University of Zurich[UZH]

Master Thesis

Master of Science in Biology/Neurosciences

Development of a High-Throughput Murine siRNA Screen to Discover Genes Involved in Regulating Levels of the Cellular Prion Protein

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14/12/2015

Preface

New knowledge is the most valuable commodity on earth. The more truth we have to work with, the richer we become.

-Kurt Vonnegut

Acknowledgements

Isaac Newton famously quipped "*If I have seen further, it is by standing on the shoulders of giants."* This thesis while largely encompassing work I have generated contains contributions from a large variety of people. I have chosen to use the pronoun "we" to reflect that while I performed a large part of the work the idea and planning as well as data analysis often included a group of people, these have been acknowledged whenever possible. I would like to thank my supervisor Bei Li for her help introducing me to all the relevant techniques used in this thesis as well as helping plan and discuss my experiments. The head of the Lab Adriano Aguzzi for providing me with a great environment to learn and grow in, as well as providing advice either directly or indirectly. I would like to thank all the members of the screen team (Bei Li, Marc Emmenegger, Daniel Patrick, Valeria Eckhardt, Elke Schaper, Valerio Berardi) for discussions about my experiments as well as advice on what experiments to conduct. I would like to thank Marc Emmenegger for assisting me with the use of the Biotek dispenser in several assays and Marc Emmenegger and Clemence Tournaire for helping create the assay run on the Labcyte access platform. The discussion is a result of various ideas interpreted by me and I would like to thank all members of the neuropathology of the USZ for their contributions. Some data is mentioned in my report that I have not contributed significantly to generating but needs to be included for continuity. I have tried to make as clear as possible when this is the case by attributing the actual author in the figure legend. The work I performed was aided by multiple technicians that enabled me to perform the experiments so easily. Therefore, I would like to thank Clemence Tournaire for her help with the robotic platforms as well as teaching me various things about the use of these platforms and about the Förster resonance energy transfer assay that we use. I would like to thank all the technicians and scientists involved in providing me with ready to use reagents such as the conjugated antibodies used in the FRET assay.

On a personal note I would like to thank my mother Monique Zurbrügg for supporting me with all my needs during this Masters and my girlfriend Man-Ying Chan for being so kind and understanding.

Lastly I would like to thank the University of Zurich for providing me with this great learning experience.

Abbreviations

AA- Amino acids
AD - Alzheimer´s disease
ALS - Amyotrophic Lateral Sclerosis
APC - Allophycocyanin
ATP - Adenosine triphosphate
BSE - Bovine spongiform encephalopathy
CAD5 - Cath.a-differentiated 5
CT-Glo - Cell-titre Glo 2.0
DMSO - Dimethyl sulfoxide
dPIA - Digital Prion Infectivity Assay
eQuIC - Enhanced real-time quaking-induced conversion
fCJD - familial Creutzfeldt-Jakob disease
FFI - Fatal Familial Insomnia
FRET - Förster Resonance Energy Transfer
GPI- Glycosylphosphatidylinositol
GSS - Gerstmann-Sträussler-Scheinker-Syndrome
HPFRET - homogenous-phase Förster resonance energy transfer
iCJD - iatrogenic Creutzfeldt - Jakob disease
KO - Knock-out
N2a - Neuro-2a
OFBS - Optimem + Fetal Bovine Serum
Parkinson´s disease - PD
PBS - Phosphate buffered Saline
PK - Proteinase K
PMCA - protein misfolding cyclic amplification
PMSF - phenylmethylsulfonyl fluoride
POSCA - Prion Organotypic Slice Culture Assay
PrP -Prion Protein
PrP^{C}-Cellular prion protein
PrP^{Sc} -Scrapie prion protein
recPrP - Recombinant Prion Protein

RISC - RNA-induced silencing complex
RML - Rocky Mountain Laboratories
rtQuIC - Real-Time Quaking Induced Conversion
sCJD - sporadic Creutzfeldt - Jakob disease
ScN2a - Scrapie susceptible N2a
siRNA - small interfering ribonucleic acid
SOD - Superoxide Dismutase
trFRET - Time-resolved FRET
TSEs- Transmissible Spongiform encephalopathy
vCJD - variant Creutzfeldt - Jakob Disease
WT - Wild type

Abstract

The prion diseases have puzzled researchers for decades. The host-encoded cellular prion protein (PrP^{C}) is present in all mammals and is normally completely innocuous. However, this protein can undergo a pathogenic conformational change and cause devastating neurodegeneration. The pathogenic prion protein (PrP^{Sc}) is an aggregate prone proteinaceous agent that causes astrogliosis, microglial activation and spongiform vacuolation. The PrP^{Sc} acts as a template that binds, converts and incorporates more PrP^{C} into growing aggregates, a process termed prion replication. Therefore, an accumulation of PrP^{Sc} happens in the brain that is thought to initiate the observed neurodegeneration.

Despite intense study there is still no treatment available for people diagnosed with prion diseases and life expectancy is often given in months once onset of disease is detected. Better understanding of the molecular pathways underlying synthesis, transport and degradation of PrP^{C} could provide novel molecular targets that will be more amenable to therapeutic targeting than PrP^{C} or PrP^{Sc} itself. Furthermore, conversion of PrP^{C} to PrP^{Sc} occurs only *in vivo* unless a massive amount of energy is expended. It is therefore likely that there are cellular factors that facilitate or even catalyze conversion, identifying these factors could prove the key to curing prion disease.

To identify factors regulating the endogenous PrP^{C} expression, we attempted to develop a high-throughput genome-wide arrayed murine siRNA screen. We were able to show that creation of a siRNA screen to interrogate genes involved in regulating PrP^{C} synthesis is feasible and created a fully automated robotic workflow to standardize screening procedures. Furthermore, we propose that the novel screening platform can be applied to identify the machinery responsible for PrP^{C} to PrP^{Sc} conversion, the molecules affecting prion replication and cell-to-cell spread of prions.

Contents

1. Background

Prion diseases are characterized by their causative agent the prion protein (PrP) which exists as the innocuous cellular isoform PrP^{C} or its pathogenic aggregating correlate PrP^{Sc}. Unlike all other known infectious agents PrP is believed to be solely proteinaceous and contain no genetic material (Prusiner 1998). Despite intense research on prion diseases and PrP there is still a dearth of therapeutic treatments.

It is known that prion diseases results from the conversion of PrP^{C} to PrP^{Sc}. PrP^{Sc} then causes a variety of pathological changes most notably Spongiosis, astrogliosis and deposition of PrP^{Sc} plaque (Glatzel et al. 2005). However, there are still a plethora of questions that remain unanswered. One of the main questions remaining in prion disease research is what genes are important for being susceptible to prion disease? It has been shown that the Prnp gene is required for mice to be susceptible to prion disease (Büeler et al. 1993). However targeting of PrP by pharmacological or immunological strategies has not provided any useful therapeutic approaches (See 1.13). Therefore alternative cellular targets are desirable. Genes that when ablated cause a down-regulation of PrP^{C} or a decrease in conversion to PrP^{Sc} would provide excellent targets, if they could be identified. Other genes of interest may be involved in clearing of PrP^{Sc} or in mediating secondary toxicity from PrP^{Sc}. These genes would most likely alter the prion disease course and are therefore termed genetic modifiers of prion disease in this thesis.

Yet genetic modifiers of prion disease remain unknown. However, there is good reason to believe that they exist. As the ex-vivo conversion of PrP^{C} to PrP^{Sc} requires large energy input to induce conversion(Saborio et al. 2001)(Atarashi et al. 2011). This indicates that there is some catalytic process happening *in vivo*. Identifying enzymes important for PrP conversion and targeting them may result in curing prion diseases. Therefore, identifying genes involved with regulating levels of PrP^{C} and conversion to PrP^{Sc} will help further our understanding of pathogenicity as well as provide novel molecular targets to potentially treat prion diseases. Identifying genetic modifiers will be the main topic of this thesis. To be able to do so we have developed a machine based high throughput RNA interference screen that allows us to systematically disrupt genes in the mouse endocytome (genes involved in the endocytic pathways).

We hope to identify genes that are important for the synthesis, trafficking and clearance of PrP^{C} and suggest ways this methodology could be used to determine genes that are important for conversion into PrP^{Sc}. Here I will first provide a thorough review of the current state of prion biology. In addition I will demonstrate that the robot based high throughput RNA interference screen is feasible to run and now allows for reliable identification of genes that when disrupted result in an up or downregulation of PrP^{C}. I will then discuss how the screen ought to be run to reliably detect genes altering the levels of PrP^{C} as well as suggest how the screen could be adapted to detect genes important for conversion of PrP^{C} to PrP^{Sc}.

1.1. Overview

The prion protein can be found in all mammals and is conserved throughout evolution but despite intense research its endogenous function and cause of PrP^{Sc} toxicity remain unclear (figure 1)(van Rheede et al. 2003)(Chiesa 2015). However, there is a general consensus that conversion of PrP^{C} to PrP^{Sc} represents a key event as neurons lacking PrP^{C} are not affected by surrounding PrP^{Sc} (Fischer et al. 1996)(Brandner et al. 1996). These two isoforms differ only in tertiary structure and PrP^{Sc} can take a variety of conformations which are thought to be crucial for pathogenicity. Therefore, depleting PrP^{C}, preventing conversion or clearing of PrP^{Sc} in prion disease may prevent disease progression and even reverse current symptoms. There are a large amount of studies that have tried these approaches but unfortunately PrP is not an easily druggable target (see 1.13). However this has not translated into clinical applications either because the method was not applicable to humans, the treatment showed no effect in humans or there was severe toxicity associated with the treatment.

In this section I will attempt to illuminate some of the key questions asked in prion biology as mentioned earlier by providing a thorough review of the prion protein: I will start with a brief explanation of why studying the prion protein is important and a historic tour of prion research, then moving on to the structure of PrP, followed by an overview of prion diseases, discussing the spread and replication of prions and finally ending with the study of non-mammalian prions and techniques used to study prion diseases as well as looking for the prion proteins endogenous role and therapeutic

treatment approaches. Finally we will address the main purpose of this thesis which is to design a screen that can identify genetic modifiers of prion disease.

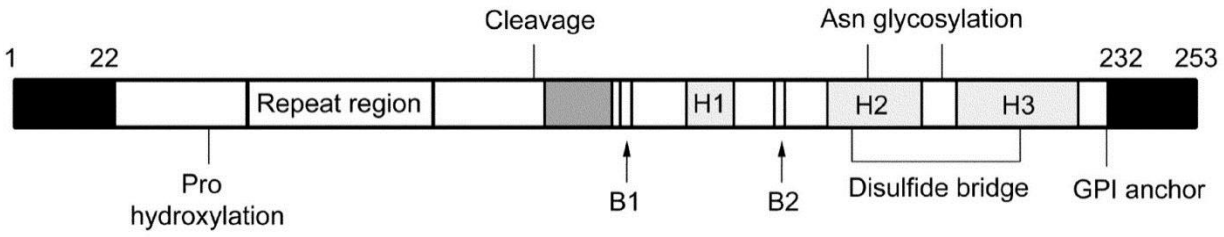

Figure 1. Representation of the human prion protein (Figure from (van Rheede et al. 2003)). Black regions represent peptide signal sequences; grey areas show hydrophobic regions with important secondary structures. In PrP there are typically three α-helices (denoted H1-H3) and two β-pleated sheets (shown as B1 and B2) also shown are the two glycosylation sites on PrP which are at residues 181 and 197 and a known cleavage site between residues 112 and 113. Finally a disulphide bridge can be observed between two cysteines at position 179 and 214.

1.2. Statement of reason for this thesis

A lack of understanding of prion proteins facilitated a series of very tragic incidents. Not understanding the resilience of prions which are resistant to protease degradation, UV radiation and traditional anti-septic methods caused the tragic infection of two young patients with CJD (Bernoulli et al. 1977). Furthermore, large amounts of patients receiving transplants have been inadvertently infected. The lack of understanding was also the root cause of the bovine spongiform encephalopathy (BSE) and variant Creutzfeldt - Jakob disease (vCJD) epidemic (see 1.9.1 acquired prion disease). It is therefore important to not only decipher these tragic events ex post facto but to get ahead and prevent these tragic deaths.

To be able to prevent future tragedies we have to gain insight into the pathomechanisms of the prion protein. To help achieve this I have reviewed briefly what is currently known about the prion protein and prion diseases. Furthermore, I helped develop a high throughput siRNA screen that will potentially allow us to gain insight into the mechanisms involved in PrP synthesis, aggregation and breakdown. We can now undertake a systematic investigation of the genome to locate key genes and mechanisms involved in prion physiology.

Additionally I would like to highlight that Prion diseases share similarities with a variety of other neurodegenerative diseases which has led to the hope that discovering mechanisms involved in prion pathogenesis may also lead to advancements in other neurodegenerative pathologies as well (Prusiner 2012).

1.3. Importance of the prion protein

There are three distinct arguments as to why studying the prion protein and understanding its physiology is of utmost importance. Primarily PrP can cause a variety of diseases (See 1.9.) Out of these Creutzfeldt-Jakob-Disease (CJD) is the most prevalent in humans. There are four known types of CJD; sporadic CJD (sCJD), Iatrogenic CJD (iCJD), familial CJD (fCJD) and variant CJD (vCJD). Sporadic CJD (sCJD) is the most common diagnosis making up about 85% of the cases in the US. The rate of sCJD is closely observed in many countries and is quiet low averaging about 1 case per million in the US, UK and Canada (University of Edinburgh 2014; Public Health Agency of Canada 2014; CDC 2014b). When citing statistics it is important to remember that every single preventable death is a tragedy and not just a number and in my research I have come across plenty of personal stories of people and relatives afflicted with this terrible disease (Minkel 2015). Furthermore, non-human prion diseases such as scrapie and more recently bovine spongiform encephalopathy (BSE) bring immense suffering to the animals afflicted and often result in culling of entire herds leading to large economic losses (see 1.8). Strict monitoring and control of afflicted sheep and cows have managed to greatly reduce the number of infected animals. Novel techniques to reduce prion diseases in farm animals such as the current effort to create PRNP KO animals (Aguzzi & Zhu 2012), are extremely useful to farmers. Lastly it has been pointed out that there are certain similarities between prion diseases and other neurodegenerative disorders such as Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis. Advancements in prion research may translate directly or indirectly to approaches curing the more common neurodegenerative diseases (Aguzzi & Rajendran 2009). Therefore, it is important to study prion diseases both to help the small part of the population affected by these diseases, as well as economic benefits that could be reaped by curing them and finally to advance science hoping that it translates to viable treatments for other diseases (see 1.9).

1.4. History of the Prion protein

The Prion protein is a biological enigma. For decades this protein has puzzled researchers and it was not until 1982 that the nature of the Prion protein was shown to be that of a self-perpetuating proteinaceous agent (Prusiner 1982)From that time on our understanding has increased immensely but yet central questions still elude us. In this thesis I hope to discuss some of the critical questions that remain in prion biology. However, any new revelations obtained must be critically integrated with the past to provide a comprehensive tapestry of understanding. Prion diseases are also referred to as transmissible spongiform encephalopathies (TSEs). TSEs are a heterogeneous group of diseases that affect a wide array of species including humans (Figure 8). Scrapie is the classical example of a TSE and reports of scrapie occurrences date back to the fifteen-hundreds (Brown 2005). There are even mentions of a scrapie like disease at the time of the romans (Kuers 1833). It is therefore clear that TSE's have been around for a very long time. Dozens of hypotheses for the cause of the disease were made such as genetic origin or sexual transmission and most prominently that of a slow virus. None of the tests conducted could confirm any of the proposed hypotheses until in 1936 Cuillé and Chelle succeeded in demonstrating prion protein transmission by injecting brain and spinal cord suspension intraocularly, epidurally, subcutaneously and intracerebrally (Schneider et al. 2008).

