoxic brain damage (11-15) or severe inflammation, such as experimental autoimmune encephalomyelitis (16, 17) or experimentally induced colitis (18) showed that pathologies were exacerbated in animals without PrP^{C} expression.

By exposing cells to various forms of stress *in vitro*, it has been demonstrated that PrP^C contributes to cellular protection by modulating different pathways. For instance, through inhibition of Bax-mediated apoptosis (19-21), or by stimulation of pro-survival signaling (22, 23) through cell-surface interaction with the extracellular co-chaperone Sti1. It has also been shown that PrP^C can contribute to increased antioxidant defense (24-26) and upon translocation to the cell nucleus to augmented AP endonuclease 1-driven DNA repair (27). There are also examples in the literature of PrP^C conferring variable (28) and even reduced (29) viability under certain conditions of stress. Despite the perplexing pleiotropy in PrP^C functions, several lines of evidence, derived from different experimental modalities, converge in highlighting the importance of the Cu^{2+}-binding N-terminal domain of PrP^C for its protective properties (26, 30, 31). Importantly, this part of PrP^C can be liberated through proteolytic cleavage in response to oxidative stress (32, 33).

PrP^C is present at relatively high levels in the testicles, epididymis and seminal fluid, and at lower levels, on the surface of ejaculated spermatozoa (34-37). It has been observed that spermatozoa derived from *Prnp*$^{-/-}$ mice are highly susceptible to Cu^{2+}-induced oxidative stress compared with wild-type mice (34), suggesting that PrP^C by virtue of its Cu^{2+}-binding properties contributes significantly to the protection of spermatozoa against ROS stress; however, not critical for fertility.

Taken together, the mechanisms of PrP^C-mediated stress protection are incompletely understood and previously assigned stress-protective roles of PrP^C have recently been questioned (reviewed in (38)), pointing to the need for reassessment and cross-validation by newly developed animal models. In the present investigation, we addressed this by examining oxidative and genotoxic stress resilience of ejaculated spermatozoa and circulating mononuclear cells derived from a naturally occurring line of goats that completely lack PrP^C (*PRNP*$^{Ter/Ter}$) in comparison with wild-type goats of the same breed (39). Animals carrying the *PRNP*^{Ter} allele do not display aberrant behavior, such as anxiety, or other clinically recognizable phenotypes. However, detailed analysis at resting state (40, 41) and under inflammatory

stress induced by lipopolysaccharide (LPS) (42) have provided data suggesting that PrP^C has a modulatory role in certain immunological pathways, such as type I interferon signaling.

Material and methods

Animals and sample material

Age- and gender-matched goats of the Norwegian Dairy Goat breed born between February–March 2016, and genotyped as either normal (*PRNP*$^{+/+}$) or PrP^C deficient (*PRNP*$^{Ter/Ter}$), were included in the study. Animals were held in a farmhouse environment and showed no signs of abnormal health issues throughout the sampling period. The study was approved by the Committee on the Ethics of Animal Experiments by The Norwegian Animal Research Authority (ID No. 8058).

Semen collection and cryopreservation

Two groups of bucks, *PRNP*$^{+/+}$ (*n* = 4) and *PRNP*$^{Ter/Ter}$ (*n* = 4) genotypes, with mean age 208 and 223 days respectively, were used. The bucks were housed at the Norwegian sheep and goat breeders AI station at Hjermstad (Norway), and allowed an acclimatization period of 2 weeks. Following a training period, semen samples were successfully collected using an artificial vagina while the bucks were mounting an estrous goat.

The volume of the ejaculates was registered, after which the spermatozoa concentration was quickly assessed by spectrophotometer in order to determine the correct dilution factor to attain a standardized concentration of 800 x 10^6 spermatozoa/ml. The ejaculates were kept at 35 °C for 10 min, before dilution to a final volume of 15 ml using AndroMed® (Minitübe, Tiefenbach, Germany) extender. After 15 min at room temperature, the ejaculates were placed in a water bath at 5 °C and kept at this temperature for 2

hrs, prior to centrifugation at 800 x g for 10 min. Some of the supernatant was carefully removed leaving the final pre-calculated volume. Spermatozoa were re-suspended by gentle mixing before filling into 0.25 ml French mini straws (IMV, L'Aigle, France). The straws were placed on ramps and cryopreserved by a cooling rate of 2 °C/min from +5 to -10 °C and from -10 to -150 °C with cooling rate of 40 °C/min, and thereafter plunged into liquid nitrogen (LN2). The straws were put in goblets and stored in LN2. When semen collection was finalized, the bucks were euthanized by an intravenous injection of pentobarbital (Euthasol vet, Richter Pharma, Austria) and tissue samples were immediately collected and treated as specified for subsequent storage and analysis.

Immunohistochemistry and immunofluorescence of testicle and epididymis

For PrP^{C} detection in the testicle and epididymis, tissues from one buck of each genotype were used. Tissues were snap frozen in liquid nitrogen and stored at -80 °C. Cryosections (12 µm) were taken of frozen tissue samples and the slides allowed to dry before further use. Tissue sections were fixed in formolcalcium prior to antibody labeling. Washing with PBS followed after each step. *PRNP*$^{Ter/Ter}$ tissue functioned as negative control.

For immunohistochemistry (IHC), an Envision anti-mouse kit (Dako, Agilent, Santa Clara, CA) was used for endogenous peroxidase blocking following the manufacturer's procedures. Goat serum was added for Fc blocking prior to incubation with primary antibodies for 45 min. For IHC, anti-prion antibodies 6H4 (mouse IgG1k, Prionics, ThermoFisher Scientific, Waltham, MA) and SAF32 (mouse IgG2b, SpiBio, Bertin pharma, Montigny le Bretonneux, France) were used, while for immunofluorescence (IF), 6H4 only was used. Secondary anti-mouse antibodies from the kit were added for 45 min, and stained with 3,3'-Diaminobenzidine (DAB), before mounting with aqueous medium. All slides were evaluated by standard light microscopy and photos were taken with a Leica EC3 camera (Leica Camera AG, Wetzlar, Germany).

For IF, goat serum was added for Fc blocking prior to incubation with primary antibodies for 3 hrs. 6H4 (mouse IgG1k) was used to detect PrP^C and c-kit/CD117 (rabbit polyclonal, Dako, Agilent) was used to detect germ cells. Secondary antibodies for PrP^C (Alexa 488 IgG1 goat anti-mouse, Molecular Probes, ThermoFisher Scientific) and c-kit (Alexa 594 IgG (H+L) goat anti-rabbit, Molecular Probes, ThermoFisher Scientific) were allowed to incubate for 1 h. ProLong™ Diamond Antifade Mountant with DAPI (ThermoFisher Scientific) was used for mounting and nuclei staining. Fluorescence was visualized with an Axio Imager 2 Research Microscope (Zeiss, Oberkochen, Germany) and images were processed in Zen (Zeiss).

Histochemistry for lipids with Oil Red O

Four bucks of each genotype were investigated. Cryosections from the testicle were fixed by a mixture of 40 % formaldehyde and 70 % ethanol 1:9 v/v for 5 min and stained by a standard protocol (Oil red O, Schmid GMBH & Co). Slides were counterstained with haematoxylin and mounted with aqueous medium before evaluation by standard light microscopy.

Western Blot

To remove seminal plasma prior to analysis, spermatozoa from one straw of each genotype were washed twice in 1 ml of PBS by centrifugation at 800 rpm for 5 min, followed by careful removal of the supernatant. Washed spermatozoa and testicle tissue samples were lysed in homogenizer buffer (Tris HCl 50 µM, NaCl 150 mM, EDTA 1 mM, DOC 0.25 %, NP40 1 %) supplemented with protease inhibitor cocktail 2x (Complete, Roche, Merck Life Science, Darmstadt, Germany). 20 µg of protein, measured by the Protein assay (Bio-Rad, Hercules, CA), were deglycosylated with PNGase-F according to the manufacturer's instructions (New England Biolabs, Ipswich, MA). 20 µg of protein and the deglycosylated samples were mixed with SDS Loading buffer and Sample Reducing agent (Invitrogen, ThermoFisher Scientific), heated for 10 min at 95 °C before separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12 %

150 Criterion gel, Bio-Rad), and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare,
151 Chicago, Il). The membrane was blocked in 5 % milk TBST, and P4 mouse anti-PrP^C antibody (Ridascreen
152 Biopharm, Darmstadt, Germany) was added in 1 % milk TBST (1:100). Secondary anti-mouse antibody
153 conjugated with Alkaline Phosphatase (AP) was used for the detection, developed with Enhanced
154 chemiluminescence (ECL) reagent (GE Healthcare) and Typhoon 9200 (Amersham Bioscience, GE
155 Healthcare).

156 $CuCl_2$ treatment of spermatozoa

157 Two straws from each buck were thawed in a water bath at 35 °C for 30 sec. The pooled straws were
158 aliquoted, and $CuCl_2$ was added and gently mixed. Three concentrations of $CuCl_2$ were used (100, 150 or
159 200 µg/ml), while no addition served as control. A stock solution of 1 mg/ml $CuCl_2$ in PBS was used in
160 combination with pure PBS to obtain the two concentrations. Semen samples were analyzed after 0, 30,
161 60, 90 and 120 min incubation at 35 °C.