However these finding went relatively unnoticed in most of the medical world. In the late 1950's Gajdusek a physician studying a curious disease called Kuru in the fore people of Papa New Guinea published an interesting paper reigniting interest in prion disease. He detailed how transmission from Kuru infected brain samples to chimpanzees resulted in an infectious neurodegenerative disorder remarkably similar to scrapie and transmissible mink encephalopathy (Gajdusek 1958)(Gajdusek et al. 1967; Gajdusek et al. 1966). This was based on observations from a veterinarian that happened to visit an exhibition of Kuru and had the idea that Kuru and Scrapie were two sides of the same coin (Hadlow 1959). From then on a search began for a transmissible agent that could be responsible for these diseases. The idea that the agent could be a protein was first formulated in 1967 but the idea was largely dismissed as it opposed current dogma (Griffith 1967). In the late 70's and early 80's evidence began accumulating that the infectious agent in scrapie was resistant to procedures that target nucleic acids but infectivity could be reduced by hydrolysis or

protein modification suggesting it was proteinaceous in nature (Prusiner, Bolton, et al. 1982). Confirmation of this hypothesis came soon after when a 27 kilo Dalton protein was found in a western blot of infected brains that was resistant to digestion by proteinase K but not present in uninfected brains (Bolton et al. 1982). Prusiner aptly named it Prion for proteinaceous infectious particle and ushered in a new era of research. However, Prusiner's discovery raised more questions than it answered as it was considered heresy that a protein devoid of nucleic acids could be a disease causing agent. This strongly opposed the central dogma of molecular biology which established that the flow of information was from DNA to RNA to proteins. It was unclear how a protein could be the cause of TSE's. Now that evidence accumulated that the infectious agents in TSE's was actually solely proteinaceous it became a race to determine how exactly the prion replicated and mediated toxicity. The next major discovery was mapping of the prion protein to the short arm of chromosome 20 in humans and mouse chromosome two (Mmu 2) and the revelation that the toxic PrP^{Sc} aggregate derives from an endogenous protein (Robakis et al. 1986). Subsequent identification of the gene coding for the prion protein (PRNP in humans and Prnp in mice) allowed for genetic manipulations of this protein (Basler et al. 1986). Büller and colleagues managed to create a Prnp KO mouse which was healthy with no obvious phenotypic abnormalities (see 1.12)(Büeler et al. 1993). The main difference between this mouse and a wild-type (WT) mouse was that this mouse was completely resistant to prion infection. This extremely important discovery meant that the Prion protein had now passed a major test. It was now established that PrP^{C} is required for pathogenicity of PrP^{Sc} supporting the idea that the disease is proteinaceous in nature.

In parallel to this leap in prion research a major epidemic was brewing. To reduce waste from animal processing agricultural scientists had developed a method in which animal waste not meant for human consumption could be compressed into protein blocks which was then fed to farm animals. This led to the bovine spongiform encephalopathy (BSE) epidemic in the UK resulting in the tragic death of around 180,000 cattle in the UK and several thousand more cows worldwide. 229 people worldwide developed variant Creutzfeldt Jakob Disease (vCJD), a new variant of CJD, by ingestion of BSE contaminated meat (figure 8) (CDC 2014a; OIE 2014). The western world was now made fully aware of the destructive effects prions could have. The epidemic was eventually brought under control by strict international guidelines.

However, a recent study found that subclinical vCJD infection may still be as high as 493 per million of population in the UK (Gill et al. 2013). This may have profound implications for preventing further spread of the disease. The thought of a large pool of subclinical carriers has serious implications for the field of blood transfusions and organ transplants as inadvertent iatrogenic infection may occur.

After having discovered the pathophysiology of the prion protein interest shifted to genetics. Genetic susceptibility factors were identified already in the early 90's. One of the most important genetic susceptibility factors is homozygosity at amino acid residue 129. There are two PRNP alleles containing either valine (V) or methionine (M) at residue 129. People who carry homozygous alleles develop prion disease at a far higher rate than would be expected from the allelic frequency observed (Palmer et al. 1991). Another predisposing mutation in the PRNP gene at codon 200 (E200K) can lead to the development of Creutzfeldt-Jakob disease at a rate about 30 times higher than normal and is present mostly in Hasidic Jews (Hsiao et al. 1991).

Even with the current BSE epidemic of zoonotic origin being averted the danger always remains that a similar crisis occurs. The fact that there appear to be large reservoirs of subclinical carriers in the population as well as the possibility of another epizootic epidemic in a different species that allows transmission to humans creates the need to better understand prion diseases. Primarily we need to understand fully how creation of BSE occurred and how to prevent a similar occurrence. While the feed that that animals now receive is more strictly regulated there appears to be the danger of creating a similar crisis in fish (Málaga-Trillo et al. 2011). Additionally, the concern is raised that some types of vCJD may be indistinguishable from sCJD (Asante et al. 2002). If this is the case we may not detect the signs of another epidemic until large amounts of cases start appearing.

George Santayana a philosopher famously quipped that "Those who cannot remember the past are condemned to repeat it" (Santayana 1905). Therefore, we need to closely scrutinize what factors lead to emergence of prion disease in the past so we can avoid the same mistakes in the future. Primarily we need to further our understanding of how to prevent spreading of prions in-between livestock. This was done by stopping feeding of meat and bone meal to farm animals. We need to ensure that there are no

other routes of transmission that would allow for creation of a large reservoir of potentially transmissible prions. We also need to understand what factors predispose people to manifest clinical symptoms or become subclinical carrier. vCJD may also be transmissible through the blood. However, we are currently not scanning our blood banks for PrP^{Sc} (see 1.11.1). A report to the House of Commons in the UK detailed some of the current short comings that may facilitate a second resurgence of vCJD because not strict enough guidelines are in place to prevent accidental human to human transmissions (Miller et al. 2014).

While looking at the history is immensely important for prevention of future outbreaks it provides relatively little guidance on what to do if these outbreaks still occur. Furthermore, it will not help us develop treatment for CJD that is of unknown or genetic etiology. To achieve treatment we must gain novel insights and further our understanding of prion biology. In the next sections I have summarized various aspects of prion biology. I will move from basic molecular and cellular prion biology to ever increasing levels of system complexity. I will end by highlighting some of the attempted approaches at treating prion diseases.

1.5. Structure of the Prion protein

The structure of the prion protein has been intensely studied. However, the structure of PrP^{Sc} remains unknown. Even determining the structure of PrP^{C} turned out to be non-trivial. Protein structure is determined primarily by amino acid sequence. We must therefore begin our analysis of PrP with the PRNP gene. The PRNP gene is located on the short arm of chromosome 20 at 20p13 in humans and on chromosome 2 in mice. Transcription results in 6 alternative transcripts, transcripts 1-5 have slightly varying length due alternative splice sites in the 5' UTR. Transcript 6 is the longest transcript but encodes the shortest protein named alternative PRP (AltPrP) and seems to be functionally different from normal PrP localizing to the mitochondrial membrane (Acland et al. 2014) [NM_001271561.1, NM_000311.3, NM_183079.2, NM_001080121.1, NM_001080122.1, NM_001080123.1]). Transcripts 1-5 all code for a protein that is 253 amino acids long.

The first 22 amino acids (AA) at the n-terminal are a signal sequence that moves the mRNA/ribosome complex to the endoplasmic reticulum mid-translation where the signal sequence is then cleaved. During translation oligosaccharyl transferase

glycosylates PrP at one or two sites and in some cases does not glycosylate it at all as this enzymes fidelity is not very high. Oligosaccharyl transferase can glycosylate amino residues 181-183 and 197-199 but does not necessarily do so leading to a mix of PrP with various glycosylation states. The last 23 AA at the c-terminal of the peptide signals for the addition of a Glycosylphosphatidylinositol (GPI) anchor in a process called glypiation and are then cleaved as well. The mature protein is now 208 AA long has been glycosylated zero, one or two times and has a GPI anchor. PrP is then transported to the Golgi and a disulfide bridge between C180 and C214 form. A DYE signal sequence at residues 144-146 targets PrP to the cell membrane where the GPI anchor tethers it to the membrane(Riesner 2003) (Figure 2.)

It has been noted that a range of different pathologies can be produced by the prion protein (See 1.8). It was thought that the glycosylation state of PrP and the glycan side chains helped determine how the disease progressed. Gel electrophoresis can be used to distinguish between no glycosylation, mono-glycosylation and di-glycosylation (Weissmann 1994). However, there are four glycosylation states and using gel electrophoresis the two different monovalent glycosylation states cannot be distinguished. Additionally, Different glycan chains cannot be distinguished using gel electrophoresis. To resolve this side chains are cleaved using PNGase F followed by mass spectrometry of the side chains.

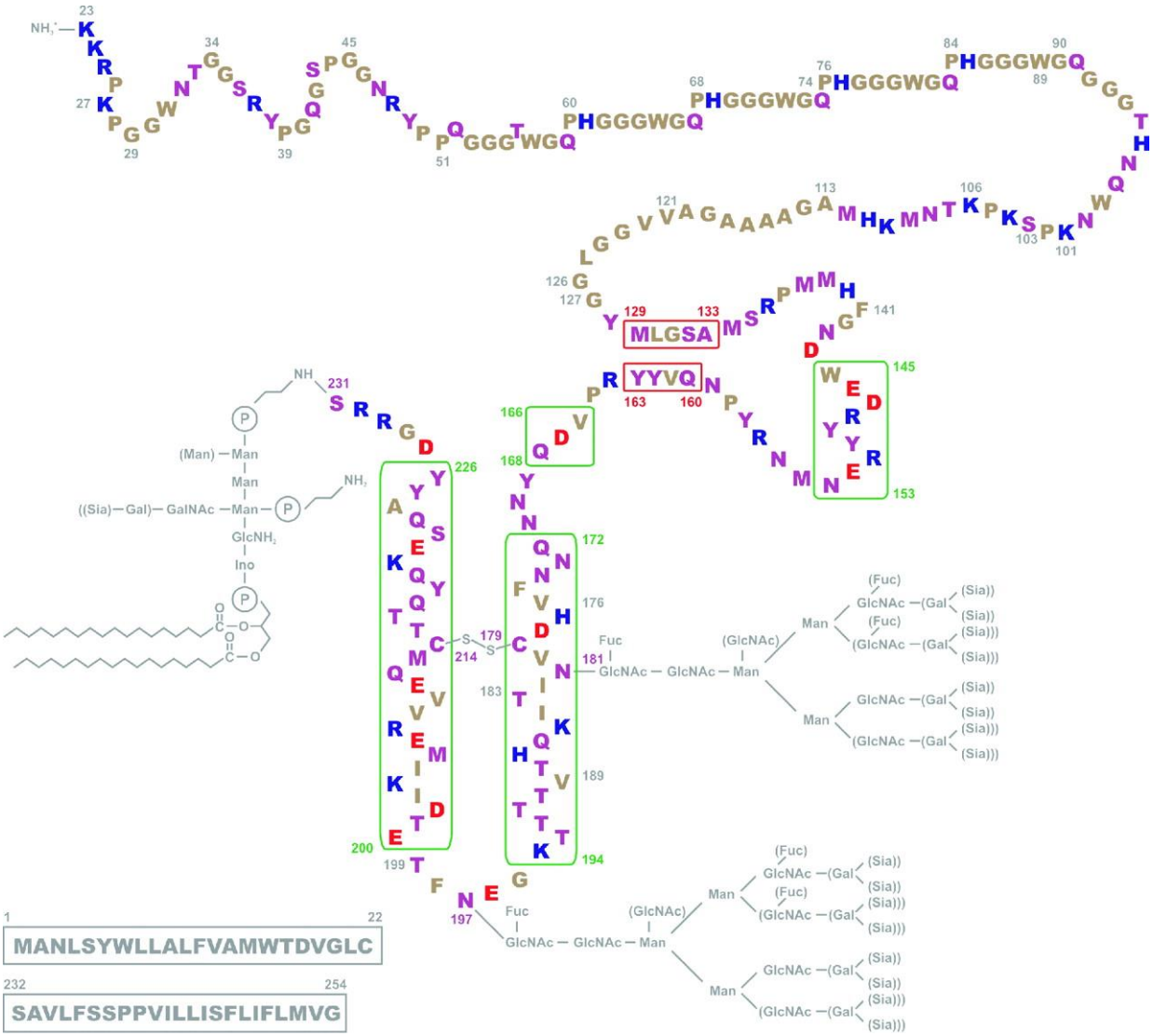

Figure 2. Amino acid sequence and major motifs in the Hamster prion protein (figure from (Riesner 2003)). Residues 34-39 and 45-50 form the double n-terminal hexarepeats. The n-terminal octarepeats can be seen between amino acids 51 and 91. Amino acids 113 to 128 form a conserved hydrophobic region. A β-strand forms between residues 129 and 133 and a second β-strand with which it interacts is located at residues 160-163. There are three α-helices at residues 145-153, 172-194 and 200-226. Residues 179 and 214 form a disulphide bridge. The two glycosylation sites mentioned are located at residues 181 and 197. Residues 232–253 form the GPI signal peptide (cleaved). Positively charged amino acids (at pH 7) in red, negatively charged in blue, neutral in magenta, hydrophobic in brown.

Single nucleotide polymorphisms in the PRNP gene, resulting in single amino acid substitutions in PrP, are critical for our understanding of prion disease. These amino acid substitutions can regulate susceptibility to prion disease, cause familial prion disease or increase risk of developing sporadic prion disease. Furthermore, three amino acid polymorphisms have been found to be protective against prion disease (Kong et al. 2004; Mastrianni 2010)The most important amino acid for prion disease is located at codon 129. There are two common alleles that code for either methionine or valine at this position. In Caucasian populations about 50% people are homozygous for methionine at codon 129. 40% of people are M/V heterozygous and 10% are valine homozygous (Mastrianni 2010). It has been shown that methionine homozygosity predisposes or shortens the incubation time for development of CJD. Furthermore, all known cases of vCJD are methionine homozygous at codon 129 and it is therefore said to be required for development of vCJD (Mastrianni 2010). Others have claimed that due to the skewed prevalence of methionine homozygotes versus valine homozygotes there is an under representation of valine homozygotes. Indeed there appear to be cases of sub-clinical vCJD in people who are VV homozygous or MV heterozygous (Ironside et al. 2006). 70% of all sCJD cases are methionine homozygous (Goldfarb et al. 2004). However there are many more amino acids substitutions that have a critical impact on disease pathogenesis and disease progression (Figure 3).

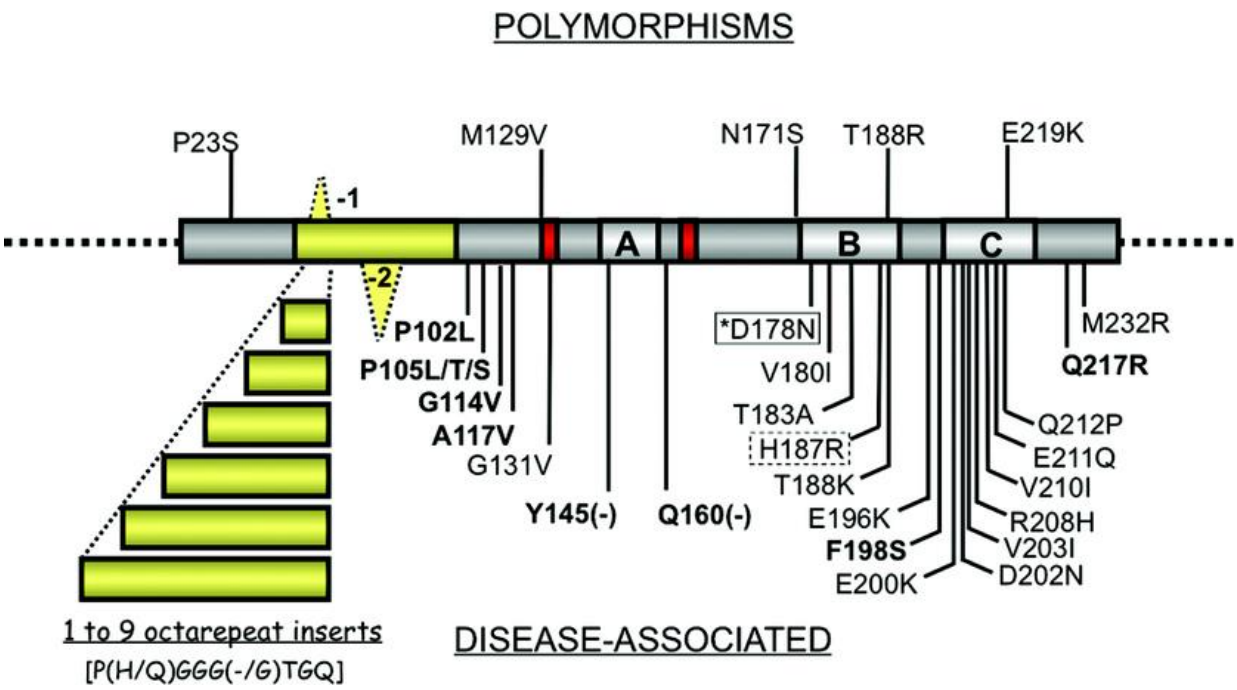

Figure 3. Representation of known mutations in PrP (Figure from (Mastrianni 2010)) relatively few mutations are present at the n-terminal of the prion protein. Importantly there can be insertions of octapeptide repeat sequences. Normally only five octapeptide repeats are present but people with this mutation have one to nine additional inserts which causes a disease similar to sCJD. The majority of mutations causing prion disease however are located in the globular c-terminus of the prion protein. A single mutation can drastically change the appearance of the disease such as the D178N mutation together with methionine homozygosity at codon 129 results in fatal familial insomnia rather than CJD. Mutations in bold are also associated with causing Gerstmann-Sträussler-Scheinker syndrome (GSS).