162 Plasma membrane and acrosome integrity analysis of spermatozoa

163 Spermatozoa plasma membrane integrity (spermatozoa viability) was assessed using Propidium iodide
164 (PI, L-7011, LIVE/DEAD®Sperm Viability Kit, Molecular Probes, ThermoFisher Scientific) to discriminate
165 between live and dead (PI positive) spermatozoa. The proportion of acrosome reacted/degenerated
166 spermatozoa was identified using the peanut (*Arachis hypogaea*) agglutinin (PNA) lectin conjugated with
167 Alexa Fluor® 488 (PNA-Alexa 488, L21409, Invitrogen, ThermoFisher Scientific). Prior to flow cytometry
168 analysis, the spermatozoa were stained for 10 min at room temperature in a PBS staining solution with a
169 final concentration of ≈ $1.5x10^6$ spermatozoa/ml, 0.47 µM PI and 49 ng/ml PNA. Four replicates of each
170 semen sample were analyzed. The reliability of the PI staining was confirmed in control samples double
171 stained with both PI and SYBR-14 (Molecular Probes, ThermoFisher Scientific). Upon staining, analysis of
172 the spermatozoa was performed using a Cell Lab Quanta TM SC MPL flow cytometer (Beckman Coulter,

Fullerton, USA). The instrument was equipped with a 22 mW argon laser with excitation at 488 nm. Data was analyzed using Cell Lab Quanta SC MPL Analysis software program (Beckman Coulter). To identify the spermatozoa, a combination of electronic volume (EV) and side scatter (SS) signals were used, as described by Standerholen et al. (43). Fluorescence detection and gating of the acrosome intact (AI) and acrosome intact live (AIL) spermatozoa was also performed according to Standerholen et al. (43).

Spermatozoa motility analysis by CASA

Spermatozoa motility analysis was performed using Sperm Class Analyzer (SCA Evolution, version 6.1; Microptic S.L., Barcelona, Spain) CASA system. 3 µl of each sample was loaded into a pre-warmed (37 °C) standardized Leja 4-chamber microscope slide (Leja products, Nieuw-Vennep, The Netherlands) and analyzed using a phase contrast microscope (Nikon Eclipse, Nicon Group, Japan) equipped with Basler digital camera (Basler Vision Technologies, Basler AG, Ahrensburg, Germany). For each semen sample (n = 4), two replicates were analyzed, and for each replicate, 8 microscopic fields were scanned, with a total of at least 500 cells per sample, and mean of the 8 fields was presented. The motility parameters analyzed were total motility and progressive motility. The instrument settings for the analysis were; spermatozoa head area between 25-75 μm^2; frame rate of 25 frames/sec; immotile spermatozoa defined with an average path velocity below 10 µm/sec.

Assessment of ATP content

The ATP content was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). This method was previously adopted for the evaluation of the ATP content in boar semen (44); however, the optimal spermatozoa number for analysis of goat semen was determined in the present study. For preparation of ATP standard curve samples, ATP disodium salt hydrate (A7699-1G, Sigma-Aldrich, Merck Life Science) was prepared in PBS to obtain the following ATP concentrations: 0, 40, 80, 200, 800 and 1000 nM. Prior to analysis, goat semen was diluted to $1.5x10^6$ spermatozoa/ml in PBS,

and 50 µl samples transferred to wells in a white 96-well microtiter plate (NUNC™, ThermoFisher Scientific). Subsequently, 50 µl CellTiter-Glo® Reagent was added to each well and the mixture was gently shaken for 2 min in a rotary shaker to induce cell lysis. After further incubation for 15 min at room temperature, bioluminescence measurement was performed using a FLUOstar OPTIMA multiwell plate reader (BMG LABTECH GmbH, Offenburg, Germany) with MARS data analysis software (Version 1.10, BMG LABTECH GmbH). Software setting was luminescence mode with gain 2900 and measurement time interval 0.5 sec. By use of the ATP standard curve, the bioluminescence value for each sample, measured in relative luminescence units (RLU), was converted to the corresponding ATP value in nM. An average of three replicates was used for statistical analysis.

ROS analysis of spermatozoa

One semen straw from each buck was thawed for 8 sec in a 70 °C water bath. The spermatozoa concentration was measured and the semen was diluted in PBS prior to staining with fluorescence markers to a final concentration of $5x10^6$ spermatozoa/ml. Hoechst 34580 (1.25 µM, Invitrogen, ThermoFisher Scientific) and Mitotracker Orange CMTMRos (MO, 0.15 µM, Invitrogen, ThermoFisher Scientific) were used to eliminate non- spermatozoa events based on DNA and mitochondrial staining, respectively. Propidium Iodide (PI, 5 µg/ml, Invitrogen, ThermoFisher Scientific) was used to discriminate between plasma membrane-intact and degenerated spermatozoa, while CellROX®Deep Red Reagent (CRR, 5 µM, Invitrogen, ThermoFisher Scientific) was used to assess levels of ROS as a measure of cellular oxidative stress.

Basal levels of ROS as well as levels after induction of oxidative stress was assessed, both in duplicate samples. Semen samples were subjected to oxidative stress with 500 µM $FeSO4{\cdot}7H_2O$ and 196 µM H_2O_2 to induce the Fenton reaction. At the same time point, the CRR and the Hoechst were added and the

samples were incubated for 15 min at room temperature, after which, the markers MO and PI were added and the samples were incubated for another 15 min.

The semen samples were analyzed on a Navios flow cytometer (Beckman Coulter) equipped with a 488 nM (blue), a 638 nM (red) and a 405 nM (violet) diode laser. The two markers MO and PI were exited using the blue laser and fluorescence emission was collected using a 560 – 590 BP filter (FL2) and a 680 – 700 nM BP filter (FL4), respectively. Hoechst 34580 was exited using the blue laser and CRR using the red laser, while fluorescence emission was detected using a 530-570 nM BP filter (FL10) and a 650-670 nM BP (FL6), respectively. The instrument was checked daily for optical alignment by running Flow-Check beads (6605359, Beckman Coulter). An unstained semen sample was included as negative fluorescence control. Compensation was performed prior to collection of data with unstained semen samples and samples stained singularly with each fluorescence marker.

The flow cytometry-generated data were analyzed in Navios or FCS express software analysis programs (Beckman Coulter). Computer-defined gates were set in a cytogram of Hoechst versus MO to identify the spermatozoa (Hoechst and MO positive). Spermatozoa viability, defined as spermatozoa with a functional mitochondrial staining (MO-positive) and intact plasma membrane (PI negative), was estimated by use of an MO versus PI cytogram. A cytogram of CRR versus PI was used to determine the proportions of spermatozoa with different levels of ROS within the viable spermatozoa population (i.e. non-viable spermatozoa were excluded, Figure 4).

Collection of PBMCs

Blood samples from animals of both genotypes were collected from the jugular vein into EDTA tubes and kept at room temperature until analysis. PBMCs were harvested and cultured as previously described (40). For the PBMC experiments, cells from individual animals were used as biological replicates. A total of four

240 animals of each genotype were included in the experiments with oxidative stressors, and the experiment
241 was repeated once with four of the same animals, two of each genotype.

242 SH-SY5Y cell culture

243 Human neuroblastoma SH-SY5Y cells (Sigma-Aldrich, Merck Life Science) were cultured and transfected
244 with a plasmid construct encoding human *PRNP* as previously described (41). Cells were allowed to grow
245 for 48 h before stress exposure.

246 Oxidative and genotoxic stress

247 PBMCs and SH-SY5Y cells were exposed to the alkylating agent methyl methanesulfonate (MMS, Sigma-
248 Aldrich, Merck Life Science) (PBMCs: 0.5 mM; SH-SY5Y: 1.5 mM) and doxorubicin (Sigma-Aldrich, Merck
249 Life Science) (PBMCs: 3 µM; SH-SY5Y: 2 µM) to induce DNA damage; methyl groups on nucleophilic sites
250 of DNA bases and double-strand breaks, respectively. To induce oxidative stress, cells were cultured with
251 hydrogen peroxide (H_2O_2, Sigma-Aldrich, Merck Life Science) (PBMCs: 75 µM; SH-SY5Y: 150 µM). MMS
252 was routinely removed from wells after 1 h and new culture media was added for recovery. Cells were
253 incubated in 5 % CO_2, at 37 °C, with their respective stressors for the designated amount of time.

254 Viability of PBMCs

255 To analyze viability after stress exposure (H_2O_2, doxorubicin, MMS) in cells with or without PrP^C, PBMCs
256 (both genotypes: n = 4; $3x10^5$ cells/well) and SH-SY5Y cells (non-transfected and transfected cells: n = 3;
257 10^4 cells/well) were cultured in a 96-well Greiner plate (Sigma-Aldrich, Merck Life Science) for a total of
258 24 hrs.

259 The average survival of the PBMCs from these four animals were included in the analysis. For SH-SY5Y
260 cells, the experiment was repeated three times. Cell survival after 24 hrs was quantified using the Alamar

Blue exclusion method and the fluorescence was read at 495 nm by Cytation 3 reader (BioTek Instruments, Winooski, VT).

To avoid the impact of differences in cell count, all stressed cells were compared to their own control, and expressed as percentage.

PRNP transcriptional expression

PBMCs were added to 6-well plates ($5x10^6$ cells/well) and exposed to MMS, H_2O_2 and doxorubicin, as described above. Cells were harvested after 1, 3, 6 and 24 hrs of exposure (1 h exposure with MMS, with recovery) and washed once with PBS containing 2 mM EDTA.

SH-SY5Y cells were plated and exposed in flasks ($7.5x10^6$ cells/flask), followed by scraping in 1 ml of PBS.

All cell pellets were frozen in -70 °C.