The majority of CJD patients do not have a mutation in the PRNP gene (CDC 2014b). It is therefore thought that while the amino acid sequence is the same, there is a difference in the tertiary structure. Determining the 3D structure of PrP^{C} and of PrP^{Sc} is of great interest as it may reveal potential pathomechanisms. Determining the 3D structure of PrP^{C} proved difficult because large quantities of high purity protein needed to be generated. This was finally overcome by the creation of recombinant PrP (recPrP) and led to the discovery of its 3D structure (Riek et al. 1996). PrP^{C} has three α helical domains at amino acids 144–154, 175–193 and 200–219 and two small antiparallel β sheets stretching from amino acids 128–131 and 161–164. In full length PrP^{C} the c-terminal domain assumes a globular conformation while the n-terminal is a flexible tail (figure 4.)

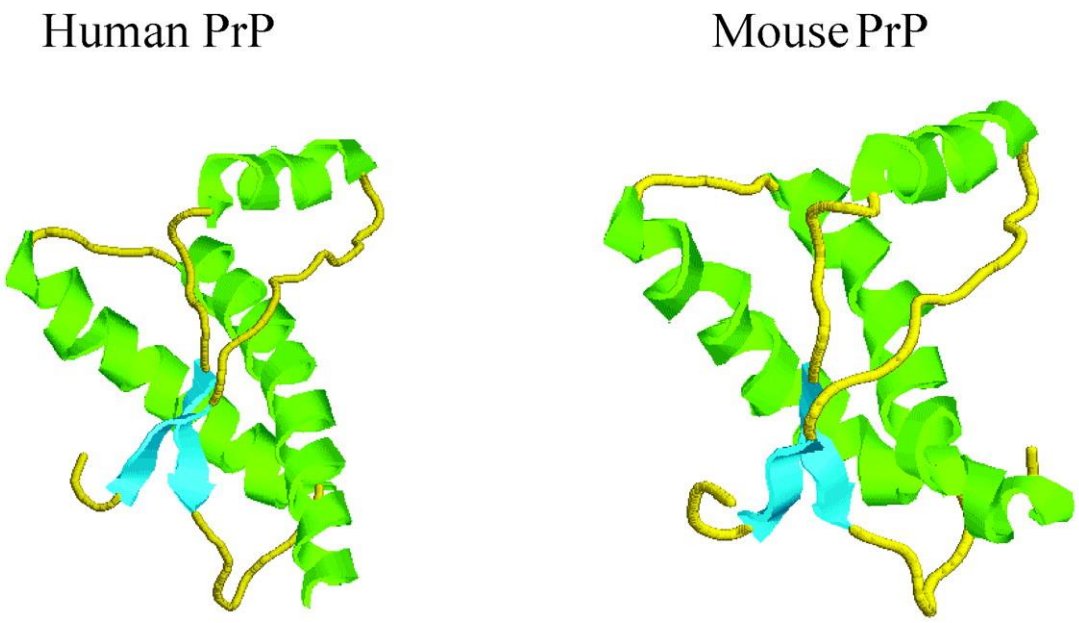

Figure 4. NMR structure of the c-terminal globular domain of recombinant human and mouse PrP^{C} Figure from ((Riek et al. 1996; Riesner 2003)). There is a high degree of homology not only in sequence but in structure between mammalian PrP. The N-terminal is unstructured and can therefore not be precisely modelled

The structures of PrP are only approximations as they were derived from recombinant PrP and therefore lack glycosylation. Additionally some areas like amino acids 166-171 show structural flexibility (Riek et al. 1996). The n-terminal tail is unstructured in solution and can therefore not be depicted. Relatively little is known about how the structure of PrP^{C} changes upon conversion to PrP^{Sc}. There is a change in the ratio of α-helices which dominate the structure of PrP^{C} to an increased amount of β-sheets or β-helices in PrP^{Sc} contributing to its insolubility (Riesner 2003). The fact that PrP^{Sc} is insoluble prevents the use of liquid state NMR and X-ray crystallography both of which need the protein of interest to be dissolved. Another large problem is that PrP^{Sc} can most likely take various but distinct conformations which cause distinct disease phenotypes (Aguzzi et al. 2007). Great heterogeneity can be seen between various prion diseases and it is still not completely clear how various prion strains mediate different phenotypic manifestations of the disease but it is thought that the great variety we see between prion diseases is encoded both in the primary and tertiary structure of PrP (Fraser & Dickinson 1973; Gambetti, Kong, Zou, Parchi & Shu G Chen 2003). Therefore the terminology of prion strains has been introduced to distinguish the exactly same infectious protein that results in an altered pathology due to a different 3D conformation. While strains tend to have conserved glycosylation

patterns there is no need for specific glycan chains to preserve strain specificity (Collinge et al. 1996)(Piro et al. 2009).

While being unable to determine the exact structure of PrP^{Sc} various attempts have been made to approximate the structure using a variety of different approaches. An early attempt was to use infrared spectroscopy followed by a reverse Fourier transformation to determine the secondary structure of PrP^{Sc}. Different secondary structures absorb different infrared wavelengths. This allowed for estimation of α-helices and β-sheets at 17% and 47% respectively (Caughey et al. 1991). This was very similar to a result obtained utilizing circular dichroism which measures differential absorption of left versus right hand polarized light. It was estimated that PrP^{Sc} was 20% α-helices and 34% β-sheets. However, using hydrogen/deuterium exchange to probe which hydrogens of PrP^{Sc} are accessible revealed that there are no α-helices and that the band observed actually correlated to a turn in-between two β-sheets (Smirnovas et al. 2011)

While prions do not form three dimensional crystals two dimensional crystals which are amenable to electron crystallography can be made. Comparison of PrP^{Sc} to the truncated form PrP^{Sc} 106 which contains only 106 residues but maintains infectivity allowed mapping of the location of the glycan sidechain. The main finding was that there is a negatively charged core region which may be involved in binding of cations. The glycan side-chains are located towards the outside of the structure and lastly it was suggested that conversion of α-helices into the unusually stable β-helical conformation is responsible for the resilience of PrP^{Sc} (Wille et al. 2002). However, these models rely heavily on inferred computation and may not actually reflect the true structure of PrP^{Sc}.

Inferring of the structure from exposed antibody epitopes has also been attempted. For a long time the large amount of homology between PrP in mammals prevented the creation of high affinity antibodies as mice were immunotolerant to PrP from other species. It was thanks to the creation of PrP knockout mice that high affinity antibodies could be created(Korth et al. 1997). The 3F4 monoclonal antibody was one of the earliest antibodies that could be used to effectively probe the structure of PrP and was created by immunizing mice with 263K Syrian hamster prions. This mouse monoclonal

antibody recognized both human and hamster but not mouse PrP (Kascsak et al. 1987). While 3F4 readily bound to PrP^{C} and guanidinium hydrochloride (GdHCl) denatured PrP^{Sc} it does not bind to PrP^{Sc} because the epitope is buried inside the molecule. This formed the basis for the conformation dependent immunoassay which allowed the differentiation of strain depending on unmasking of the 3F4 epitope by different sensitivity of strains to GdHCl (Safar et al. 1998). While this was a great way of probing the structure of PrP^{Sc} to determine different strains it was limited by the fact that only one epitope is targeted.

This also led to the discovery that the conformational stability of the prion is inversely related to its infectivity with strains showing low conformational stability being highly infectious and having a low incubation period in mice (Colby et al. 2009). It has been posited that the less stable forms of PrP^{Sc} more easily form monomers that can then cause further aggregation of PrP^{Sc}. This concurs with other results as it was shown that the protease sensitive forms of PrP^{Sc} have shorter incubation times for both naturally occurring and synthetic prions and the fact that rapidly progressing prion diseases tend to produce fewer plaques (Safar et al. 1998; Colby et al. 2010). This is a crucial point indicating that prion diseases do not arise through the inability of the cell to breakdown protease resistant strains but that the conformational stability and the pathogenicity are inversely related. More likely the cellular clearance mechanisms are not capable of clearing the aggregates rapidly enough leading to cell death and deposition of aggregates in extracellular space.

Our lab created a comprehensive set of antibodies against PrP consisting of 19 monoclonal antibodies designated POM1 – POM19 (Polymenidou et al. 2008). This set of antibodies allowed for probing changes in various regions of PrP as it changed from PrP^{C} to PrP^{Sc}. It was found that POM1 binding to the c-terminal caused toxicity very similar to that observed after prion infection. The theory is that the binding of the antibody to a specific region caused a conformational change similar to the structural change occurring when PrP^{C} is converted to PrP^{Sc}. Additionally, POM2 could rescue toxicity exerted by POM1. The n-terminal flexible tail is therefore an important mediator of POM1 toxicity (Sonati et al. 2013).

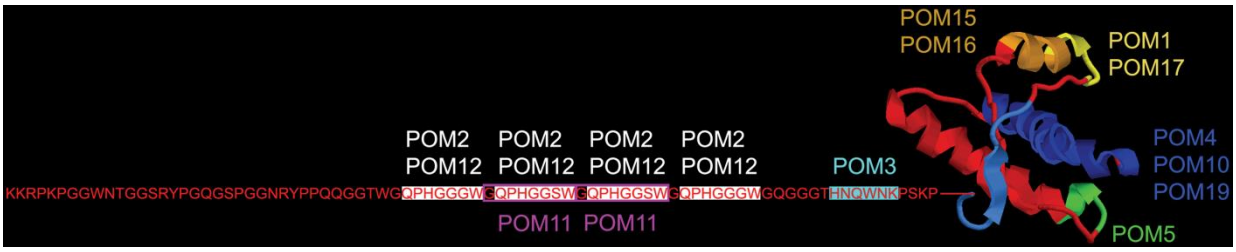

Figure 5. Summary of binding sites of POM antibodies on the PrP (figure from (Polymenidou et al. 2008)) POM 2 and POM 12 bind within the octarepeat region. POM 11 binds to a mouse specific variant of the octapeptide repeat sequence. POM 3 binds the carboxy proximal part of the n-terminal flexible tail near the centre of the molecule. POM 17 and POM1 recognize the beginning of the helix one region and POM 15 and 16 the carboxy proximal part of helix 1. POM 5 recognizes the beginning of Helix 2 and POM 4, POM 10 and POM 19 bind the Helix three region.

So to summarize what is known about the structure of PrP^{C}, it is a 253 amino acid long protein coded by a single exon. The mature protein is 208 amino acids long with 3 alpha helices towards the c-terminal which is globular in nature and a flexible n-terminus tail. It is processed through the secretory pathway and eventually anchored in the membrane via a GPI anchor. It may carry none, one or two n-linked glycan's. There are over 52 different varieties of glycan's that can be attached to PrP^{C} (Weissmann 2009). PrP^{Sc} can vary greatly in structure which is thought to encode the specific prion strain. In conclusion it can be said that even with a wide array of studies performed we are still unable to conclusively elucidate the structure of PrP^{Sc} partially due to its insolubility but also because PrP^{Sc} can take a variety of conformations which may account for the variety of observed strains.

1.6. Prion protein trafficking, processing and degradation

Developing an understanding of how PrP is processed by the cell is vital to understand disease pathogenesis. PrP^{C} is trafficked through the secretory pathway and anchored in a lipid raft on the plasma membrane using a GPI-anchor. PrP can then undergo either α-cleavage or β-cleavage. PrP that has undergone α-cleavage is thought to not be capable of conversion to PrP^{Sc} anymore and expression of the c-terminal fragment obtained by α-cleavage acts as a dominant negative inhibitor of prion replication (Westergard et al. 2011). PrP that has undergone β-cleavage on the other hand is capable of propagating prion infection as the fragment remaining on the cell surface still displays amino acids critical to prion replication(Fischer et al. 1996). Lastly PrP can be cleaved *in vivo* by ADAM 10 in a process known as shedding. ADAM 10 results

in nearly full length PrP being released into the extra-cellular space. The exact implications of this are unclear but shedding of PrP from the membrane is most likely protective in prion disease (Enari et al. 2001). Furthermore, it was found that mice expressing anchorless PrP are partially resistant to prion disease but instead develop a form of cerebral amyloid angiopathy (Chesebro et al. 2010). However, another mouse that also expressed anchorless PrP was found to be more susceptible to PrP infection with quicker incubation times and even developed prion disease spontaneously (Stohr et al. 2011). Therefore, the importance of PrP being anchored to the membrane for conversion to PrP^{Sc} is still unclear.

As with all proteins there is a gradual protein turnover where old proteins are degraded and new ones synthesized to replace the lost protein. PrP is endocytosed and exists in a state of flux between the membrane surface and endocytic vesicles (Figure 6) (Harris 2003). These endocytic vesicles can be recycled or degraded by the lysosomal pathway and may be a second major site of prion replication (Borchelt et al. 1992). In about 1-5% of internalized PrP vesicles PrP undergoes proteolytic cleavage near residue 110 (α-cleavage) and the cleavage product is secreted from the cell (Harris 2003). PrP endocytosis is primarily done by clathrin coated vesicles. If clathrin mediated endocytosis is disrupted by expressing a dominant negative dynamin I mutant then PrP^{C} accumulates in unfissioned endocytic vesicles just below the cell surface. Furthermore PrP is localized in Rab5 positive early endosomes. Interestingly, PrP does not follow standard GPI-endocytosis pathway which is clathrin independent (Lakhan et al. 2009). It was shown that PrP-GFP endocytosis and GFP-GPI endocytosis occur separately (Magalhães et al. 2002). This leads to the question as to how and why PrP is endocytosed differently from other GPI anchored proteins and whether this presents a viable target for therapeutic approaches by perhaps accelerating endocytosis of PrP^{C}. It must be noted that PrP also lacks membrane domains for interaction with membrane adaptor proteins or direct interaction with clathrin. Therefore, the question of how PrP initiates clathrin mediated endocytosis also needs to be resolved (Harris 2003). Copper which has been show to interact with the octarepeat sequence of PrP can cause endocytosis of PrP (Pauly & Harris 1998). The physiological implications of PrP binding and undergoing endocytosis in response to copper are unknown. It is still not clear whether this effect represents a physiological mechanism or a curious side-effect. While PrP is mainly degraded by the lysosomal

pathway it was suggested that it can also be leaked into the cytosol by retrograde translocation where it is broken down by the proteasome as proteasomal inhibitors increased the levels of PrP (Nunziante et al. 2003). Later experiments found contradictory results and it was suggested that proteasomal degradation does not occur as proteasomal inhibitors also cause an increase in the rate of prion protein synthesis (Drisaldi et al. 2003). A highly surprising finding was that even PrP^{Sc} in very high titres is readily cleared as Prnp KO mice inoculated with prions become non-infectious after a fortnight. This famously led Charles Weissman to state that the only reliable way to disinfect prions is to inject them into Prnp KO mice (Falsig et al. 2012).