RNA and DNA isolation, RT-qPCR

Total DNA was isolated from PBMCs and SH-SY5Y cells using a DNeasy Blood and tissue kit (Qiagen, Germantown, MD). Total RNA was isolated from frozen cell pellets using an RNeasy mini plus kit (Qiagen), following the manufacturer's instructions and quantified using a NanoDrop-1000 Spectrophotometer (ThermoFisher Scientific), prior to cDNA synthesis using SuperScript™-III reverse transcriptase, dNTPs mix, First-Strand Buffer, DTT, RNAse OUT™ (Invitrogen, ThermoFisher Scientific) and 500 ng of total RNA.

Quantitative PCR was performed using a LightCycler 480 Sybr Green I Master mix (Roche Holding AG, Basel, Switzerland) and run on a LightCycler 96 System (Roche), with the following primers *PRNP* (goat) F: GTGGCTACATGCTGGGAAGT, R: AGCCTGGGATTCTCTCTGGT; *PRNP* (human) F: CTGCTGGATGCTGGTTCTCT, R: GTGTTCCATCCTCCAGGCTT. See Malachin et al. for further details (41).

DNA damage analysis: LC-MS/MS quantification of 8-oxo(dG) and 7-m(dG)

DNA samples were digested by a mixture of nuclease P1 from *Penicillium citrinum* (N8630, Sigma-Aldrich, Merck Life Science), DNaseI (04716728001, Roche) and ALP from *E. coli* (P5931, Sigma-Aldrich, Merck Life Science) in 10 mM ammonium acetate buffer pH 5.3, 5 mM MgCl2 and 1 mM CaCl2 for 30 min at 40 °C. The samples were methanol precipitated, supernatants were vacuum centrifuged at room temperature until dry, and dissolved in 50 µl of water for LC/MS/MS analysis. Quantification was performed with an LC-20AD HPLC system (Shimadzu, Kyoto, Japan) coupled to an AB Sciex API 5000 triple quadrupole (McKinley scientific, Sparta, NJ) operating in positive electrospray ionization mode. The chromatographic separation was performed with the use of a Supelco Ascentis Express C18 2.7 µm 150 × 2.1 mm i.d. column protected with a Supelco Ascentis Express Cartridge Guard Column (both from Ascentis, Sigma-Aldrich, Merck Life Science) with an Exp Titanium Hybrid Ferrule (Optimize Technologies Inc., Oregon City, OR). The mobile phase consisted of A (water, 0.1 % formic acid) and B (methanol, 0.1 % formic acid) solutions. The following conditions were employed for chromatography: for unmodified nucleosides—0.13 ml/min flow, starting at 10 % B for 0.1 min, ramping to 60 % B over 2.4 min and re-equilibrating with 10 % B for 4.5 min; for 8-oxo(dG)—0.14 ml/min flow, starting at 5 % B for 0.5 min, ramping to 45 % B over 8 min and re-equilibrating with 5 % B for 5.5 min. For mass spectrometry detection, the multiple reaction monitoring (MRM) was implemented using the following mass transitions: 252.2/136.1 (dA), 228.2/112.1 (dC), 268.2/152.1 (dG), 243.2/127.0 (dT), 284.1/168.1 [8-oxo(dG)].

Transcriptomics

For transcriptomic analysis, PBMCs were harvested from 8 *PRNP*$^{+/+}$ and 8 *PRNP*$^{Ter/Ter}$ age-matched animals and RNA was isolated. RNA samples of high quality were shipped to Beijing Genomics Institute (BGI), Hong Kong. Paired-end sequencing was conducted on an Illumina HiSeq 2000 with 91 bp read-length, retrieving 5G clean data per sample. Raw data were analyzed using EdgeR (45). For further details of the study protocol and analysis see Malachin et al (41).

Gene expression of enzymes involved in antioxidant defense and DNA damage repair were analyzed, specifically those involved in base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and double-strand break (DSB) repair.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.07 (Graphpad, La Jolla, CA). Statistical significance was evaluated by multiple t-tests using the Holm-Sidak correction and p values < 0.05 were regarded significant.

Results

PrP^C is abundantly expressed in the testicle and present on spermatozoa

To evaluate the physiochemical properties of PrP^C in the testicle and spermatozoa, we performed Western Blot (WB) analysis from *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ bucks (Figure 1A). In *PRNP*$^{+/+}$ samples not treated with PNGaseF, high molecular weight bands were present, apparently dominated by diglycosylated, full-length PrP^C. After PNGase-F digestion, full length PrP^C at 27 kDa and a band of approximately 18 kDa were recovered from both spermatozoa and testicular tissue derived from the *PRNP*$^{+/+}$ animals. The 18 kDa band corresponds well with a C-terminal fragment known as the C2 fragment derived after proteolytic processing of PrP^C in the octapeptide repeated sequence. Two further bands, particularly prominent in the preparations from spermatozoa with corresponding molecular masses at about 22 to 24 kDa, could represent PrP^C truncated from the C-terminus. Further studies are needed to clarify this.

Analysis by IHC on testicles from *PRNP*$^{+/+}$ bucks (Figure 1B, C) showed strong interstitial PrP^C staining in a pattern suggesting that both Leydig cells and connective tissue express PrP^C. Along the basement

membrane of the seminiferous tubules, a distinct PrP^C-negative zone was noted consisting of the basement membrane and probably also the myoid cells. Inside the seminiferous tubules, PrP^C was distributed from the basement membrane through to the lumen in between the spermatogenic cell nuclei that in most developmental stages of the seminiferous cycle (46) was surrounded by a weakly stained or non-stained zone, indicating that the cytoplasm of the immature spermatogenic cells contains little PrP^C. The staining pattern indicated that the Sertoli cells harbor PrP^C in their cytoplasm, including along the rim of basally located vacuoles and in cytoplasmic projections towards the center of the tubuli. PrP^C was not detected by IHC on tissue sections from *PRNP*$^{Ter/Ter}$ bucks (Figure 1D). Immunofluorescent staining for PrP^C and c-kit (CD117) with DAPI for identification of nuclei (Figure 2A-C) confirmed the pattern of PrP^C distribution in the testicles of *PRNP*$^{+/+}$ bucks. The c-kit$^+$ spermatogonia close to the basement membrane showed weak PrP^C immunostaining, while the strongest signals were found in aggregates of small vesicles on the luminal side, likely cytoplasmic projections from early spermatids (cytoplasmic lobes) or residual bodies inside Sertoli cells. Immunolabeling was also seen through the tubular wall between cells to the basement membrane with the conspicuous abluminally located PrP^C-decorated vacuoles (Figure 2G), as also noted by IHC. Histochemical staining with Oil Red O (ORO) suggests that the PrP^C-labelled vacuoles contain lipids, described as a normal constituent of Sertoli cells (Figure 2H,I) (47). Lipid vacuoles were found at various stages. Interestingly, in association with the largest vacuoles, a significant number of small vesicles were present, some even outside the tubule in the basement membrane or the interstitial tissue. In stage 8 (Figure 2H), small vesicular ORO-stained structures were also located among the elongated spermatids, while after the release of the spermatids, in stage 1-2, less prominent ORO staining was present towards the lumen (Figure 2I). In *PRNP*$^{Ter/Ter}$ bucks, PrP^C was not detected, whereas the c-kit labeling (Figure 2D-F) and ORO staining (data not shown) was similar as in the testicles of the *PRNP*$^{+/+}$ bucks. The expression pattern of PrP^C in epididymis was established by IHC and IF (Supplementary figure 1), both showing a strong staining of round basal cells within the epididymal epithelium and interstitial

connective tissue. Smooth muscle cells stained weakly for PrP^C. Tissues from *PRNP*$^{Ter/Ter}$ bucks were negative by both methods.

Spermatozoa from *PRNP*$^{Ter/Ter}$ and *PRNP*$^{+/+}$ bucks show similar stress responses

To assess whether spermatozoa cells suffer from loss of PrP^C during an increase in ROS stress, we analyzed acrosome intactness, semen ATP levels and motility in $CuCl_2$-treated and non-treated spermatozoa from *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ bucks (Figure 3). There was a clear dose- and time-dependent effect of $CuCl_2$ in all experiments.

The acrosome intactness of non-treated spermatozoa did not differ between the genotypes and was consistent at ≈80 % acrosome intact and ≈40 % spermatozoa with intact acrosome live throughout the length of the experiment (120 min, data not shown). Treatment with 100 (Figure 3A), 150 or 200 (data not shown) µg/ml CuCl, on the other hand, resulted in a distinct decline in intact spermatozoa. Importantly, there were no differences between the genotypes (Significance tested by multiple t-test with Holm-Sidak correction). In living spermatozoa, intactness seemed to increase during the first 30 min in both treated and non-treated cells, most likely due to a gradual decrease in permeability that stabilizes during incubation (Figure 3B).

Non-treated spermatozoa cells had a slow decline in ATP with high levels at 120 min (data not shown), and the effect of CuCl treatment was therefore dramatic (Figure 3C). When treated with 100 µg/ml CuCl, all ATP was gone after 60 min (Figure 3C); however, when the dosage was increased to 150 µg/ml the ATP levels were almost not detectable already at 30 min (data not shown).

Similar findings were observed after motility tests. Motile spermatozoa from both genotypes declined slowly during a 120 min interval. The starting motility at time 0 was 30.6 % and 36.0 %, for *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ respectively. $CuCl_2$ severely affected the spermatozoa motility, which dropped to low levels after 60 min (100 µg/ml) (Figure 3D).