Many questions about PrP endocytosis, trafficking and degradation are therefore still unresolved. For example how does PrP initiate endocytosis via clathirin coated pits and why does copper induce PrP endocytosis? What are the relative contributions of the lysosomal and proteasomal degradation pathways in respect to PrP^{C} and PrP^{Sc}? If the proteasomal pathway is important for degradation of PrP^{C} or PrP^{Sc} then perhaps a viable strategy would be to increase the rates of ubiquitination preventing PrP^{Sc} aggregation. Increased rates of PrP^{C} degradation could also increase the asymptomatic period experienced by patients suffering from prion diseases. Discovering genes that change the level of endogenous PrP^{C} could represent a first step in uncovering the detailed mechanisms of the prion protein lifecycle. This in turn could lead to novel therapeutic targets.

PrP degradation and turnover rates are relatively fast and even PrP^{Sc} is eliminated from Prnp KO mice in a matter of days. It is therefore likely that this innate clearance system is impaired or cannot clear new prions as fast as they are generated in prion disease. It was found that Prnp mRNA has a half-live of 7 hours in N2A cells (Pfeifer et al. 1993). PrP^{C} and PrP^{Sc} have half-lives 5 and 30 hours to degrade in N2A cells respectively (Borchelt et al. 1990; Peretz et al. 2001). Notably very varying half-lives have been reported *in vivo* (Safar et al. 2005). This poses the question how one of the most resilient proteins known can be rapidly degraded and cleared *in vivo*. It can be assumed that targeting mechanisms that cause increased clearance of PrP^{C} or PrP^{Sc} may provide valuable therapeutic targets for prion diseases.

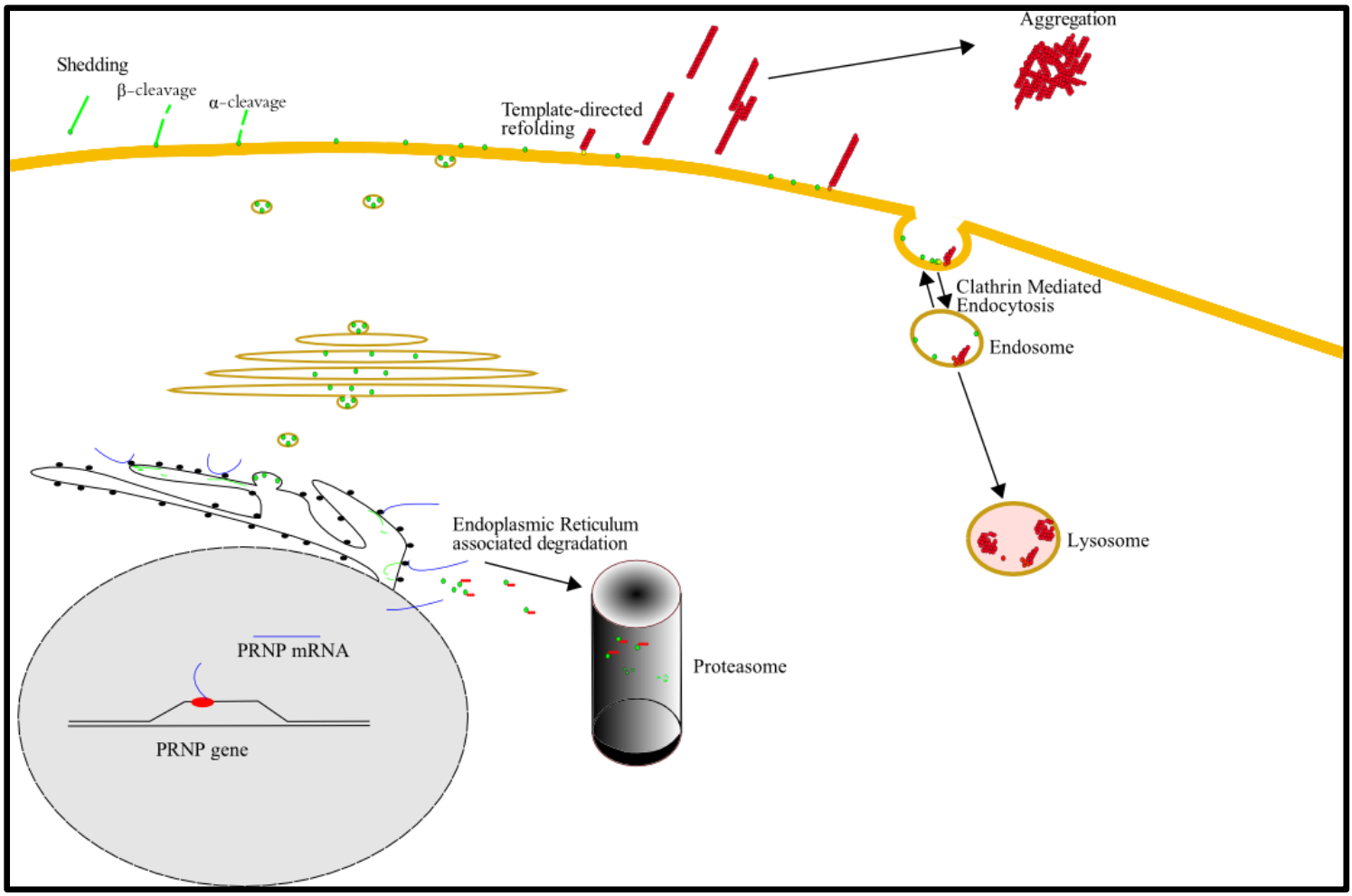

Figure 6. The cellular prion protein processing pathway. The PRNP gene located on 20p13 in humans is transcribed to form PRNP mRNA which then undergoes splicing to yield five different mature mRNAs. The mRNA is exported through the nuclear pores and binds a ribosome. The first 22 amino acids signal the mRNA ribosome complex to transition to the endoplasmic reticulum (ER). PrP is then translated directly into the ER and the last 23 amino acids are cleaved in the process of glypiation. Inside the ER PrP also undergoes folding where it acquires its typical C179-C214 disulphide bond and maybe glycosylated at residues 181 and 197. PrP is then either sent to the Golgi via the secretory pathway or undergoes endoplasmic reticulum associated degradation (Ma & Lindquist 2001). The golgi mainly provides quality control for protein folding but may also act as a conversion site for PrP^{Sc}. The DXE signal sequence at codons 144-146 ensures that the prion protein is processed via the exocytosis pathway and is sent to the cell membrane where it anchors to lipid rafts. PrP anchored on the surface of the cell membrane provides the major site for prion conversion (Enari et al. 2001; Priola & McNally 2009). PrP can then undergo α or β-cleavage or be shed from the membrane. PrP undergoes clathrin mediated endocytosis and gets processed through the lysosomal pathway.

1.7. Prion de novo generation and replication

After having reviewed the lifecycle of PrP^{C} an important question that remains is how exactly does PrP^{C} get converted into PrP^{Sc}? Historically many different theories have

been proposed. Two main theories called template directed refolding and seeded nucleation are discussed here (Aguzzi 2005). In Seeded nucleation there appears to be spontaneous conversion of PrP^{C} into PrP^{Sc} this occurs at a low rate. The PrP^{Sc} monomer is then stabilized by interaction with another PrP^{Sc} monomer and slowly causes an exponential growth into aggregated plaques. The forming of plaque is thought to be the rate limiting step and may explain why sporadic prion disease is so rare but inoculation with prions is highly infectious. It also explains why prion diseases occur later in life. Aging has been shown to slow cellular metabolism perhaps leading to a decreased clearance of PrP^{Sc} monomers. Spontaneous conversion of PrP^{C} into PrP^{Sc} is almost impossible to detect *in vivo* as the PrP^{Sc} monomer if it does exist as described would be degraded rapidly. However, a series of experiments proved that a synthetic 55 residue peptide could be misfolded and cause prion generation when injected intra-cranially into mice (Tremblay et al. 2004). This indicates that stabilization of the misfolded prion protein by the 55 residue peptide is paramount to allowing accumulation of misfolded protein. Once a small stable aggregate has been formed newly formed PrP^{Sc} can be incorporated into it very rapidly. After enough PrP^{Sc} monomers are incorporated into this seed it will break forming many smaller seeds thus causing accelerating PrP^{Sc} inclusion. This model matches the time course observed in prion disease with a long incubation period relating to the initial formation of the seeding aggregate and its growth and dispersion. However, prove that PrP^{C} can convert to PrP^{Sc} spontaneously *in vivo* is missing. Again discovering genes that aid conversion of PrP^{C} to PrP^{Sc} is of major importance. Additionally, there have to be mechanisms for clearance of PrP^{Sc}.

An alternate process called template-directed refolding (Figure 7) requires an exogenous source of PrP^{Sc}. In template-directed refolding PrP^{Sc} both as a monomer and in its aggregated form can interact with a monomer of PrP^{C} and cause its conversion by providing a thermodynamically favourable intermediate conformation. This has been shown to be the case in yeast prions which require Hsp 104 for prion propagation (Chernoff et al. 1995). No equivalent enzyme has been detected in mammalian prion disease. It is speculated that PrP^{Sc} is that enzyme itself but as conversion does not happen spontaneously ex-vivo there may be a chaperone or other protein that are required for prion propagation in humans. A potential problem with this model is that it does not explain the long incubation periods seen in prion

diseases. If this model were true an exponential increase should be seen pretty quickly as each PrP^{Sc} monomer should convert multiple PrP^{C} monomers. Starting with only 1 PrP^{Sc} monomer will result in 1.07×10^{9} PrP^{Sc} molecules after 32 doublings. Incubation periods lasting several months at which initial seeding concentration are very high therefore pose the question of how slow the conversion is. The problem may be resolved by the fact that PrP^{Sc} can initially be cleared preventing over aggregation. The mechanism that leads to clearing of PrP^{Sc} and why PrP^{Sc} fails to be cleared during prion disease is still unknown. So overall template directed refolding fails to address two major questions. Primarily the pathogenesis of the disease is not addressed as PrP^{c} can never convert to PrP^{Sc} as in seeded nucleation. Secondly it does not explain why prion disease occurs late in life and why the disease progression has a long pre-clinical phase with a rapid and violent clinical phase.

To address the first objection we need to assume that an exogenous factor leads to prion disease. Very little is known about the exact factors which lead to sporadic prion diseases and while environmental links cannot be excluded there is no convincing evidence that environmental factors are the cause of sporadic prion disease. Most sporadic cases are not linked back to infection with prions which may indicate that either there is an unknown source of prions in our environment that we have failed to detect or that there can be spontaneous conversion of PrP^{C} into PrP^{Sc} supporting the seeded nucleation hypothesis. Two hypotheses can be formulated to explain the origin of prion disease. I) There is an exogenous source of prions which we have not detected this is unlikely but not impossible as genetic variation may obfuscate any clear relationship between environment and prion disease. II) PrP^{C} can convert into PrP^{Sc} spontaneously and then become stabilized by crosslinking of monomers that somehow evade degradation possibly related to impaired protein processing with increasing age, these monomers then facilitate further conversion possibly via an enzymatic process. Of course a combination of both models is also possible where spontaneous conversion happens but under normal circumstances the misfolded protein is degraded before it can aggregate.

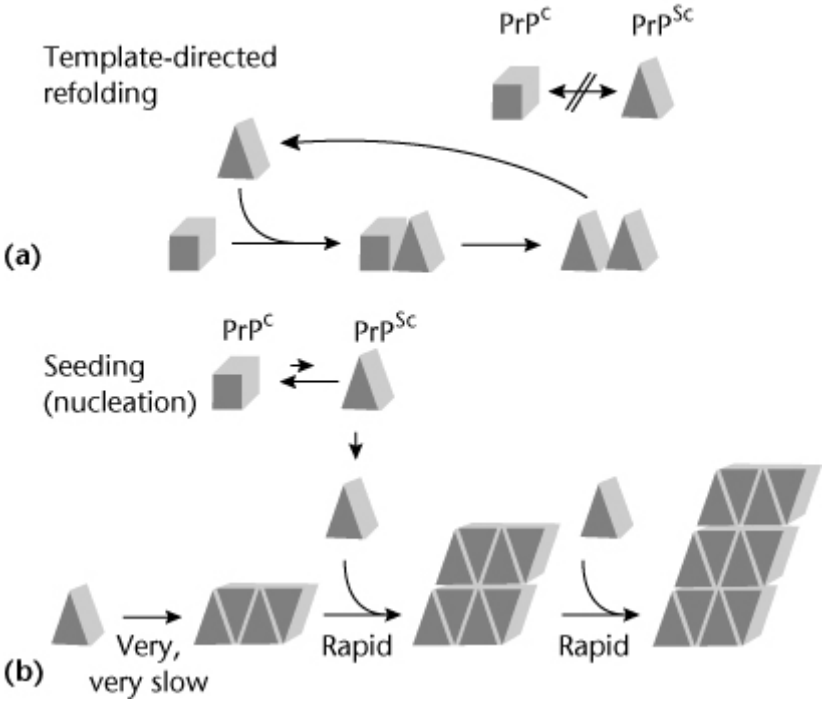

Figure 7. Two of the possible mechanisms by which PrP aggregation can occur (taken from (Aguzzi 2005)). a) template directed refolding in which PrP^{Sc} is needed for conversion of PrP^{C}. b) Seeded nucleation in which conversion to PrP^{Sc} occurs spontaneously however the monomer is degraded readily and inclusion into the seed is slow.

As it can be seen there are still various important questions that need to be addressed about PrP^{Sc} generation and replication. Developing a high-throughput screen allows us to determine what factors such as enzymes and chaperones are required for conversion and will aid in our understanding of how these diseases originate and progress.

1.8. Traditional Prion disease

Just as our knowledge of the prion protein has grown so has our knowledge about the diseases it causes. Traditionally prion diseases only denoted diseases caused by aggregation of the prion protein. The discovery that other neurodegenerative diseases may also be caused by self-replicating aggregating proteins has questioned this assumption. However, for the sake of clarity we will use prion diseases only referring to diseases caused by the prion protein, diseases which are prion-like are instead referred to as prionoid (Aguzzi & Rajendran 2009).

While prion diseases affect a wide variety of livestock and wild animals we will be mainly concerned with human diseases. This is due to the fact that these are better researched but also that the approaches to curing prion diseases are fundamentally different in livestock animals. In animals selective breading for prion disease resistance is feasible and even encouraged. Furthermore, culling of infected livestock can prevent the spread of disease and careful management of infected individuals is enough to reduce levels of observed disease to almost negligible levels. As of 2014 for example there are only about a dozen incidence reports of BSE worldwide (OIE 2014). Levels of scrapie have also decreased significantly with only 18 confirmed cases in the US in 2014 (USDA 2014). However, caution is still needed as surveillance of infected individuals in less economically developed countries is still poor. Additionally, the risk of a novel epidemic of zoonotic origin developing cannot be completely excluded as prion susceptibility of various livestock's used is not clearly known. A recent study has found that various fish can act as prion reservoirs and it is still unclear what enables prions to cross the species barrier (Málaga-Trillo et al. 2011). For obvious reasons methods applied in livestock are not amenable to use in humans. Furthermore, the majority of prion disease cases are sporadic making it difficult to anticipate and prevent. A large variety of prion diseases affecting both humans and animals are known now (Figure 8). For ease of reading the human diseases have been divided into two sub-categories of acquired and non- acquired diseases. Non-acquired diseases can be further sub-divided into genetic and sporadic. This artificial divide is to ease the discussion of prevention and treatment of the diseases later on.