373 ROS detection in spermatozoa

374 To evaluate whether spermatozoa without PrP^C react differently to oxidative stress induced by H_2O_2 in
375 combination with $FeSO_4$, we measured the basal ROS levels as well as the ROS levels after induction of
376 oxidative stress (Figure 4). Incubating spermatozoa for 30 min with 500 µM $FeSO4{\cdot}7H_2O$ and 196 µM H_2O_2
377 did not induce cell death as there were no differences in viability before and after exposure (data not
378 shown). A shift in fluorescence signal reflecting different levels of ROS in *PRNP*$^{+/+}$ vs *PRNP*$^{Ter/Ter}$
379 spermatozoa was detected in all regions (Figure 4 C and F). Approximately 51 % of the *PRNP*$^{+/+}$
380 spermatozoa fell into the K region, indicating high ROS levels, as compared to only 11.42 % of the
381 *PRNP*$^{Ter/Ter}$ spermatozoa, after induction of oxidative stress. The results indicate that *PRNP*$^{+/+}$ spermatozoa
382 accumulated slightly higher levels of ROS than *PRNP*$^{Ter/Ter}$ cells but even so, the viability was similar
383 between the genotypes.

384 Comparable viability of peripheral blood mononuclear cells with and without PrP^C expression upon
385 cellular stress exposure

386 Considering that spermatozoa cells are highly specialized cells expressing only limited sets of proteins, we
387 decided to include other cell types in the study of PrP^C's role in *in vitro* stress resilience. Thus, we measured
388 the relative viability 24 hrs after treatment with methyl methanesulfonate (MMS) (1 h with 23 h recovery),
389 H_2O_2 and doxorubicin (both 24 h treatment) in primary peripheral blood mononuclear cells (PBMCs) from
390 both genotypes, and human neuroblastoma cell line SH-SY5Y cells and hu-PrP SH-SY5Y cells expressing
391 moderate levels of PrP^C. Surprisingly, viability was significantly higher in *PRNP*$^{Ter/Ter}$ PBMCs after both H_2O_2
392 and MMS exposure (Figure 5A). With doxorubicin, PBMCs from both genotypes reacted similarly. No
393 significant differences were detected between SH-SY5Y and hu-PrP SH-SY5Y cells (Figure 5B). The *PRNP*
394 mRNA expression levels in PBMCs increased slightly after doxorubicin treatment (Supplementary Figure
395 2A); however, after 24 hrs of doxorubicin, the expression was downregulated. No major regulation of

PRNP expression was observed with the other stressors. In both hu-PrP SH-SY5Y and SH-SY5Y cells, a consistent but moderate upregulation of *PRNP* expression was observed with all stressors (Supplementary Figure 2B-C).

No differences in DNA damage levels after oxidative and genotoxic stress

The 7-m(dG) lesions induced by MMS exposure increased dramatically after MMS treatment in both cell types. Importantly, there were no differences between the genotypes (Figure 6). The accumulation of lesions was most profoundly seen in SH-SY5Y cells, although the levels of 7-m(dG) after 1 h (data not shown) and 24 hrs were similar, indicating that the cells had reached a maximum lesion threshold already after 1 h.

H_2O_2-induced oxidative stress yielded a similar amount of 8-oxoG lesions in both genotypes (Supplementary Figure 3), indicating that the presence of PrP^C is not essential to maintain the DNA damage-repair response. Notably, in PBMCs, the major enzymes involved in base-excision repair (BER), nucleotide-excision repair (NER), double-strand break repair (DSB) and mismatch repair (MMR) displayed no significant difference in mRNA expression levels between the two genotypes (Supplementary Figure 4 A-D). No differences were detected in mRNA expression levels for antioxidant enzymes either (Supplementary Figure 4 E).

Discussion

The expanding literature dealing with putative physiological functions of PrP^C has recently been comprehensively reviewed (38, 48, 49). Some of the methodological challenges inherent to various animal models, particularly concerning genetic confounders have also been elaborated (50). In the current

417 research study we use an excellent animal model for prion research; dairy goats that completely lack PrP^C,
418 caused by a nonsense mutation affecting codon 32 in the PrP^C reading frame (39).

419 The potential value of goats without PrP^C for breeding purposes, specifically to combat scrapie in goats,
420 or for the production of "prion free" bio-products depends not only on the production parameters,
421 general health and fitness of the animals, but also their fertility and reproductive capacity in breeding
422 systems using artificial insemination (AI) with frozen semen. In view of data from mice demonstrating that
423 PrP^C significantly protected spermatozoa against metal (Cu^{2+})-induced oxidative stress (34), we assumed
424 that spermatozoa from bucks homozygous for the *PRNP*^{Ter} mutation would display increased stress
425 sensitivity possibly leading to reduced viability after storage in liquid nitrogen.

426 Analysis of ejaculated spermatozoa and samples from testicles with WB demonstrated that *PRNP*$^{Ter/Ter}$
427 bucks completely lack PrP^C, whereas *PRNP*$^{+/+}$ bucks show a significant presence of PrP^C. The protein is
428 predominantly di-glycosylated, with somewhat heavier molecular masses in the testicle as compared with
429 spermatozoa preparations. Upon deglycosylation, full length PrP^C (27 kDa) and a band with an estimated
430 molecular mass that corresponds to the C2 cleavage product (18 kDa) of PrP^C, are recovered from both
431 preparations. The C2 fragment stems from ß-cleavage of PrP^C, known to be stimulated by oxidative stress,
432 probably cleaving goat PrP^C between His88 and Gly89 (51). Since the mab used here (P4) binds to amino
433 acids 95-105 (52), it detects the C2 fragment of PrP^C, but not the C1 fragment generated by α-cleavage,
434 which most likely occurs around amino acid 115 in goat PrP^C (53, 54). If the 18 kDa band shown here stems
435 from ß-cleavage of PrP^C, it could indicate that this cleavage occurs more frequently in the testicle and
436 spermatozoa, compared to brain, in which the C2 fragment is at low levels. Moreover, it has been shown
437 that PrP^C can be liberated from the cell membrane by cleavage close to the C-terminus, generating a
438 protein species about 4-6 kDa smaller than full length PrP^C due to the loss of the GPI anchor (51, 55). This
439 might be of relevance in interpreting the two distinct bands with molecular mass between 22-24 kDa
440 observed in preparations from spermatozoa. However, as demonstrated in several previous studies (34,

441 56), detailed epitope mapping of PrP^C species from the male genital organs and spermatozoa is
442 challenging, and conflicting data have emerged. Although interesting, solving this task was outside the
443 scope of the current investigations.

444 By IHC and IF, PrP^C was found most abundantly in Sertoli cells, including in large cytoplasmic Oil-Red-O
445 (ORO)-positive lipid vacuoles, particularly prominent in the periphery of the seminiferous tubules. Ford et
446 al (35) showed that an intense PrP^C -positive immunostaining could be detected in lipid droplets shed from
447 the spermatozoa as part of their maturation process. These droplets are phagocytosed by the Sertoli cells
448 and transformed into small phagolysosomal, ORO-positive lipid vesicles (47), along the abluminal aspects
449 of the tubuli in the present study. As the distribution of ORO-positive residual bodies seem to overlap with
450 the granular PrP^C-staining, it is likely that the residual bodies contain PrP^C. Whether PrP^C in residual bodies
451 stems from the phagocytosed spermatid-released cytoplasmic droplets, produced by the Sertoli cell itself,
452 or a combination of the two, remains to be investigated. The large lipid vacuoles in the basal aspect of the
453 Sertoli cells were present in almost all stages of the goat spermatogenic cycle (57) and may stem from the
454 degradation of apoptotic spermatogenic cells (47) and/or residual bodies (58). The latter structures were
455 positive for PrP^C and ORO, and given that the spermatogenic cells were mostly PrP^C negative, a co-
456 transport of PrP^C and lipids from the residual body stage to the larger vacuoles seems possible. Smaller
457 vacuoles, maybe from degradation of the larger vacuoles, were observed to disseminate across the
458 basement membrane, similar to the way immunogenic antigens are transported (59). Studies have found
459 *PRNP* mRNA in several developmental stages of spermatozoa and in Sertoli cells (35, 37).

460 PrP^C has been detected in the Sertoli cells of immature goat bucks (130 days post conception) and in
461 ejaculated buck spermatozoa (60). Levels of *PRNP* mRNA were high in buck testicular tissue from birth
462 and gradually decreased to reach a steady level at puberty. This observation might suggest that PrP^C in
463 the testicle serves roles that are not specific to spermatogenesis. This is in contrast to the prion-like
464 protein Doppel (Dpl), encoded by *Prnd*, which is expressed at low levels in sexually immature bucks, before

raising sharply in expression towards puberty, after which it remains at a high level (36, 61, 62). Interestingly, genetic knockout of *Prnd* renders male mice infertile (63), whereas absence of PrP^C apparently has no direct effect on male fertility, neither in mice nor in goat bucks (7).

It has been proposed that PrP^C is present in spermatocytes and spermatids; although absent in the earlier spermatogonia (36). Our data demonstrate high PrP^C levels in Sertoli cells, raising the intriguing possibility that PrP^C on spermatids could originate from the Sertoli cells. Studies of PrP^C in spermatozoa and in fluids along the ram genital tract revealed that the epididymal fluid contained significant levels of what appeared to be soluble, highly glycosylated forms of the prion protein (64). The authors proposed that some of PrP^C species present on ejaculated spermatozoa could be acquired from the seminal fluid during ejaculation. While our investigations have not focused on seminal plasma or epididymal fluids, IHC and IF analysis of the epididymis showed that PrP^C is present in cells below the columnar epithelium, which itself appeared negative. However, the inter-tubular connective tissue was strongly positive for PrP^C, whereas the smooth muscle stained substantially weaker for PrP^C. Although, these observations do not rule out secretion of PrP^C from the epididymal epithelium, it clearly illustrates that PrP^C appears to serve functions in the epididymis that are unrelated to production and release of PrP^C into the seminal fluid.