Disease	Host species	First reference in medical literature	Mechanism of pathogenicity	References
Scrapie	Sheep and goats	1500's	Infection in genetically susceptible animals. Transmission can be horizontal between sheep especially if parturition occurs inside the living area. Vertical transmission occurs mainly between the ewe and her offspring and it has been shown that both fetal membranes and milk passed from mother to offspring can cause infection.	Brown, 2005; OIE, 2012
Bovine Spongiform Encephalopathy	Cows	1986	It is now accepted that the BSE epidemic was caused by a decision to feed cows concentrated protein made meant and bone meal (MBM) which was contaminated by prions, it is thought that a spontaneous mutation may have been responsible for the original transformation.	Pain, 1987; of Worth Matravers, Bridgeman and Ferguson-Smith, 2000
Chronic wasting disease	elk, mule deer, white-tailed deer, black-tailed deer, and moose	1967	Suggested to be by interchange of saliva either during feeding or during social interactions	Williams and young, 1980
Transmissible Mink Encephalopathy	Mink	1947	Thought to result from ingestion of BSE infected meat.	Baron, 2007; Sikorska, Guiroy and Bessen, 2009
Feline Spongiform Encephalopathy	Cats	1990	Feeding of BSE contaminated beef	Legget 1990
Exotic ungulate Encephalopathy	greater kudu, nyala, gemsbok, common eland, Arabian and Scimitar Oryx,Ankole-Watusi cow, American bison.	1990	Feeding of BSE contaminated beef	Marcella, John and Regan, 2009
iatrogenic Creutzfeld Jakob disease (iCJD)	Humans	1974	contaminated growth hormone, dura mater grafts and other transplants from undiagnosed CJD patients	Brown et al., 2012
new variant Creutzfeld Jakob disease (vCJD)	Humans	1996	Consumption of BSE infected meat	Will et al., 1996; Hill et al. 1997
Kuru	Humans	1958	Ritual cannibalism leading to ingestion of infective agent	Gajdusek and Zigas, 1958
familial Creutzfeldt Jakob disease (fCJD)	Humans	1924	Mutations in the PRNP gene	Gambetti, 2003; Meggendorfer, 1930; Kirschbaum,1924
Fatal Familial Insomnia (FFI)	Humans	1986	D178N mutation in the PRNP gene with methionine at codon 129	Gambetti, 2003; Chokroverty, Hening, and Walters 2003
Gerstman-Straeusler-Scheinker disease (GSS)	Humans	1936	P102L, P105L/T/S, G114V, A117V, Y145(-), Q160(-), F198S, Q 217R mutation in PRNP found in majority of cases	Gambetti, 2003
Typical sporadic CJD (sCJD)	Humans	1920	Somatic mutation in PRNP gene, Stoichometric reaction caused by chaotic nature of living systems	Gambetti, 2003; Jakob, 1921; Creutzfeldt 1920
Sporadic fatal insomnia (SFI)	Humans	1999	Unknown nature maybe a somatic mutation in the PRNP gene as hypothesized in sCJD	Gambetti, 2003, Parchi et al., 1999
Variably protease-sensitive prionopathy	Humans	2008	Sporadic much like vCJD but carriers are homozygous for valine at codon 129/ absence of PrP Sc	Gambetti, Puoti and Zou, 2011

Figure 8. Different diseases caused by the prion protein. Prion diseases affect animals (seen in green) and humans (other colours). Human diseases can be subdivided into acquired diseases (red), genetic diseases (blue) and finally sporadic diseases with unknown etiology (Purple).(Brown 2005; OIE 2012; of Worth Matravers et al. 2000; Williams & Young 1980; Baron et al. 2007; Brown et al. 2012; Hill et al. 1997; Gajdusek 1958; Kong et al. 2004; Meggendorfer 1930; Kirschbaum 1924; Creutzfeldt 1920; Gambetti et al. 2011; Pain 1987; Liberski et al. 2009; Leggett et al. 1990; Contreras et al. 2008; Will et al. 1996; Parchi et al. 1999)

While prion diseases can be subdivided into various sub-categories the unifying pathology is something they all share. The quintessence of prion disease is threefold. Primarily there is large spongiform vacuolation of the brain tissue. There is observable astrogliosis and finally there is the characteristic deposition of PrP^{Sc} in the form of prion plaques (Figure 9) (Glatzel et al. 2005)

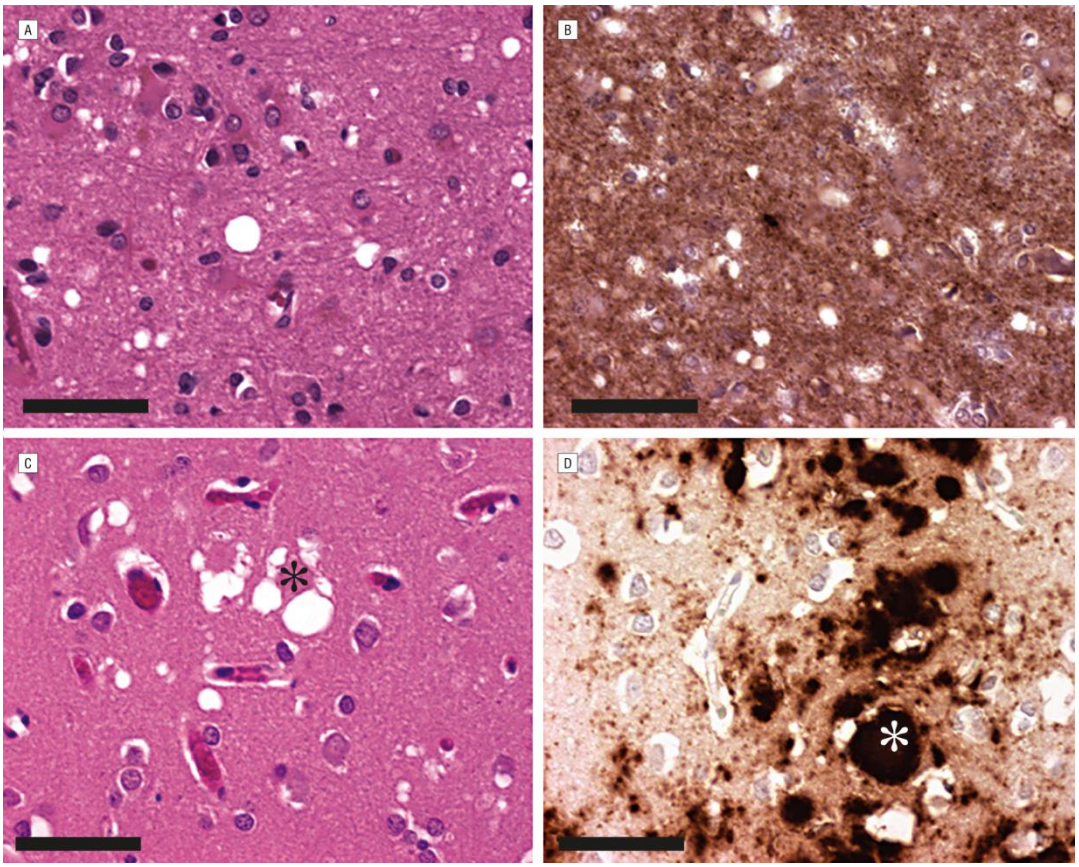

Figure 9. Neuropathological symptoms of prion diseases (Figure taken from (Glatzel et al. 2005)). Panels A and C show tissue from sporadic and variant CJD respectively stained with haematoxylin and eosin typical spongiform vacuolation as well as astrogliosis can be observed. Panels B and D show antibody staining against the prion protein showing the plaque like formations. The tissue was again obtained from sporadic and variant CJD patients.

1.8.1. Acquired prion diseases

All prion diseases are infectious. However a distinction has been made between patients that have a de novo case of Prion disease and patients that have been infected by a known carrier who transmitted the disease to them. Luckily human prion diseases are not as easily transmissible as more common diseases such as influenza. There are a variety of ways in which they can pass from a carrier to host. Three prion diseases that propagate by human to human transmission are known. The first being Kuru the infamous disease devastating the tribes of Papa New Guinea, which was contracted by post-mortem ritual cannibalism (Gajdusek et al. 1966). Secondly Iatrogenic CJD is contracted by contaminated growth hormone and dura mater grafts, with a small amount of cases resulting from contaminated surgical utensils, corneal grafts, gonadotrophic hormone contamination, and blood transfusions from blood contaminated with vCJD (Brown et al. 2012). Lastly vCJD can be acquired by ingestion of BSE contaminated beef by susceptible people (Collinge et al. 1996). Incidence rates of these diseases vary greatly. However overall it can be said that acquired prion diseases have been one of the great success stories of prion research. Kuru had reached epidemic proportion with about 200 deaths per year in the south fore tribe. After the ban on ritual cannibalism rates dramatically decreased and no new cases are known. Only 11 deaths occurred between 1996 and 2004 (Collinge et al. 2006). Similarly iCJD rates have dropped to nearly zero avoiding a large scale outbreak mainly due to the astute observations of some great physicians, undoubtedly saving many lives (Brown et al. 2012). Lastly variant CJD rates have also decreased to near zero due to efficient scientific intervention in public policy again saving many lives. While decrease in the prevalence of acquired prion diseases is remarkable a self-critical evaluation is also needed to prevent future disasters.

A simple blood test needs to be devised to allow checking for PrP^{Sc} in blood donations. This milestone has fortunately already been reached. The development of Enhanced real-time quaking-induced conversion (eQuIC) is based on quaking induced conversion of PrP^{C} to PrP^{Sc}(Orrú et al. 2011; McGuire et al. 2012). While prions are generally present in a variety of bodily fluids such as saliva and blood the concentrations are usually too low for detection. However, eQuIC claims to be able to detect miniscule quantities of PrP^{Sc} (2 attograms per ml). This allows for high throughput screening of samples with high sensitivity and very high specificity. This

assay provides a useful screening tool for blood banks to prevent the use of PrP^{Sc} contaminated blood. Furthermore, it may allow for selective culling of infected animals rather than of entire herds.

Another reason for the decrease in iatrogenic transmission was stringent inactivation procedures which have been introduced after it was clear that prion diseases may survive standard equipment cleaning techniques. Due to the quick thinking there were only a total of 4 incidences of iCJD due to contaminated neurosurgical instruments (Brown et al. 2012). Currently the WHO has a set of recommendations how to prevent prion contamination and how to decontaminate tissue and instruments contaminated with PrP^{Sc} (Rutala & Weber 2010). A significant problem is that most of these protocols are not amenable with sensitive medical equipment such as endoscopes or biological tissue meant for transplantation.

While the rate of infection for acquired prion diseases is extremely small the issue remains that once the host is inoculated there are no available treatment methods. This should be cause for concern while premonitions of a large scale prion disease outbreak have so far not come to pass there is still the fear of latent infection. In a large scale survey of appendix tissue in the UK it was found that as many as 1 in 2000 may harbour the pathogenic PrP^{Sc} protein (Gill et al. 2013). This makes it all the more important that screening procedures are put into place to ensure no accidental transmission of CJD occurs.

1.8.2. Genetic prion diseases

This group of prion diseases is composed of familial CJD (fCJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker-Syndrome (GSS). These diseases are caused by a variety of mutations in the PRNP gene (Kong et al. 2004; Mastrianni 2010)However despite similar etiology the symptoms and pathology can display remarkable heterogeneity.

fCJD is caused by mutations in the PRNP gene that promotes spontaneous conversion of PrP into its infectious form. The genotype of the mutation determines the observed phenotype and symptoms can range greatly from supranuclear palsy to hallucinations depending on the mutation (Gambetti, Kong, Zou, Parchi & Shu G. Chen 2003). The Age of onset is also determined by the genotype. Histopathological

observations in these diseases include typical spongiform appearance of tissue, astrogliosis and neuronal loss.

GSS is defined by an earlier onset but a slower progression than fCJD. Symptoms include clumsiness, dysarthria, dysesthesia and dementia. Diagnosis can be confirmed by genetic testing as most patients seem to have the Pro102Leu mutation (Arata et al. 2006); however other mutations are known (Mastrianni 2010).

FFI as its eponym suggests is characterized by disturbances in the sleep wake cycle, dysautonomia and motor impairments and there is prominent degeneration of the thalamus (Cortelli et al. 1999). It is caused by the D178N mutation in the PRNP gene (Mastrianni 2010). While genetic diseases accounts for less than 10% of all observed prion disease cases, study of families with genetic prion diseases have been indispensable to understanding of the prion protein and associated diseases. Furthermore, it is likely that treatments effective against genetic prion diseases will also yield benefits to patients suffering from sporadic prion diseases.

1.8.3. Sporadic prion diseases

The majority of prion diseases cases are defined as sporadic CJD. Sporadic CJD can be divided into six categories sCJDMM1, sCJDMV1, sCJDVV2, sCJDMV2, sCJDMM2 and sCJDVV1 depending on the amino acid at codon 129 of the PRNP gene and the fragment size observed in proteinase K digestion western blot. All of these categories have certain similarities, but strikingly there is variable pathology observed between these groups even though the pathogenic mechanism is highly similar (Gambetti, Kong, Zou, Parchi & Shu G. Chen 2003). This may be due the fact that conformational changes in the infectious prion protein can determine pathology and different prion strains have been shown to have a consistent phenotype (Aguzzi et al. 2007). This large heterogeneity in disease phenotype displays the ability for small structural changes in the PRNP gene to apparently affect pathology.

1.9. Amyloidogenic Prion like (prionoid) diseases

Neurodegenerative diseases as diverse as Alzheimer's disease (AD), Parkinson's disease (PD), Tauopathies, Amyotrophic lateral sclerosis (ALS) and CJD share a unifying characteristic, aggregation and deposition of amyloid protein with high β-sheet content. Furthermore, they seem to share a variety of secondary pathomechanisms

such as excitotoxicity, astrocytosis, microglial activation and reactive oxygen species metabolism (Dong et al. 2009). However, marked differences are also apparent, unlike with bona-fide prion diseases like Kuru and BSE we do not see large epidemics of AD and PD caused by secondary transmission of an infectious agent. While some of these proteins show a certain amount of transmissibility they are not as infectious as traditional prion diseases. Therefore, these self-aggregating non-infectious agents were defined as prionoids making their similarity to the prion protein clear but showing their lack of transmissibility under normal circumstances (Aguzzi & Rajendran 2009).

Prionoid	associated disease	transmissibility	sources
β-Amyloid	Alzheimer's disease, cerebral amyloid angiopathy, sometimes present in lewy body dementia and inclusion body myositis	Shown that brain homogenates from AD patients can cause AD in APP overexpressing mice	Meyer-Luehmann et al., 2006
α-Synuclein	Parkinson's disease, Lewy body dementia and multiple systems atrophy	Shown that pre-formed fibrils can cause seeded aggregation in cultured cells, infection of non-transgenic mice cause PD like pathology in mice	Luk et al., 2012; Volpicelli-Daley et al., 2014
Tau Protein	Progressive supranuclear palsy, frontotemporal dementia and parkinsonism linked to chromosome 17, corticobasal degeneration, Gangliocytomas, Picks disease	Can spread from seeded brain region to neighbouring brain regions in mice overexpressing murine tau	Lasagna-Reeves et al., 2012; Masuda-Suzukake et al., 2013
TDP-43	Frontotemporal dementia-TDP, Amyotrophic lateral sclerosis	Shown that brain homogenates from FTLD-TDP patients can cause seeded aggregation in SH-SY5Y cells	Nonaka et al., 2013
PolyQ	Huntington's disease	PolyQ can cause seeded aggregation in non-neuronal cells	Ren et al.,2009

Figure 10. A list of known prionoid diseases. While the majority of prionoids only show molecular transmissibility the possibility that they show inter-individual transmissibility cannot be excluded. (Meyer-Luehmann et al. 2006; Luk et al. 2012; Volpicelli-Daley et al. 2014; Lasagna-Reeves et al. 2012; Masuda-Suzukake et al. 2013; Nonaka et al. 2013; Ren et al. 2009)

So while most prionoids can spread from cell to cell, it has only been shown for α-Synuclein that there is the possibility of infection from an exogenous source(Luk et al. 2012). However, research in prion disease may eventually lead to viable treatments of other neurodegenerative disease in two ways. First there may be a common pathway utilized by all these diseases in which case discovering genes involved in prion protein pathogenesis would also be implicated in other neurodegenerative diseases. ADAM 10 for example is important for cleavage of both the amyloid precursor protein (APP) and PrP. In that case treatments for prion diseases would also be directly useful for prionoid diseases. Secondly methodologies developed to study the aggregation and treatment of prions may be transferred to the study of prionoids.

1.10. Non-mammalian prions

While prions and prionoids found in mammals are pathogenic there are other forms of life that may utilize prions as a "bet-hedging system" to allow for a greater diversity of microbial phenotypes (Newby & Lindquist 2013). For example in fungi prion proteins are transferred both horizontally to sexual partners and vertically to offspring. This involves a whole system that ensures that viable oligomeric seeds are transferred to the partner or offspring with high fidelity. Most of these seeds then form an amyloid fibre consisting of a β-helix structure running perpendicular to the axis of the fibre. These fibres are very stable and resistant to denaturation by chemicals and heat. They can be visualized by addition of hydrophobic dyes such as thioflavin T and congo red (Newby & Lindquist 2013).

Prions in yeast may serve functions similar to plasmids becoming activated in times of stress to protect the yeast cell. Yeast prions can confer resistance to azole based antifungals at the cost of not growing as fast in media (Suzuki et al. 2012). Another beneficial trait is conferred by the PSI^{+} prion. This prion is formed from a translation termination factor and allows the ribosome to read through stop codons therefore producing novel variations of proteins. This allows for a quantum leap in evolution in stress situations at the risk of also producing new toxic or non-functional proteins. However, the colony is far more likely to survive even though a lot of individuals will die due to toxic mutations. Furthermore, some prions are induced by specific stressors. GAR+ is a yeast prion induced by a factor produced by bacteria inhabiting the yeast environment. This will cause a switch from mainly glucose based metabolism to utilizing other carbon sources (Jarosz et al. 2014). Using prions allows microbes to

have a robust inheritable system to respond to novel stressors but they can also be reversed easily. These traits are especially useful during stressful times but are a distinct disadvantage during other times. While loss of function mutations are the most common they still occur quite infrequently and therefore being able to use prions to regulate certain traits is desirable as they can mimic loss of function of genes but under certain environmental conditions express those genes again. Furthermore the self-templating feature of amyloids and their structural properties can be exploited to perform tasks such as creating scaffolds or coating surfaces. It is also likely that many prion-like proteins and mechanisms still await discovery. Therefore it is important to note that while prions are regarded as pathogenic in humans they execute important physiological tasks in other organisms (Newby & Lindquist 2013).

1.11. Disease models for prion disease

The majority of clinical trials fail in phase II because the drugs which have looked so promising in preclinical models show no efficacy in humans. It is therefore important to choose a model system that is appropriate for the chosen task. There is an inverse correlation between complexity of the model system chosen and the volume of data obtained. So for a complex model like the mouse it takes months or even years to obtain data and it is extremely labour intensive to obtain relatively few results. Using a cell culture system on the other hand can yield tens of thousands of data points. However, cell culture systems are very different from a functioning brain. The cells replicate and many cell lines do not show toxicity in response to prion infection (Mahal et al. 2007). Furthermore, spreading mechanisms through the brain or interaction between different cell types present in the brain cannot be studied. Therefore, the experimental system needs to be carefully selected to ensure that the system is capable of faithfully replicating the variable you wish to study. An increasing level of complexity is recommended in which you start by identifying promising targets and then validate this reduced number in more physiological systems such as the mouse, the Syrian hamster or even macaque monkeys. We will discuss some of the most widely utilized systems and discuss their strengths and limitations.

1.11.1. Cell Free replication and detection of Prion protein

It was long thought that prion replication was a property of the living cell; in fact the hypothesis of a slow virus requires a cell based replication system. However in 1994 Kocisko and colleagues managed to achieve cell free replication of the prion protein

(Kocisko et al. 1994). From this initial discovery two major techniques emerged the Real-time quaking-induced conversion (rtQuIC) which is based on shaking of infected brain homogenate together with recombinant PrP^{C} followed by a ThioflavinT readout (Atarashi et al. 2011). A version of this assay is now utilized in the clinic with high sensitivity and specificity using patient's cerebrospinal fluid (McGuire et al. 2012). This is a major achievement as rtQuIC allows for pre-mortem diagnosis of CJD. However the problem of being able to detect CJD before onset of clinical symptoms still remains. The second major assay used is the protein misfolding cyclic amplification (PMCA) which uses sonication of aggregates from infected brain homogenate together with non-infected brain homogenate to cause conversion of PrP^{C} to PrP^{Sc} and thus can amplify low concentrations of prion proteins (Saborio et al. 2001). PMCA and rtQuIC allow for detection of PrP even in miniscule concentrations. However, they are very limited in functionality as they do not faithfully mimic the physiological mechanisms of prion replication and therefore do not reveal viable pathways for targeting them. Cell free replication assays provide further proof that the infectious agent is purely proteinaceous (polyanion RNA was required for conversion but this was sequence independent and more likely related to the need for a polyanion (Wang et al. 2010)).

1.11.2. Cell based assay systems

Cell based assays have revolutionized prion research. Before their use mice had to be inoculated with prions five months prior to experiments and the clinical phenotype had to be assessed. Typically it would require 60 mice to detect the titre of a single prion sample. Therefore, it typically took one and a half to two years between designing an experiment and obtaining results (Prusiner 1998). There are a variety of cell lines that are susceptible to prion infection. One of the earliest attempts occurred in 1970 by Clarke and Haig using cells derived from prion infected mice (Clarke & Haig 1970). They argued that if PrP did not replicate in the cultured cells then the infectivity titre should decrease however they observed an increased infectivity after about every fifth passage of cells. A major breakthrough occurred when labs started using immortal cell lines and infecting them using brain homogenate of infected mice then creating a clone library and checking which clone line is best infected by different prion strains. Creation of clone libraries led to the discovery of the ScN2A line which is a clone of a mouse neuroblastoma cell line (N2A) that showed high susceptibility to the rocky mountain laboratories (RML) strain of prions. Cell based screens have been both a

blessing and a curse. For unknown reasons some cell lines show high affinity for a certain type of prion strain but are immune to others, the Cath.a-differentiated 5 (CAD5) cell line is one of the few that shows susceptibility to a wide range of prions. This difference in infectivity is so large that different cell lines can be used to distinguish the prion strain (Mahal et al. 2007). Additionally infectivity titres can be different for example PK1(Mallucci et al. 2003) cells are a sub-clone of ScN2a but show up to 1000 times higher infectivity than their parent cell line (Klöhn et al. 2003). It is unclear why different cells are susceptible to different prion strains.

A large problem with using prion infected cells is that prion infected cell lines also do not show signs of malaise after infection and are therefore unlikely to be useful for determining how prions induce toxicity. The major upside of cell models is their high throughput capability. The standard scrapie cell assay was the first major breakthrough in cell based assays as it allowed for automated detection of prion titres making it about ten times as fast and two orders of magnitude lower in cost than animal based assays. However this method is still not fast enough for truly high throughput screening taking 14 days to run and utilizing a 96 well format (Klöhn et al. 2003). The digital prion infectivity assay (dPIA) can offer a much quicker solution (Li et al. 2012). The dPIA relies on a homogenous phase, time resolved Förster resonance energy transfer (FRET) readout of APC and europium linked to POM1 and POM19 respectively. This assay can be performed in 384 well plates. An assay with such high throughput capabilities enables all sorts of interesting studies such as decontamination protocols, drug screening and in our case siRNA screens. The major advantage of using cell based systems therefore is the very high throughput capability and the ability to replicate the prion protein in a physiologically relevant manner.

1.11.3. Prion Organotypic Slice Culture Assay (POSCA)

Cells are useful for performing prion titrations. However, using cells reveals very little about the physiological interactions occurring in prion infected brains. Furthermore, prion infected cell cultures show very little or no cytotoxicity. It is therefore important to have a system which does not require a great quantity of mice and provides direct access to the brain but is still useful for determining prion interactions in a physiological system (Falsig & Aguzzi 2008). The prion organotypic slice culture assay model has already been used to elucidate some mechanisms of prion neurotoxicity finding that calpain inhibitors could uncouple prion replication from its neurotoxicity

(Sonati et al. 2013). Furthermore, the slices can be more easily manipulated than rodent models; drugs can be applied directly and do not need to cross the blood brain barrier. Additionally, in-situ imaging of the slices is possible allowing for multiple imaging time-points of the same slice rather than having to sacrifice a certain number of mice for each time-point.

1.11.4. Rodent models

Some of the earliest prion experiments used mice as model systems. The original way to detect infectivity titres was to make serial dilutions of the "scrapie agent" and injecting batches of mice with them. This was very laborious and required a large amount of animals to be sacrificed. Prusiner showed that the incubation time of the prion is correlated with the prion titre (Prusiner, Cochran, et al. 1982) and therefore greatly reduced the number of mice needed to determine prion titres. Mice are very time intensive to work with and the long incubation time discouraged many researchers from studying prion diseases. Syrian hamsters were often used as an alternative to mice as they had shorter incubation times. However, care is needed as mouse and Syrian hamster prion strains show distinct properties and may not be comparable (Prusiner et al. 1990). The main advantage of using mice instead of hamsters was the ability for genetic modification. Creating a $Prnp^{-/-}$ mouse showed that endogenously produced PrP is required for prion infection (Büeler et al. 1993). A plethora of PrP KO mice have been created and their phenotypes have been documented rigorously. Both the Zurich I and Edinburgh I remarkably had no overt phenotype except that they were immune to inoculation with PrP^{Sc}; however a series of phenotypes can be shown after intensive study. These include perplexing peculiarities such as reduced mitochondrial number in the CA1 region of the hippocampus, altered neuronal excitability and neuroprotection (see 1.12).

The lack of a major phenotype was fairly surprising as it was thought that the prion protein would be highly important. Other PrP KO mice most notably Nagasaki I showed profound cerebellar neurodegeneration with corresponding ataxia. This paradox was resolved when Weissman and Aguzzi pointed out that a different technique for the creation of these mice meant that "doppel" a gene downstream of PrP was now ectopically expressed and was responsible for the neurodegeneration (Weissmann & Aguzzi 1999). Knock-in and transgenic mice are also very useful for studying prion diseases. Transgenic mice are still used routinely in prion research.

Most prominently the TGA20 mouse has been utilized for various purposes. The TGA20 mouse was created by using a half genomic construct in which the large 6-12kb large downstream intron was deleted to facilitate mutagenesis. This resulted in 60 copies of the Prnp gene to be inserted and an eightfold increase in the level of PrP. The main useful property that these mice have is that they have greatly reduced inoculation time. After inoculation with RML prions average survival for tga20 mice was 68±10 days, wild-type mice on the other hand required 166±8days. This means that this mouse line allows for about two and a half times faster results making them useful at accelerating experiments (Fischer et al. 1996). Knock in mice refer to mice in which the endogenous gene has been replaced by homologous recombination rather than by random genomic integration. While increased expression of PrP decreases incubation time of the disease it does not causes a sporadic occurrence of the disease phenotype in the mouse. Yet the majority of prion diseases in humans are not caused by infection but are either genetic or occur sporadically and are of unknown etiology. Transgenic mice expressing PrP alleles with known prion causing mutations often fail to develop prion disease. A variety of mouse models in which the genes are inserted by homologous recombination were created to address this issue. Both FFI and CJD mice with causative mutations were successfully made and showed the corresponding clinical phenotype (Jackson et al. 2013). Furthermore, these mice are known to be homologous disease models rather than isomorphic making them perhaps the gold standard to test potential prion treatments on.

1.11.5. Macaque and new world monkeys

While using mice and hamsters is good for studying the concept of prion diseases there are some very crucial issues. Primarily there is a species barrier to transmission meaning that amplification of human prion species is inefficient in mouse and vice versa. This problem can often be overcome by expressing human PrP^C in mice. There are other unknown factors that affect the level of prion replication which have been suggested to be properties of the intact cell (Herva & Weissman 2012). It is therefore imaginable that even if human PrP^C is expressed in a transgenic or knock-in mouse there may still be differences in susceptibility to prions due to altered cellular mechanisms. Therefore, while macaques should be used sparingly they are still indispensable due to the great genomic homology with humans and should be used for purposes such as establishing the risk of consuming a specific prion strain for humans (this may be relevant in the case of atypical scrapie or any other prion disease

that may develop in livestock). Furthermore, Macaques may also be useful for confirming pre-clinical treatment efficacy against human prion diseases such as CJD.

1.11.6. Humans

While infecting humans with prions is not allowed, the study of humans with sporadic diseases or more commonly familial cases of the prion disease is allowed. We have gained tremendous insight from sequencing data from individuals afflicted with prion diseases. Studying human prion diseases has specific benefits and drawbacks. The largest benefit is that humans are not really a model but the actual system of study, so any results obtained are likely to be directly relevant in other humans as well. We have gained a large amount of knowledge by studying mutations in the PRNP gene (see 1.5). Genome wide association studies further enabled us to identify genes that may be relevant to prion disease in humans (see 1.14). The running of clinical trials or treatment of patients under compassionate care has allowed us to glimpse at some of the potential problems with extrapolating data from other model systems to humans. Therefore while the experiments which we can perform in humans are very limited they are indispensable for eventually developing a working therapy against prion disease.

Model system	Benefits	Downsides
Cell free Prion assays	Useful for quick diagnostic test that is extremely sensitive and highly specific.	Non-physiological system not suitable for developing treatments for Prion diseases.
Cell based assays	Amenable to high throughput, can be used to study physiological mechanisms of prion replication and conversion to PrP^{Sc}.	No human cell lines available. Unknown variability between cell lines and using immortalized cell lines for a long time can introduce a wide variety of mutations.
Prion Organotypic slice culture assay	Shows physiological interactions of cells. Can be used to study mechanisms of prion neurotoxicity. Higher throughput than using mice.	Still requires animal use brings ethical and legal problems associated with.
Mouse	Can study the effect on brain, creation of homologous disease models, great for testing potential therapies on.	Very slow to work with. Difficult regulatory and ethical concerns.
Macaque monkey	Closest model system to human, most likely to yield clinically relevant results	Great ethical concerns, Very expensive, very labour intensive
Humans	Actual species at which most therapies and research is aimed.	Very limited amount of experimental manipulations are allowed.

Figure 11. A list of disease models used to study the prion protein. As can be seen there are advantages and disadvantages to all models. The model chosen needs to reflect the experimental purpose and careful thought should be put into planning which model is used.

1.12. Endogenous role of the prion protein

The function of the prion protein has been an enigma in prion research. There is perhaps no other protein that has been linked to such a diverse array of specific functions ranging from modulation of t-cells to synapse formation (Isaacs et al. 2006; Steele et al. 2007). One of the major problems in studying physiological function is that the knockout mice used in most experiments were created by mating C57BL/6 with 129/Ola and therefore have a mixed genetic background. These mice were then back-crossed typically for about 10 generations ensuring that most loci in these mice will be of a BL/6 background. However, the genes flanking the Prnp locus will segregate non-randomly meaning that a region of up to 50 mega base pairs flanking the Prnp locus contains genes isogenic to 129/OLA mice (Nuvolone & Aguzzi 2015). It is impossible to address all proposed functions of the prion protein therefore we will focus on the functions which have a considerable amount of evidence in their favour. Comprehensive studies of the Prnp KO mouse have revealed a wide variety of

phenotypes. However, the most notable phenotype is the absence neuronal degeneration upon attempted inoculation with prions because the mice are resistant to prion infection and propagation.

1.12.1. Copper binding

A very interesting ability of the prion protein is for the ability of the four octarepeat regions to bind Cu^{2+} and Zn^{2+} (Brown, Qin, et al. 1997). Furthermore a variety of studies revealed that binding of copper causes rapid and reversible endocytosis of PrP and targeting to the early endosome and golgi (Pauly & Harris 1998). While PrP can bind copper at physiological levels it is not clear whether it has an effect on levels of copper in the brain as they are not correlated with PrP expression level (Waggoner et al. 2000). Therefore it is not clear whether copper binding serves a physiological role or is a curious artefact.

1.12.2. Oxidative stress

It was found that Zurich I mice had a higher proportion of oxidized proteins (B. S. Wong et al. 2001). This may be due to decreased levels of Cu/Zn Superoxide dismutase (SOD) however there was no evidence of altered SOD levels in KO mice (Waggoner et al. 2000). Cu/Zn SOD is the dominant isoform in mammals and is responsible for converting harmful oxidative species into hydrogen peroxide which can then be turned into water and oxygen by catalase (Brown, Schulz-Schaeffer, et al. 1997). It is interesting to note that the octapeptide repeat region of the PrP gives it SOD like activity itself (Brown et al. 1999). An absence of phenotype observed in KO mice may therefore be due to the other SOD isoforms masking any strongly observable phenotype. Indeed as PrP is highly conserved throughout the phylogenetic tree it is possible to imagine that it served an initial very important function like reducing oxidative stress but now shares this responsibility with the newer SOD isoforms. However crossing SOD and Prnp KO mice yielded no detectable difference (Hutter et al. 2003).