Analyses of acrosome intactness, viability, ATP levels and motility after oxidative stress induction with $CuCl_2$ showed no differences between the genotypes in their ability to handle this stressor. The dramatic reduction in parameters are in line with other studies on oxidative stress in spermatozoa (65). Our finding differ from results reported by Shaked and colleagues (34); namely that Cu^{2+} exposure caused motility loss at a faster rate in spermatozoa from *Prnp* KO mice. This apparent discrepancy could be due to different experimental procedures or represent true species differences. For instance, Shaked at al studied spermatozoa extracted from the epididymis, whereas we studied ejaculated spermatozoa that have undergone routine dilutions and freezing in liquid nitrogen. Epididymal mouse spermatozoa are immobile, but motility can be established by dilution in specific buffers (66). In our preparations of spermatozoa that

had undergone cryopreservation, approximately 30-40 % of the spermatozoa were recorded as motile. We observed, as expected, a gradual decline in ATP levels and motility in untreated controls for both genotype groups during the 60 min incubation. From a breeder's point of view, it can be concluded that all fundamental spermatozoa parameters as recorded in cryopreserved semen, appear unaffected by the loss of PrP^C in the goat buck.

In order to explore further *in vitro* stress resilience, we analyzed accumulations of ROS in spermatozoa from $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ bucks. Interestingly, we observed that a larger proportion of viable spermatozoa from $PRNP^{+/+}$ bucks fell into the "high ROS" categories, demonstrating that under these experimental conditions, higher numbers of PrP^C-containing spermatozoa contained higher ROS levels, while still remaining viable. This surprising observation indicates that in mature spermatozoa, PrP^C appears not to contribute to ROS scavenging capacity. This is in contrast to previous observations of increased levels of ROS in various diploid PrP^C-deficient cell lines (67-70).

We wanted to test whether our observation of complete lack of PrP^C-mediated stress protection could be a peculiarity of spermatozoa prepared for AI. In order to achieve this, we exposed goat PBMCs with and without PrP^C and human neuroblastoma SH-SY5Y cells expressing different levels of PrP^C to different types of genotoxic and oxidative stress. Interestingly, upon exposure of cells to doxorubicin, an inducer of DNA double strand breaks, H_2O_2 for induction of ROS stress, and methyl methanesulfonate (MMS) for introduction of methyl adducts, we were unable to detect any stress-protective effect of PrP^C in terms of cellular viability in any of the cell types. Moreover, there was no effect of PrP^C on levels of 7-m(dG), neither before nor after treatment with MMS. Our data appear to be in line with the conclusion drawn by Castle and Gill (38), in their recent review, that a direct stress-protective function for PrP^C remains unproven. In accordance with previous observations, the SH-SY5Y cells increased their expression of endogenous PrP^C in response to severe stress (27). This effect was less consistent in PBMCs, although a clear induction was evident after treatment with doxorubicin. Our data do not provide support for the concept that PrP^C is

513 critically important for DNA repair, as reported by Bravard and colleagues (27). They observed decreased
514 survival of SH-SY5Y cells without PrP^C after treatment with MMS and that DNA lesions were increased in
515 the absence of PrP^C. Further investigations are needed to clarify this issue.

516 Taken together, our data, derived from different cell types and under a variety of stressful conditions,
517 show that *in vitro* there appears to be no stress-protective effects of PrP^C. The findings are in contrast to
518 studies that have shown that PrP^C can positively influence survival of cells exposed to xanthine oxidase
519 (24, 71), H_2O_2 (72, 73), and paraquat (30, 74). It has been suggested that antioxidant enzyme activities
520 such as glutathione reductase (73) or SOD-1 (71) are reduced in the absence of PrP^C. Other studies have
521 not found changes in the enzyme activities of glutathione peroxidase, catalase and Cu/Zn SOD (24). It has
522 also been proposed that PrP^C stimulates the expression of antioxidant enzymes (73, 75) and that this could
523 partly explain the protective effect of PrP^C against oxidative stress. However, RNA sequencing data from
524 PBMCs derived from goats with or without PrP^C do not support this notion, since we were unable to detect
525 any differences between genotypes in expression of major DNA repair enzymes or a panel of enzymatic
526 antioxidants.

527 Interestingly, increased levels of oxidative DNA damage have been detected in cells without PrP^C during
528 normal physiological states (76). Higher levels of oxidated lipids and proteins were reported in the CNS
529 (77, 78) and peripheral structures (77) of *Prnp* KO mice, reflecting a higher oxidative load. These scenarios
530 are likely to have yielded higher levels of enzymes involved in these repair processes (79); however, we
531 were unable to detect any major differences in neither DNA damage-repair enzymes nor enzymatic
532 antioxidants in PBMCs from $PRNP^{+/+}$ and $PRNP^{Ter/Ter}$ animals.

533 In conclusion, our observations of PBMCs, spermatozoa and SH-SY5Y cells after induction of different
534 forms of severe cellular stress suggest that PrP^C is not directly protective against these stressors *in vitro*.

This, however, does not rule out that PrP^C could serve protective functions *in vivo*, particularly during inflammation, as suggested in several studies in mice (11, 17, 18, 80), and goats (42, 81).

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Figure legends

Figure 1: PrP^C is expressed in testicular tissue and spermatozoa

PrP^C was detected in testicle and spermatozoa by Western blot (WB) (A) and Immunohistochemistry (IHC) (B-D) using the P4 and 6H4 antibodies, respectively.

In WB (A), glycosylated full-length PrP^C is detected in both spermatozoa and testicular tissue. After deglycosylation with PNGaseF, several bands are visible on both preparations, including bands of approximately 25 kDa and 18 kDa molecular mass, probably corresponding to full length PrP^C and a C-terminal fragment, respectively. Further bands with apparent molecular mass of 22-24 kDa are particularly prominent in samples from spermatozoa. No PrP^C could be detected in samples from *PRNP*$^{Ter/Ter}$ animals.

By IHC (B: Stage 1, C: Stage 3), a distinct PrP^C staining of seminiferous tubules with positive Sertoli cells, including the rim of vacuoles (white arrowheads) was observed. Spermatogonia (B, black arrowheads) and spermatocytes (C, black arrowheads) appeared negative for PrP^C. Testicular tissue from *PRNP*$^{Ter/Ter}$ bucks (D) are completely unstained, but similar in architecture and the presence of vacuoles (white arrowheads). The basement membrane of seminiferous tubuli is indicated by dots, and the lumen by asterisk. B-D: x400.

Figure 2: PrP^C is distinctly present in lipid vesicles in seminiferous tubules

Immunofluorescence analysis of testicular tissue demonstrates the presence of PrP^C (A, green), and the stem-cell marker c-kit (B, red), cell nuclei counterstained with DAPI (blue) and merged (C) in *PRNP*$^{+/+}$ bucks. Spermatogenic stem cells positive for c-kit$^+$ were distributed along the basement membrane and appeared to express low levels of PrP^C. However, PrP^C was prominently present in vesicles on the luminal aspect of tubules and in Sertoli cell vacuoles (arrowheads) along the periphery of the tubules, as highlighted by digital magnification (G) of rectangle depicted in A. Both the luminal vesicles and peripheral vacuoles (arrowheads) contain lipids, as shown by ORO-staining (H, I). Testis from *PRNP*$^{Ter/Ter}$ bucks is negative for PrP^C and show similar distribution of c-kit and harbor similar vacuoles (arrowheads) as *PRNP*$^{+/+}$ bucks (D-F). Asterisk: Lumen of seminiferous tubules. x400 (all except G which was digitally magnified to x1000).

Figure 3: Expression of PrP^C does not increase viability upon induced oxidative stress in spermatozoa

Acrosome-intact spermatozoa (A), acrosome-intact viable spermatozoa (B), ATP levels (C) and motility (D) were measured by flow cytometry in thawed spermatozoa from *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ bucks (both *n* = 4) at 0, 30, 60, 90 and 120 min after $CuCl_2$-induced (100 µg/ml) oxidative stress. Values are shown as mean ± SEM. (Significance tested by multiple t-test with Holm-Sidak correction).

Figure 4: ROS levels in spermatozoa indicates no protection from PrP^C

ROS levels were measured by flow cytometry in *PRNP*$^{+/+}$ and *PRNP*$^{Ter/Ter}$ spermatozoa after treatment with H_2O_2 and $FeSO_4$ for 30 min. Plots from controls (A and B) and oxidized (C and D) spermatozoa are shown for both genotypes (A and D: *PRNP*$^{+/+}$, B and E: *PRNP*$^{Ter/Ter}$).

Based on fluorescence intensity, spermatozoa were gated into regions G, H, I and K, of which region K represents spermatozoa with the highest ROS levels.

Mean basal (C) ROS levels and mean ROS levels after H_2O_2 and $FeSO_4$ treatment (F) in both genotypes is shown (*n* = 4). Values are shown as mean ± SEM. ** indicates $p < 0.005$, *** $p < 0.0005$. (Significance tested by multiple t-test with Holm-Sidak correction).