1.12.3. A Prion receptor

There have been many receptors which have been said to bind and be activated by the prion protein such as the laminin receptor (Rieger et al. 1997). The idea originates from the fact that if truncated versions of the prion protein are created and expressed in Prnp KO mice then the mice had varying types of pathology. It was suggested that this may be due to PrP activating an unknown receptor. In Prnp KO mice this receptor

is constitutively active and therefore little pathology is observed. However truncated versions of PrP could bind to the receptor and inactivate it in a dominant negative fashion leading to prominent demyelination akin to Schmerling syndrome. This could explain some of the neurotoxicity of PrP^{Sc} because it can also inactivate the prion receptor (Steele et al. 2007). Peripheral demyelination is also observed in Prnp KO mice but the mechanisms leading to demyelination are still unclear (Bremer et al. 2010). A prominent possibility would be a prion receptor present on Schwann cells similar to a trophic receptor responsible for maintaining peripheral myelination. Therefore while no receptor has been identified for the prion protein its existence and involvement in myelination now seems plausible.

1.12.4. Long term potentiation and synaptic functioning

It was reported that the prion protein is required for normal synaptic functioning (Collinge et al. 1994). Collinge and colleagues concluded that mice have disrupted GABA A mediated fast inhibition and impaired long term potentiation. However, other studies failed to find reduced inhibitory post synaptic current in Prnp KO mice and instead found abnormal NMDA receptor activity and that PrP normally prevents NMDA receptor over activation (Khosravani et al. 2008). In concordance with these results some cognitive deficits were found. Spatial learning is impaired in Prnp KO mice (Criado et al. 2005). Additionally, Prnp KO mice did not show increased locomotor activity in response to MK-801 treatment (Coitinho et al. 2002). Lastly there is also decreased anxiety response after foot shock treatment and decreased stress response after forced swimming trials (Nico et al. 2005). In combination these results suggest that the cellular prion protein is involved in neuronal signalling. There is growing evidence for the involvement of PrP in NMDA receptor signalling and NMDA mediated excitotoxicity has been indicated in a wide variety of neurodegenerative diseases including CJD (Dong et al. 2009; Khosravani et al. 2008).

1.12.5. Neuroprotection

Many studies support the claim PrP has a neuroprotective function. PrP protects neurons from ischemic damage in hypoxic brain tissue and PrP^{C} is up regulated in the ischemic brain tissue. Furthermore, lesion sizes caused by infarct are significantly greater in Prnp KO mice (McLennan et al. 2004). It is unclear whether PrP plays a direct role as an anti-oxidant defence protein or modulates glutamatergic signalling to prevent excitotoxicity or if there is a different mechanism altogether. A significant

amount of research points towards the fact that pro-survival pathways are decreased in Prnp KO mice (Spudich et al. 2005).

These observations are intriguing as they offer a possible explanation to the fact that onset of sporadic prion diseases increases drastically as late age is reached. Older patients typically have higher levels of oxidative stress and decreased cerebral circulation. Therefore if PrP production is increased in aged individuals this may contribute to the formation of plaques. An interesting experiment would be to determine whether mice which have substantially reduced blood supply to the brain perhaps by carotid artery clamping are more susceptible to prion protein infection or show shorter incubation times.

Prnp KO mice have been crossed to other neurodegenerative model including Huntington's, Parkinson's and Alzheimer's. No significant impact was observed (Steele et al. 2007). This argues against the claim that PrP is protective or contributes to other neurodegenerative diseases. However, PrP^{C} protects neurons against Doppel toxicity. Truncated version of PrP from amino acids 32-121 cause Schmerling syndrome which mimics doppel toxicity causing Purkinje cells to die. However, reintroduction of wild-type PrP protects neurons against the toxic effect. The critical region of the prion protein for protection was mapped to amino acids 94-134 (Baumann et al. 2007). This can be interpreted as evidence of a neuroprotective effect. Alternatively, it can be theorized that PrP may inhibit its own function something which has been shown for a variety of proteins such as P53 (Ko & Prives 1996), Snare and WASP proteins and many others (Pufall & Graves 2002). In this model misfolded or truncated PrP causes neurotoxicity by over activation of the effector due to lack of auto-inhibition. This would explain why addition of PrP protects neurons from truncated PrP because full length PrP can bind to the PrP receptor preventing activation. There is no evidence that supports the claim that PrP inhibits its own function and it does not possess a domain that would be typical of auto-inhibition.

1.12.6. Summary of the endogenous roles of the prion protein

The most robust phenotype observable in Prnp KO mice is their resilience to inoculation with prions. The majority of phenotypes observed in the Prnp KO mice are very mild and have often been difficult to replicate, possibly because they are a genetic artefact (Steele et al. 2007). The best documented case for a phenotype

(except their resistance to prions) is the peripheral demyelination that Prnp KO mice undergo. The extent and cause of peripheral demyelination is not yet clear but perhaps relies on interaction with a g-protein coupled receptor (Bremer et al. 2010). However, targeting PrP^{C} may still be a viable treatment as the benefits of slowing the disease far outweigh the problems caused by the demyelination.

1.13. Creating novel therapeutic approaches

A multitude of potential chemicals have been described as being effective at slowing or curing prion diseases in preclinical models. However, no known drug demonstrated clear efficacy in clinical trials or the trials were aborted due to severe side-effects. Complex considerations need to be taken into account when extrapolating data from various scientific publications. Many compounds that have shown promise in cell-free and cell based systems have proven disappointing when subsequently tested in animal models. Many of the therapeutics tested in-vivo show limited efficacy (Trevitt & Collinge 2006). Furthermore, variable methods of inoculation being either peripheral by intra-peritoneal or central by intra-cranial injection can obfuscate infection studies. Lastly, treatment in pre-clinical trials is often started post-inoculation. However, treatment in patient's only starts at a late disease stage as diagnosis only happens after onset of clinical symptoms. We need therapeutics that are effective after PrP^{Sc} is already present in the central nervous system. Unfortunately the majority of potential compounds showed very little efficacy after central pathology has occurred (Trevitt & Collinge 2006). Therefore, some essential considerations should be made when developing drugs against prion disease.

Primarily we should consider potential targets. A large problem with rational drug design thus far has been a lack of viable targets. Most therapies are aimed at PrP^{C} and PrP^{Sc}. Targeting PrP^{Sc} may seem like a logical option to prevent and reverse progression of prion diseases. However it was recently noted that the relationship between PrP^{Sc} and disease progression is not linear with PrP^{Sc} titres peaking before onset of clinical symptoms. This would indicate that PrP^{Sc} is not responsible for toxicity (Sandberg et al. 2014). However other interpretations are possible, the paper does not analyze individual brain areas and therefore prion titres in specific brain areas may still be rising. The paper also does not consider the possibility that the toxicity is a result of a protective mechanisms being overwhelmed in which the protective mechanism at first prevents neurodegeneration but is then overwhelmed after a certain timespan.

The relationship between levels of PrP^{C} and prion disease progression on the other hand are very clear with ablation of the Prnp gene providing complete resistance to prion disease (Büeler et al. 1993). Targets other than PrP^{C} and PrP^{Sc} have been sparse (See 1.14) therefore the majority of treatments developed have targeted PrP in some form.

We must also consider the time of administration. Proposed therapeutic interventions must show that they are still effective when administered at a time point where there are observable symptoms. The method of administration is another critical factor. The brain is very difficult to target because it is encased in the skull and the neurons are protected by the blood brain barrier. Therefore pharmacokinetics of potential compounds need to be carefully evaluated.

1.13.1. Antibodies

There have been various attempts to target the prion protein using monoclonal and polyclonal antibodies. One of the earliest attempts showed that there is 100 fold reduction in infectivity after incubation of PrP^{Sc} with an antibody (Gabizon et al. 1988). A possible explanation for the effect was that the antibody produced steric hindrance preventing the conversion of PrP^{C} to PrP^{Sc} (Horiuchi & Caughey 1999). Furthermore, it was suggested that the antibodies prevent conversion of PrP found in lipid rafts as antibodies are unable to enter the cell (Kim et al. 2004). Later studies using KDEL tagged intrabodies found that they interfere with lysosomal degradation of PrP and conversion to PrP^{Sc}. This prevented prion formation both *in vitro* and *in vivo* (Vetrugno et al. 2005). Great success at abolishing PrP propagation *in vitro* lead to the hope that PrP specific antibodies could be utilized to treat patients (Enari et al. 2001). However, attempts to target PrP^{C} in the hippocampus via antibodies resulted in high levels of toxicity (Solforosi et al. 2004). It was observed that binding location of the antibodies may trigger a conformational change. Antibodies targeting the c-terminal but not the n-terminal of PrP result in toxicity. The flexible tail of the prion protein is an important mediator of toxicity as antibodies targeting the n-terminal prevented the toxicity of antibodies targeting the c-terminal. The n-terminal mediates creation of reactive oxygen species and activation of calcium dependent proteases that underlie neurotoxicity (Sonati et al. 2013). However, other studies using stereotactic injection into the mouse hippocampus have found no signs of neurotoxicity (Klöhn et al. 2012).

The marked discrepancy is most likely a result of the use of different antibodies as well as models used.

Antibodies highlight the problems faced trying to develop anti-prion therapeutics. Variations in prion strain, model of investigation used, reagents utilized, method of administration and outcome measures obfuscate clear comparative interpretation of results. The great heterogeneity of methodologies possible for investigation highlights the mechanistic diversity of the pathologies we observe. Antibodies continue to be a tantalizing treatment option as they show great efficacy. However, advancements in antibody therapy needs to be closely mirrored in early detection methods of prion disease as studies that applied antibodies after clinical onset of the disease showed a greatly reduced efficacy (Trevitt & Collinge 2006). Recent clinical trials with antibodies against the Alzheimer´s related amyloid protein Aβ42 have dampened high hopes that targeting the misfolded protein by using antibodies would lead to significant improvements (Doody et al. 2014; Salloway et al. 2014). One of the most important aspects seems to be time point of administration. With ever increasing ability for early diagnoses of these disease antibody treatments may eventually become viable. Furthermore the mechanisms behind antibody mediated toxicity need to be understood in far greater detail to ensure that the treatment delivered is safe.

1.13.2. Vaccinations

A major leap forward in prion disease therapy would be to induce an immune response against PrP^C. However, this strategy bears considerable risk such as disrupting the physiological function of PrP^C and eliciting a major immune response in the central nervous system which can cause a variety of secondary complications. However, the severity of prion diseases may still warrant a vaccination if it is known that someone is afflicted by prion disease. There have been multiple attempts to induce an immune response against native PrP^C utilizing a wide variety of methods. Most of these have shown little success when subsequently tested for their efficacy (Heppner & Aguzzi 2004). There is tolerance to induction of an anti-prion immune response using normal PrP. Most attempts have used recombinant PrP or PrP peptides to elicit immune responses. However, it was not determined in the majority of these studies whether there is also activity against native PrP^C and many of the studies did not subsequently test for an anti-prion effect *in vivo* (Heppner & Aguzzi 2004). As discussed previously passive immunization by injection of antibodies seems more easily achievable and

may be safer. However, a group of researchers recently managed active immunization by injecting attenuated salmonella bacteria expressing murine PrP this extended the incubation period or protected the mice from infection by oral administration of 139A prions (Goñi et al. 2005). Furthermore using a similar method but using cervid PrP led to partial protection from chronic wasting disease demonstrating that a vaccine can be effective at preventing prion disease (Goñi et al. 2015). The rarity of prion diseases, potential vaccine side effects and public opinion on vaccinations all make it unlikely that there will ever be a large scale program to vaccinate humans against prion diseases. Animals however stand to benefit very soon if vaccinations become available.

1.13.3. Polyanionic compounds

This is a group of negatively charged molecules that have been shown to increase the incubation time of prion diseases (Trevitt & Collinge 2006). Endogenous poly-anions include glycosaminoglycans (GAGs) such as heparin sulfate and chondroitin sulfate: This class of drugs has been shown to interact with amyloidogenic diseases increasing incubation time and decreasing the levels of PrP^{Sc} (Díaz-Nido et al. 2002; Caughey & Race 1994). However, in cell free conversion systems glycosaminoglycans promote the formation of PrP^{Sc}. It has been suggested that this discrepancy arises from the fact that administered GAGs compete with endogenous GAGs in cellular systems. In cell free systems on the other hand they help mediate conversion (C. Wong et al. 2001). Various derivatives of GAGs have been tested and many have yielded slight benefits. However, the benefits were often associated with toxic side effects and not suited for use in humans. Amyloid dyes such as congo red may mimic the activity of GAGs (Priola & Caughey 1994). Therefore these dyes were also studied for their anti-prion activity but had smaller efficacy than GAGs and large toxicity. However, derivatives of congo red were synthesized and have shown some promise in reducing conversion of PrP^{C} to PrP^{Sc} (Sellarajah et al. 2004). Another anionic compound with light structural similarities to Congo red is suramine. Suramine was tested for efficacy at reducing PrP aggregation. Surprisingly, it was found that suramine caused aggregation of PrP but these aggregates were sensitive to proteolytic digestion and non-infectious. Furthermore, treatment of TGA 20 mice suramine resulted in increased disease incubation time. The authors conclude that suramine causes re-routing of PrP to the lysosome preventing it from being at the cell surface causing faster degradation of PrP as well as preventing conversion to PrP^{Sc} (Gilch et al. 2001).

While polyanions represent an interesting class of drugs their efficacy seems very dependent on the time point of administration. It is therefore unlikely that these drugs will be useful in treating prion disease. Analyzing how they interact with PrP to prolong incubation periods on the other hand may yield novel insights into prion disease progression.

1.13.4. Polyene antibiotics

An early study by Amyx et al. in 1984 identified amphotericin B as a drug that causes prolongation in the incubation period of peripherally infected animal models (Amyx et al. 1984). This delay was significant only when the drug was administered peripherally and pre-symptomatically (Pocchiari et al. 1987). A whole range of follow up studies reviewing various antibiotic compounds have been performed finding some highly significant reductions in survival time (Minkel 2013b). However, there was only one study ever conducted using escalating doses of amphotericin B in humans. An escalating dose ending at 1 mg/kg was administered after clinical diagnosis of two patients. Consistent with Pocchiari's findings there was no significant prolongation in survival time if the drug was administered after clinical onset of the disease (Masullo et al. 1992). The fact that this drug class is unlikely to be of use after appearance of clinical symptoms combined with the fact that most of them have high levels of toxicity if administered chronically makes these unattractive therapeutics to treat prion disease with. However, the mechanism by which these drugs cause a prolongation in survival time has never been thoroughly clarified and may warrant further investigation (Adjou et al. 2000)).

1.13.5. 2-aminothiazoles

This class of small molecule prion therapeutics was discovered during a high throughput ELISA screen (Ghaemmaghami et al. 2010). Two lead compounds designated IND24 and IND81 were able to double the survival time of mice infected with RML and ME7. However, the two compounds did not increase the survival time of mice containing human PrP inoculated with CJD brain homogenate (Berry et al. 2013). This highlights one of the many problems in the prion field which is our lack of understanding of prion strains. Furthermore, Berry et al. (2013) address the problem of prions developing resistance to certain anti-prion drugs. This phenomenon has yet to be characterized in detail but it is thought that slightly different conformations of PrP^{Sc}

may be more or less susceptible to treatment with anti prionoid drugs. Therefore, conformations that are less susceptible will be selected for. This may still be favourable as altered conformations may show less favourable replication kinetics resulting in a slowing of disease progression. However, it does pose a considerable problem for curing prion infections as treatment resistant reservoirs may remain and replicate even after treatment. However, these data also offer hope for finding drugs that substantially increase the life expectancy and quality of life of patients as it has been shown that it is possible in mice.

1.13.6. Tricyclic and phenothiazine compounds

These classes of drugs were thought to be highly effective at treating prion disorders both quinacrine and chlorpromazine showed promising anti prion properties *in vitro* (Korth et al. 2001). However, their in-vivo efficacy was unclear, chlorpromazine shortened the incubation time with intra-peritoneal inoculation but lengthened it with intra-cranial inoculation the reason for this effect is unknown but may be due to biased study design and small number of samples (Roikhel et al. 1984). A follow up study on the work done by Korth subsequently found no effect of chlorpromazine on concentration of PrP^{Sc} in the spleen of infected animals (Korth et al. 2001). Quinacrine which showed greater efficacy at lower doses was utilized for treatment of humans and seemed to yield great improvements in the one patient who was treated (Minkel 2012). There were a large variety of mechanisms proposed by which quinacrine could have this effect but there is little evidence to conclusively prove or disprove them (Amaral & Kristiansen 2001). Furthermore, the patient who was treated with quinacrine still died three months after commencement of treatment. Phenothiazine's are a class of drugs that show potential promise at reducing levels of PrP^{Sc} during a high throughput screening testing a library of over 2000 drugs and natural products (Kocisko et al. 2003). A recent study aimed at identifying novel styryl-based and tricyclic compounds that are nontoxic and prolong the incubation time of prion disease. They identified two imaging compounds 23l and 59 as well as two tricyclic compounds trimipramine and fluphenazine. These compounds all prolonged the incubation period and reduced level of spongiform change and astrogliosis in mice (Chung et al. 2011) but no follow up studies were done.