Figure 5: Lack of PrP^C expression does not decrease cellular viability

Peripheral blood mononuclear cells (PBMCs) with and without PrP^C expression, and human neuroblastoma SH-SY5Y cells with very low PrP^C levels (mock-transfected) and moderate PrP^C levels (stably transfected with human PrP^C), were treated for 24 h with doxorubicin (PBMCs 3 µM, SH-SY5Y 2 µM) or H_2O_2 (PBMCs 75 µM, SH-SY5Y 150 µM), or methyl methanesulfonate (MMS) (PBMCs 0.5 mM, SH-SY5Y 1.5 mM) for 1 h, with 23 h recovery. Viability in PBMCs (*n* = 4) (A) and SH-SY5Y cells (*n* = 3) (B) (relative to controls) with and without PrP^C expression after induction of cellular stress with doxorubicin, H_2O_2 and MMS is shown, assessed by the Alamar Blue assay using Cytation 3. Values are given as mean ± SEM. * indicates $p < 0.05$. (Significance tested by multiple t-test with Holm-Sidak correction).

Figure 6: Similar accumulation of the methyl adduct 7-meG in PBMCs and SH-SY5Y cells with and without PrP^C expression after treatment with MMS

PBMCs (*n* = 3-5) (A) and human neuroblastoma SH-SY5Y cells (*n* = 2) (B) with and without PrP^C expression were exposed to MMS for 1 h, with 23 h recovery. Levels of 7-meG were assessed 24 h after exposure. Data are given as mean ± SEM. (Significance tested by multiple t-test with Holm-Sidak correction, all p-values > 0.05).

Figure 1

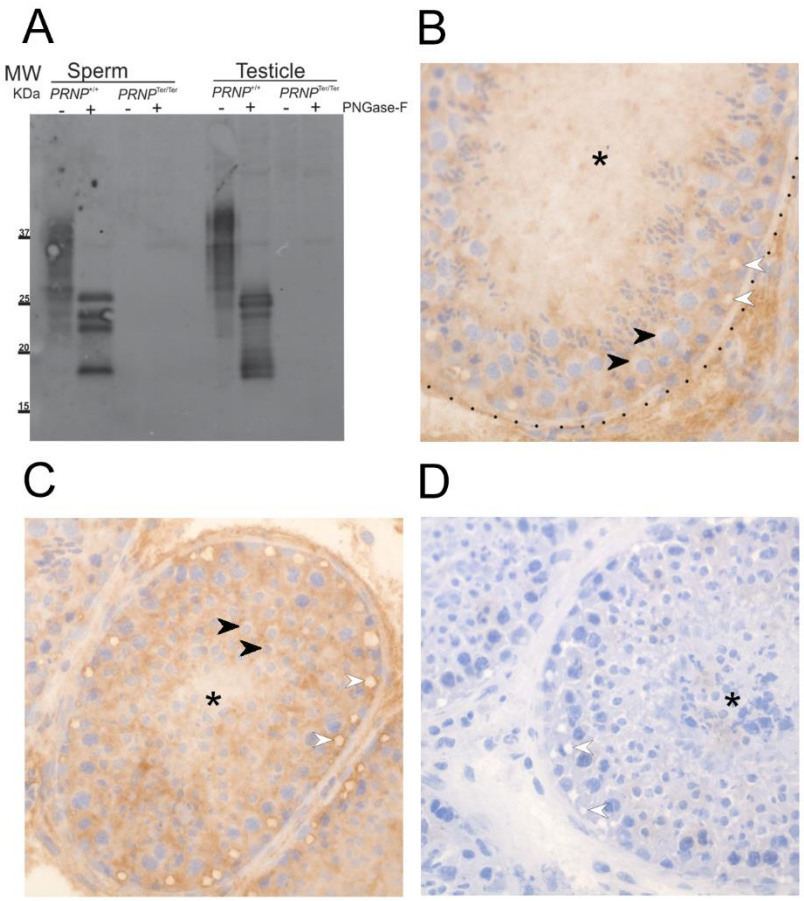

A
B
C
D
MW
KDa
Sperm
Testicle
PRNP+/+
PRNPTer/Ter
PNGase-F
37
25
20
15

Figure 2

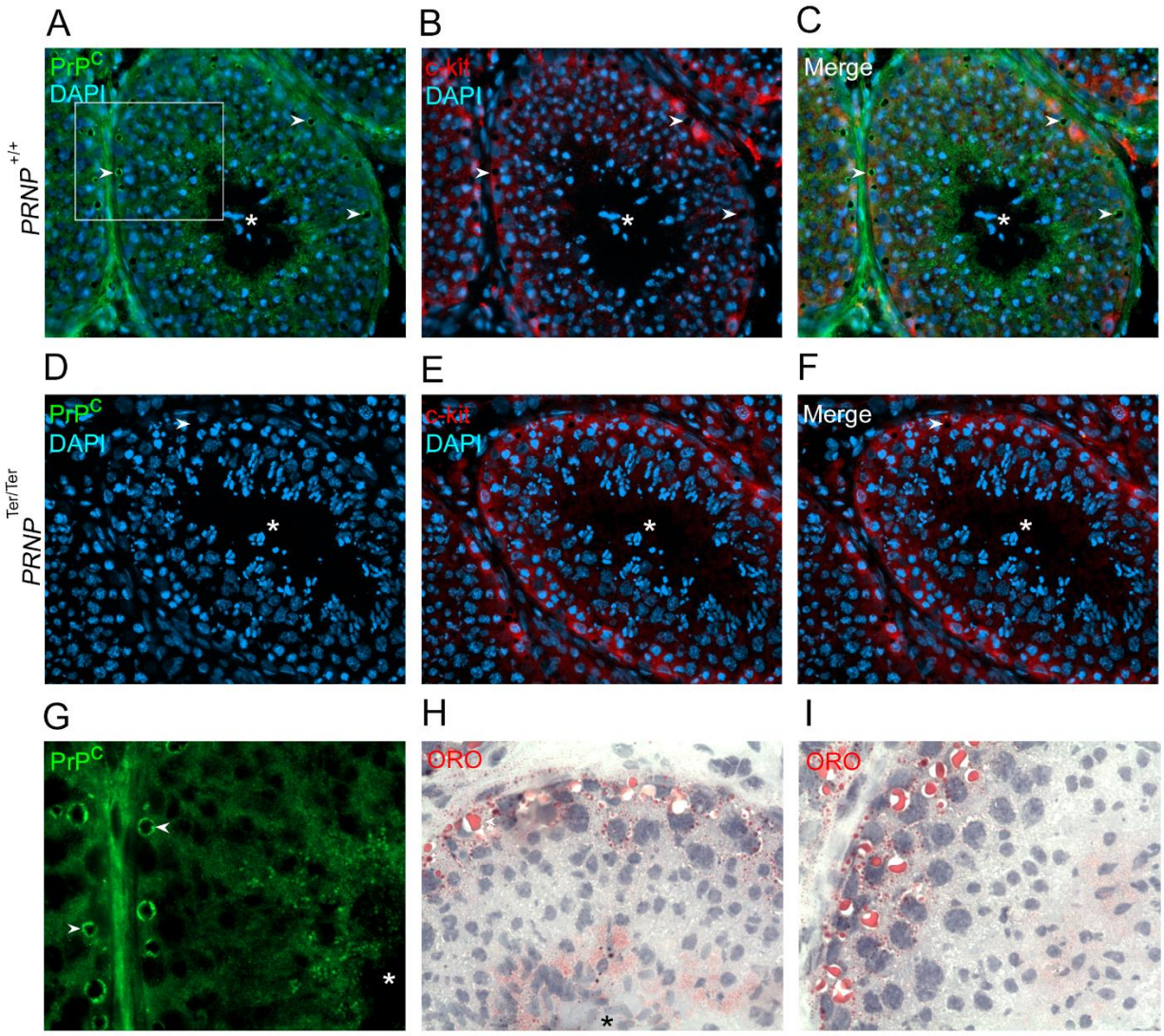

A
B
C
PrPC
DAPI
c-kit
DAPI
Merge
PRNP+/+
D
E
F
PrPC
DAPI
c-kit
DAPI
Merge
PRNPTer/Ter
G
H
I
PrPC
ORO
ORO

Figure 3

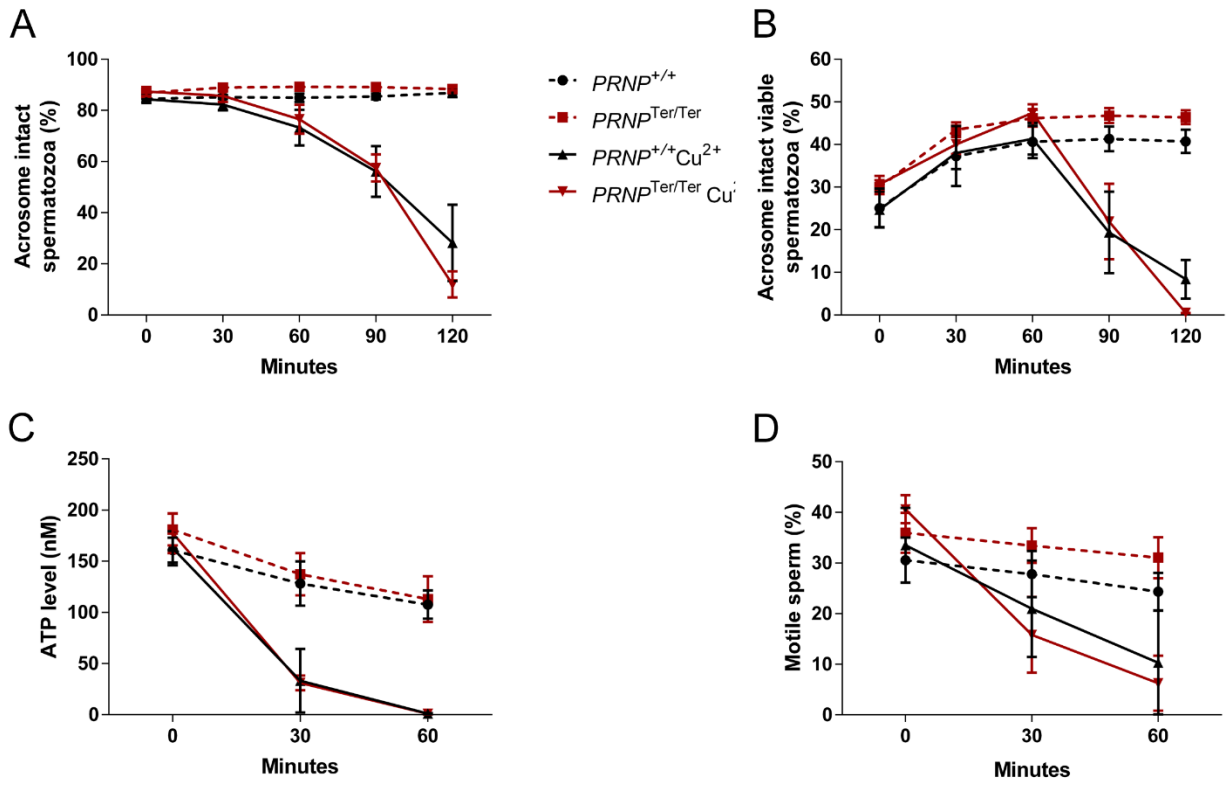

A
Acrosome intact spermatozoa (%)
100
80
60
40
20
0
0
30
60
90
120
Minutes
PRNP+/+
PRNPTer/Ter
PRNP+/+Cu2+
PRNPTer/Ter Cu
B
Acrosome intact viable spermatozoa (%)
60
50
40
30
20
10
0
0
30
60
90
120
Minutes
C
ATP level (nM)
250
200
150
100
50
0
0
30
60
Minutes
D
Motile sperm (%)
50
40
30
20
10
0
0
30
60
Minutes

Figure 4

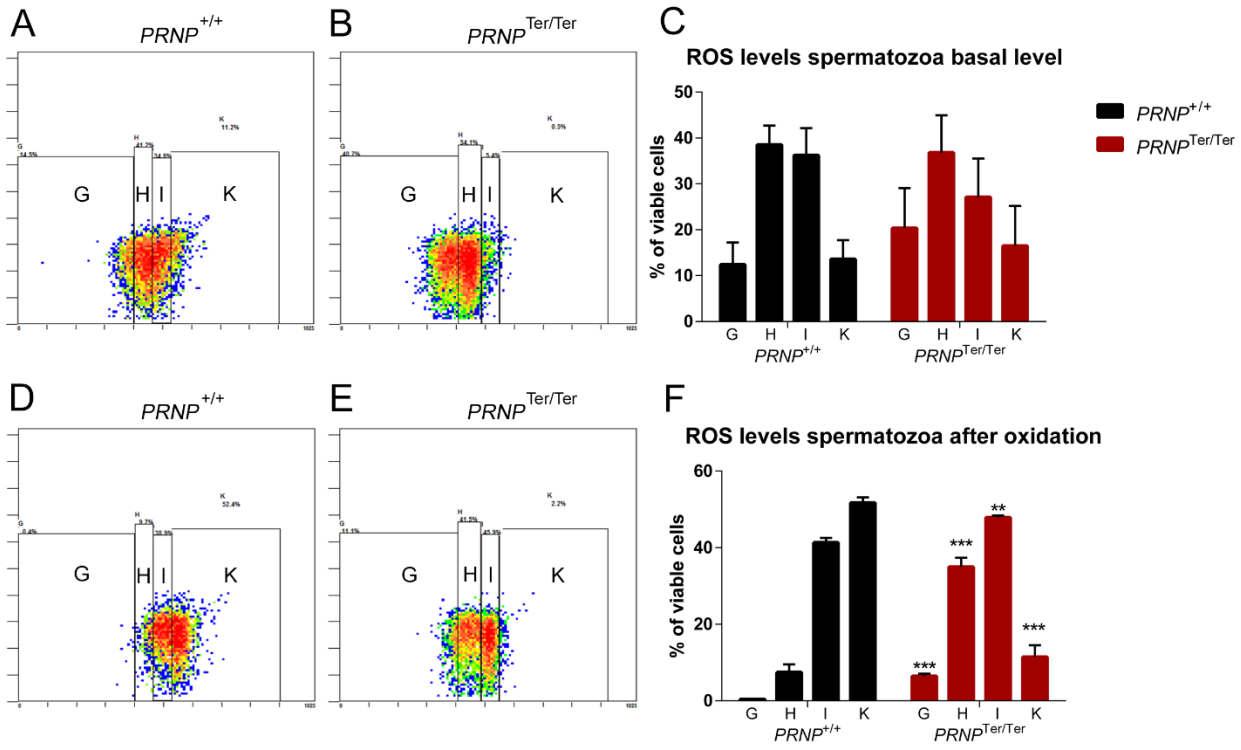

A
PRNP+/+
B
PRNPTer/Ter
C
ROS levels spermatozoa basal level
% of viable cells
PRNP+/+
PRNPTer/Ter
G H I K
D
PRNP+/+
E
PRNPTer/Ter
F
ROS levels spermatozoa after oxidation
% of viable cells
***
**

Figure 5

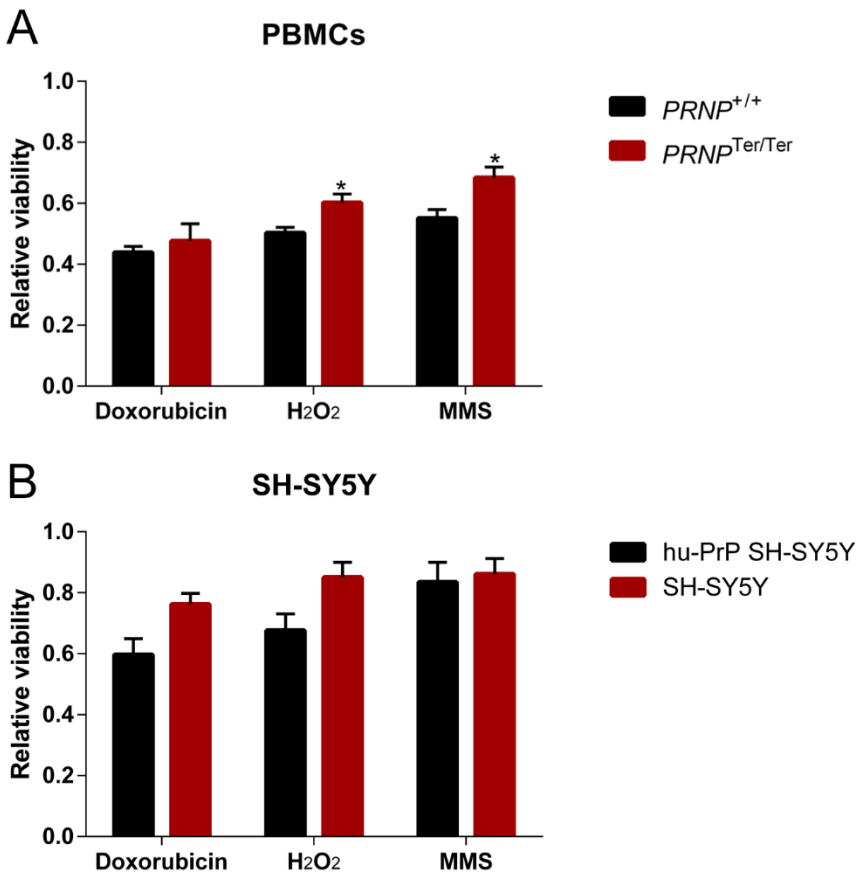
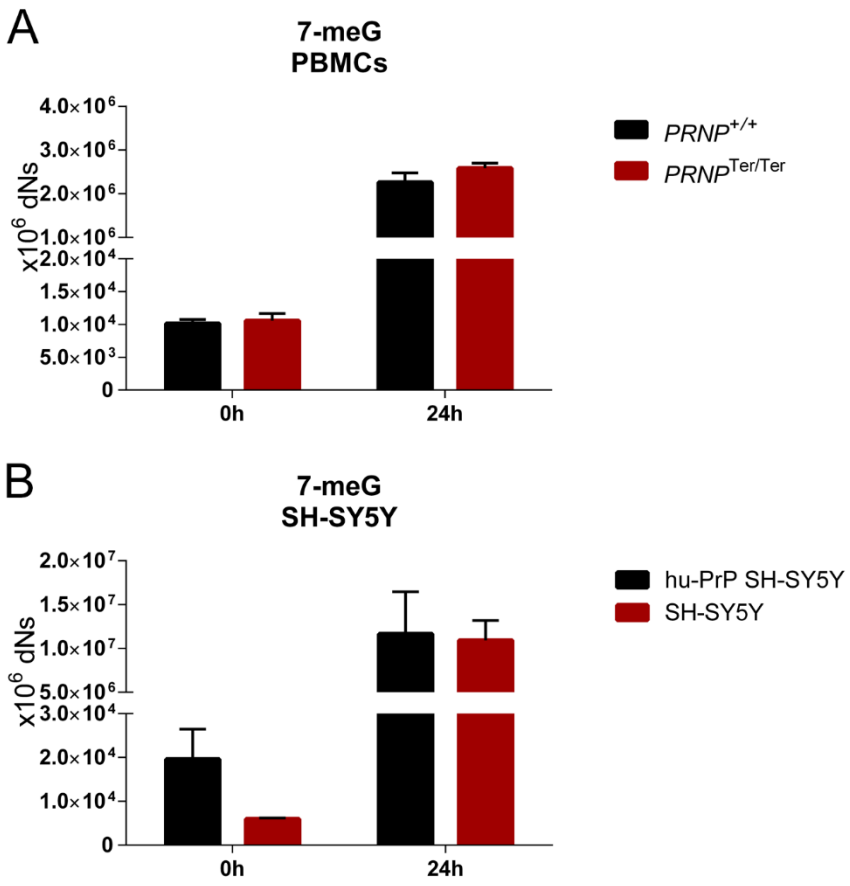

A
PBMCs
Relative viability
1.0
0.8
0.6
0.4
0.2
0.0
Doxorubicin
H2O2
MMS
*
*
PRNP+/+
PRNPTer/Ter
B
SH-SY5Y
Relative viability
1.0
0.8
0.6
0.4
0.2
0.0
Doxorubicin
H2O2
MMS
hu-PrP SH-SY5Y
SH-SY5Y

Figure 6


A
7-meG
PBMCs
4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0
x10^6 dNs
0h
24h
PRNP+/+
PRNPTer/Ter
B
7-meG
SH-SY5Y
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
3.0×10^4
2.0×10^4
1.0×10^4
0
x10^6 dNs
0h
24h
hu-PrP SH-SY5Y
SH-SY5Y

Supplementary figure legends

Supplementary figure 1: PrP^C is expressed in epididymis

PrP^C expression in epididymis was detected by IHC (A and B) and IF (C and D) using the 6H4 antibody. IHC of *PRNP*$^{+/+}$ (A) tissue showed a strong staining of PrP^C in the layer of basal cells below the columnar epithelium lining the epididymal duct. Strong staining was detected also in the interstitial connective tissue whereas the smooth muscle cells exhibited a weak PrP^C staining. IF confirmed the staining pattern (C), with PrP^C (green) and DAPI (blue). *PRNP*$^{Ter/Ter}$ tissues were negative both by IHC (B) and IF (D).

Supplementary figure 2: Minor upregulation of *PRNP* after cellular stress in SH-SY5Y cells

Peripheral blood mononuclear cells (PBMCs) and human neuroblastoma SH-SY5Y cells with and without PrP^C expression were incubated with doxorubicin or H_2O_2 for 1, 3, 6 and 24 h, or with MMS for 1 h, with 0, 2, 5 and 23 h recovery. *PRNP* mRNA levels in PBMCs (*n* = 3-4) (A), Hu-PrP SH-SY5Y cells (*n* = 4) (B) and SH-SY5Y cells (*n* = 4) (C), measured in controls and at 1, 3, 6 and 24 h after treatment, are shown as fold change compared to control cells. Values are shown as mean ± SEM. (Significance tested by multiple t-test with Holm-Sidak correction, all p-values > 0.05).

Supplementary figure 3: H_2O_2-induced oxidative stress creates equal amounts of 8-oxoG lesions in PBMCs and SH-SY5Y cells with and without PrP^C expression

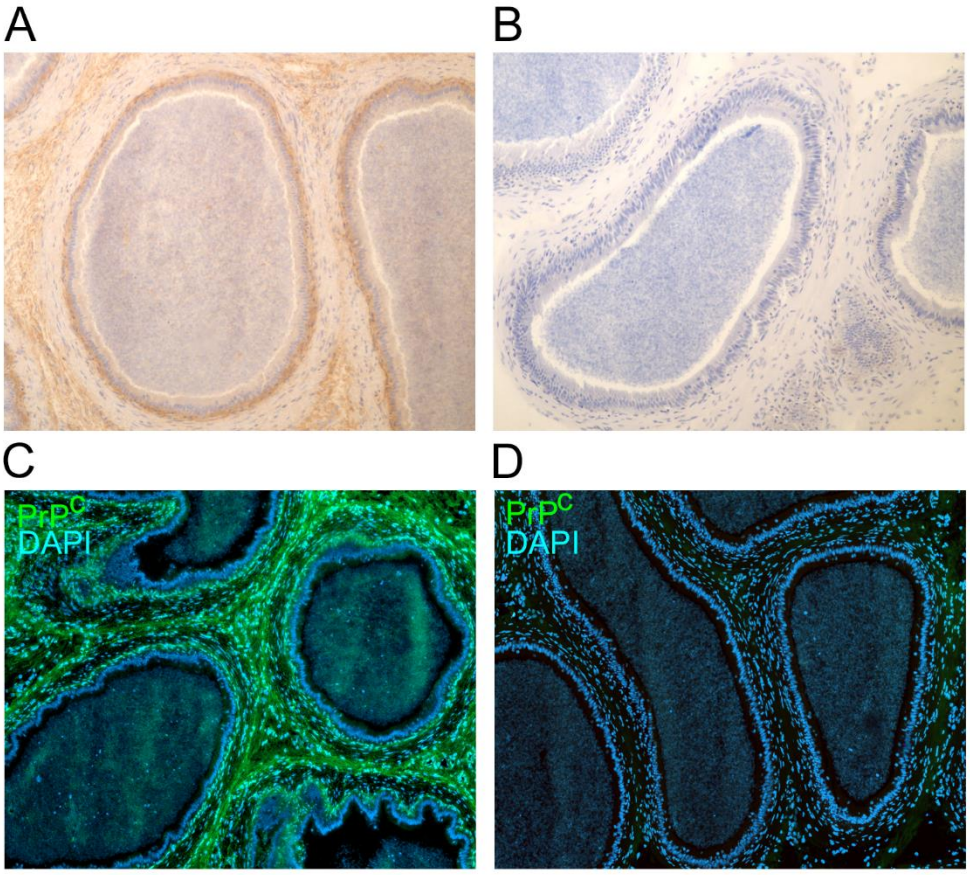
PBMCs (*n* = 3-5) (A) and SH-SY5Y cells (*n* = 1-3) (B) with and without PrP^C expression were incubated with H_2O_2 for 24 h. The measured amount of oxidized bases (8-oxoG) assessed by LC-MS/MS is shown. Values are shown as mean ± SEM. (Significance tested by multiple t-test with Holm-Sidak correction, all p-values > 0.05).

Supplementary figure 4: Lack of prion protein does not affect mRNA levels encoding DNA damage-repair enzymes and antioxidant enzymes

RNA sequencing was conducted on peripheral blood mononuclear cells (PBMCs) with and without PrP^C expression. Transcriptomic analysis revealed no differences in the levels of major enzymes involved in the base excision repair (BER) (A), mismatch repair (MMR) (B), double strand break (DSB) (C) and nucleotide excision repair (NER) (D) pathways of DNA damage repair, and the levels of antioxidant

enzymes (E) (n = 8, mean ± SEM, all $p > 0.05$). (Significance tested by multiple t-test with Holm-Sidak correction).

Supplementary figure 1



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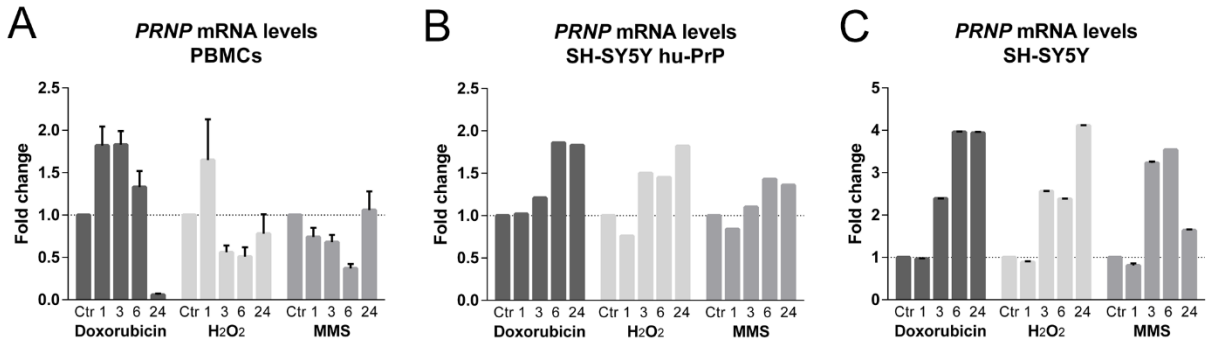

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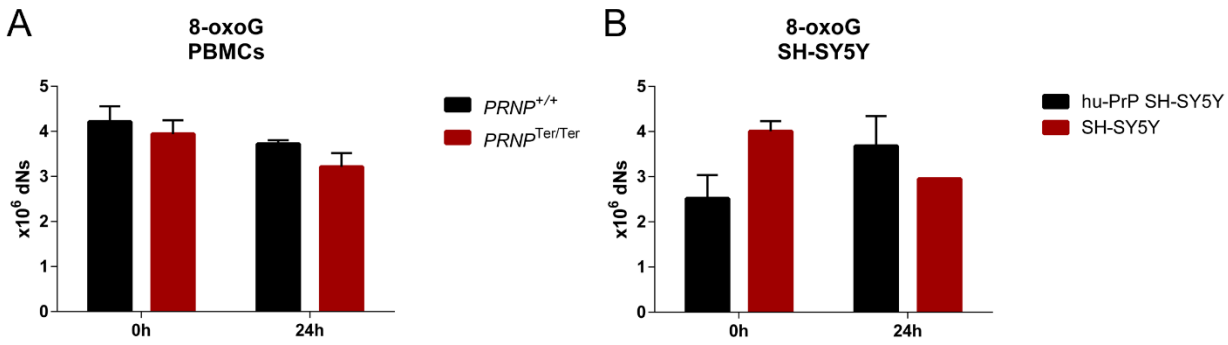

Supplementary figure 4