1.13.7. Dimethyl sulfoxide

Dimethyl sulfoxide (DMSO) is a commonly used solvent as it dissolves both polar and non-polar compounds. Therefore, it is a common choice to use DMSO as a solvent to apply anti-prion compounds. This is problematic as DMSO itself has been shown to alter disease progression. Administration of DMSO ad-libitum to hamsters 55 days after intra-peritoneal inoculation significantly prolonged the incubation time till disease onset by about 10% (Shaked et al. 2003). Furthermore, when the brain homogenate of hamsters, sacrificed at different time points, was analyzed for levels of PrP^{Sc} there was a large reduction in the level of PrP^{Sc} in the DMSO treated animals. It is known that DMSO has an effect on amyloid proteins (Hanai et al. 1979). Therefore, drugs which are dissolved in DMSO need to have careful controls set up testing them against animals treated with DMSO only to establish their efficacy.

1.13.8. Aptamers

Nucleic acid aptamers are short strands of oligonucleotides that are selected from randomly generated pools. They show the ability to bind proteins with a similar or even higher affinity than antibodies. However due to the easy generation of novel aptamers, their small size, their stability and the ability to modify aptamers they show significant advantages over antibodies (Keefe et al. 2010). Furthermore, they are non-toxic and non-immunogenic which could be a large advantage in prion therapy compared to antibodies. Humanization of antibodies by replacing the constant region of murine antibodies with human sequences and more recently full humanization where only the complementarity determining region of the antibody is still of murine origin has been used to avoid an immunogenic response in antibody therapies. However, the problem remains that these antibodies loose efficacy due to clearing by the immune system (Harding et al. 2010). RNA aptamers that were synthesized against PrP showed efficacy at limiting conversion of PrP^{C} to PrP^{Sc} in a cell culture system (Proske et al. 2002). Furthermore, recent advancements in the field of aptamer creation and selection have helped increase the speed at which these aptamers can be created. A novel aptamer created using a new technique termed Selex has again shown that levels of PrP^{Sc} can be reduced by up to 50% *in vitro* (Mashima et al. 2013). However what is really needed for aptamers to be considered as viable treatment is evaluation of their efficacy *in vivo*. Aptamers may also be useful in diagnostics as their easy ex-vivo selection process and coupling to fluorophores that become unquenched after binding of target protein allows rapid and reliable detection (Xiao et al. 2012).

1.13.9. Conclusion of therapeutic approaches

A large variety of different compounds were tested for their efficacy against prion disease. Many of these treatments showed promise when assessed in cell free conversion systems or in cell based assays. However, follow up studies in animals have often been disappointing showing no efficacy (Trevitt & Collinge 2006). The most promising therapeutic approaches have cleared either PrP^{C} or PrP^{Sc} after inoculation. Antibody therapies are limited by needing to start therapy before the onset of clinical symptoms as well as toxicity (Aguzzi et al. 2007; Sonati et al. 2013). Large progress has been made at understanding the pathophysiology of prion disease. However, there are still no viable therapeutic approaches currently available. Better understanding of how the levels of prion protein in the cell are regulated and genes involved in regulating levels of prion protein may aid in finding novel therapeutic targets that show greater efficacy with smaller toxicity.

1.14. Approaches to finding genetic modifiers of prion disease

Genetic modifiers of prion disease have been predicted as cell free prion formation requires a great energy input (Saborio et al. 2001; Atarashi et al. 2011; McGuire et al. 2012). As cells do not have access to that amount of energy an enzymatic process must be at work. In yeast this protein could be identified by plasmid screening. It was found that HsP104 was required for prion propagation (Chernoff et al. 1995). Furthermore, many genes may regulate the levels of PrP^C and thus modify susceptibility to prion disease. Various attempts have been made at identifying mammalian genes involved in prion physiology.

Three major methods have been utilized to determine genes. Quantitative trait locus analyses in mice models were done to locate loci at which genetic modifiers of prion disease are located (Lloyd et al. 2002; Stephenson et al. 2000). While these studies identified various loci identification of actual genes was not performed. A linkage peak on chromosome 11 looked especially promising but follow up studies were all negative or inconclusive. Analysis of transcriptional changes *in vivo* post prion infection identified various genes that were transcribed at different levels in mice after prion infection (Riemer et al. 2000; Riemer et al. 2004; Hwang et al. 2009). However, in more stringent studies using an *in vitro* approach studying transcriptional changes in three murine cell lines found that there were no significant changes in transcriptional regulation following prion infection. The authors concluded that changes observed in previous studies were most likely due to secondary effects of prion infection (Julius et al. 2008). However, it has to be noted that the lack of differential gene expression may represent the non-physiological nature of in-vitro systems. For example the change in mRNA transcription may be a response to immune factors such as cytokines released by microglia which are absent in neuronal cultures. Third, there were various genome wide association studies performed. These detected the obvious single nucleotide polymorphisms found in the PRNP gene but did not find any other significant associations between prion disease and various genes (Mead et al. 2012). However some hits which were near significance such as the retinoic acid receptor beta. These hits were validated later on. Additionally, a variety of genes have been implicated by the most recent GWAS studies (Lloyd et al. 2013). The gene coding for the metabotropic glutamate receptor 8 is the most recent gene implicated in prion disease

by a GWAS (Sanchez-Juan et al. 2014). Despite some success there is the need for more comprehensive approaches allowing us to examine the function of every gene. The golden standard for finding modifiers of prion disease is the knockout (KO) mouse model. Any gene that is to be considered must demonstrate that when knocked out it changes the incubation time of prion disease or even prevents it as was shown for Prnp (Büeler et al. 1993).

Recent improvements in high throughput screening combined with techniques that allow for knockdown of genes have made it possible to systematically investigate entire genomes for genes that contribute of protect from prion disease. These assays have already been successfully used for high throughput drug screenings finding drugs and pharmacophores which modulate prion disease progression. A FRET assay has been developed and has been used to identify drugs which decrease PrP^C expression (Karapetyan et al. 2013). A high-through-put ELISA screen has identified several chemical groups that may lead to development of drugs decreasing PrP^C levels (Silber et al. 2014). Therefore the opportunity presents itself to combine these advancements in high throughput screening techniques with improvements in genetic manipulation to conduct a large scale genetic screen to identify modifiers of prion disease.

1.15. Introduction to RNAi Screens

RNA interference screens offer the possibility to interrogate the entire genome for genes important in disease or specific cellular processes (Hasson et al. 2013). RNAi screens are the result of over a decade of research on RNA mediated gene silencing. It all began with examination of how double-stranded RNAs (dsRNA) can result in potent and specific interference with gene expression in a sequence dependent manner in C.Elegans (Fire et al. 1998). At first it was hypothesized that the injected RNA could hybridize with the mRNA and therefore inhibits translation or targets it for degradation. However, single stranded RNA was found to not cause silencing of the target gene (Fire et al. 1998). Furthermore, the silencing effect lasted a lot longer than the RNA transcripts which were injected are stable for. It was then found by two teams simultaneously that the silencing effect of the dsRNA depended on cleavage into 21-23 nucleotide fragments (Zamore et al. 2000; Hammond et al. 2000). These small RNA fragments were named small interfering RNAs (siRNAs). The discovery that

silencing was dependent on siRNAs was a big step forward as it allowed application in mammalian cells as introduction of long dsRNA elicits an interferon response in mammalian cells (Sen & Blau 2006).

The question remained what caused the cleavage of the dsRNA and how it results in mRNA silencing. Bernstein and colleagues (2001) found that high speed centrifugation could separate these two functions. The RNA-induced silencing complex (RISC) was responsible for mRNA silencing and was lost during centrifugation while the endonuclease remained. The endonuclease responsible was given the name dicer (Bernstein et al. 2001). To identify the nature of the RISC biotin labelled siRNA was used to co-precipitate two proteins (Argonaute 1 and 2) with the siRNA (Martinez et al. 2002). It was shown that Argonaute 2 is responsible for cutting the mRNA strand thus causing its degradation. Therefore RNAi depends on short RNA fragments made by dicer to guide the RISC complex to the complementary mRNA allowing Argonaute 2 to cleave the mRNA.

1.15.1. Advantages and disadvantages of siRNA screens

As explained above siRNA screens depend on RNA induced gene silencing. This is mediated by the RNA-induced silencing complex (RISC). If exogenous or endogenous double stranded RNA is introduced the RISC will cause gene knock-down in a sequence specific manner by destroying mRNA containing complementary base pairs of the introduced siRNA (Elbashir et al. 2001). This allows for control of protein expression at the mRNA level and provides tremendous possibilities for interrogation of genomic function as well as siRNA based therapeutics. The construction of siRNA libraries has enabled the systematic study of large sets of genes. Already siRNA screens have yielded important findings in diverse biomedical fields (Mohr et al. 2010).

However, care is needed in interpreting the results of primary siRNA screens as many pitfalls are possible. SiRNA screens can be performed in two different ways. Pooled screenings use a viral library that express short hairpin RNA (shRNA) so that one cell will on average contain one type of shRNA resulting in the silencing of one gene. A selecting condition is then introduced that in some way mimics the disease of interest for prion disease addition of brain homogenate of an infected mouse could be used. A large proportion of cells will then die however some cells will contain shRNAs that

protects them from death by silencing a gene that allows for or enhances the progression of prion disease. PCR amplification followed by RNA sequencing of the shRNA present in the target cells can then be performed allowing identification of genes that protect cells from death. Another possible pooled approach is that two sets of cell libraries are created one undergoes the experimental condition while the other remains as a reference set. Again the RNAi strands used are identified by RNA sequencing or microarray analysis. The levels of a specific RNA sequence in the reference versus the experiment set now reveals how important that gene is in protecting from the experimental condition. Pooled approaches have the benefit of being relatively quick and not as work intensive in comparison to non-pooled approaches. The disadvantage of the pooled screen is that they cannot be used to identify non-selecting conditions.

1.16. Introduction summary

This introduction has taken us from the PRNP gene to the primary structure of the prion protein and finally to the 3D conformation of PrP. We have seen that a plethora of single nucleotide polymorphisms can cause a variety of different prion diseases. We discovered that the prion protein most likely encodes information in its structure and that this leads to variable pathology. Prion diseases can be separated based on the method in which they are acquired and further subdivided by specific disease manifestation observed as well as their staining pattern on western blot and codon 129 polymorphisms. Furthermore, a variety of neurodegenerative diseases may actually be prionoid meaning that they rely on self-templating of a misfolded protein catalyzing the conversion of normal protein resulting in amyloid plaque formation. We shortly discussed that prions may have developed in cells as a sort of bet-hedging mechanism to rapidly induce large phenotypic changes allowing a population of cells to survive in case of environmental catastrophe. We looked at current models in which we can research prion disease. We discussed some of the endogenous roles PrP^{C} may have *in vivo*. We also discussed some of the prominent pharmaceutical approaches that have been attempted so far and what their shortcomings are. Lastly, we discussed how there are most likely genetic modifiers of prion disease and how these can be found as well as their importance for developing novel therapies.

As can be seen from this brief overview a large amount of research has been dedicated to studying the prion protein and diseases associated with it. However, there are still important questions which are left unanswered. First it is still unclear if, how and why PrP^{C} is spontaneously converted to PrP^{Sc}. PrP^{C} conversion may represent a random stochastic event however the fact that PrP^{Sc} can be cleared from Prnp KO mice indicates that the clearing mechanism is impaired in sporadic prion diseases. The fact that the conversion also happens readily *in vivo* suggests that a variety of host proteins collaborate in PrP^{C} to PrP^{Sc} conversion. The identities of these are still not known and identifying them may eventually lead to viable treatments for prion diseases. Furthermore, the mechanisms for breakdown of Prion aggregates are not known and it is not clear why PrP^{Sc} can be cleared or decontaminated in Prnp KO mice but not if there is PrP present. Perhaps the rate of conversion of PrP^{C} to PrP^{Sc} overwhelms the clearing mechanisms but this is not concordant with thermodynamic predictions of prion aggregation in which the initial step is extremely slow. The question is then raised as to why there are incubation periods of several hundred days in wild type mice and low dose injection of prions causes sub-clinical disease in which animals do not develop terminal disease but oscillate between disease state and healthy state including reversible pathological symptoms despite having high prion end titres (Thackray et al. 2002). This suggests that there is a homeostatic control mechanism for removing PrP^{Sc} which is inactivated or overwhelmed in prion disease and that at low dose inoculation the mechanism is close to failing but manages to recover.

Lastly, we briefly described that we expect various genes besides the Prnp gene to be involved in prion disease and attempts at finding them. This is the starting point of the thesis in which we utilized a high-throughput semi-automated machine based siRNA time resolved FRET assay to discover novel genes involved in regulating the levels of PrP^{C} and genes aiding in the conversion between PrP^{C} and PrP^{Sc}.

2. Aims

The Prion protein is one of the most studied proteins in biology. However, there are no treatments available for prion diseases. Like many neurodegenerative diseases finding treatment has been difficult due to the inaccessibility of the brain as well as lack of pathomechanistic understanding. Finding drugs that slow down or even reverse the progression of prion diseases like CJD would be a tremendous achievement and also offer hope for finding better treatments for other neurodegenerative diseases. The main question that needs to be answered is why previous studies have failed to find therapeutic approaches. A large amount of studies have been performed looking for viable treatments (See 1.13). However, the methodology used to find these targets is highly divergent and the mechanism by which a certain drug acts is often poorly understood. It is therefore very difficult to identify potential pharmaceutical targets *in vivo* due to the large amount of information available but also due to the variability in the reported results. Additionally, it is difficult interpreting the results from various prion disease models and extrapolating the data to predict efficacy in humans as can be seen from the IND compounds (Berry et al. 2013). Furthermore, genes involved in the prion protein pathway that regulate levels of PrP^{C}, trafficking of PrP^{C}, conversion into PrP^{Sc} and clearance of PrP^{Sc} are still not known.

It is therefore our aim to clarify which genes are involved in the PrP lifecycle and conversion to the pathogenic stereoform. We hope that better understanding genetic modifiers of prion diseases will lead to more specific treatment approaches by revealing molecular therapeutic targets. To fulfil this aim we have developed a high throughput small interfering RNA (siRNA) screen to sequentially disrupt genes in the mouse genome. Using homogenous phase time resolved FRET assays we are able to determine the levels of PrP^{C} and PrP^{Sc} in a high throughput manner. By sequentially determining levels of PrP^{C} and PrP^{Sc} in wells containing siRNAs targeting different genes we can infer which genes regulate levels of cellular PrP and which genes aid or inhibit conversion to PrP^{Sc}. This screen attempts to find genetic modifiers of prion disease which can then be assessed in follow up studies for clinical relevance to prion disease with the hope of discovering novel molecular targets in prion disease.

2.1. Summary of aims

We can therefore identify two clear aims of this study.

1. Identify genes involved in regulating levels of PrP^{C}.
2. Identify genes involved in PrP^{C} to PrP^{Sc} conversion and PrP^{Sc} clearance.

Our screen will reveal a huge variety of genes many of them should form active biological pathways. Therefore, while our screen is suitable for discovering primary hits, bioinformatic analysis and validation of the primary hits by either a second round of screening or other techniques are needed to confirm the findings.

2.2. My contributions

For the initial round of screening I assisted in preparing all the reagents and buffers. I helped with the reformatting of the siRNAs to the assay plates. I assisted Bei Li in running the screen by preparing the robotic platform as well as various things required during the screen. The main analysis of the screen was done by Bei Li alone. Then under the supervision of Bei Li, I performed all the experiments to improve the murine siRNA –based PrP^{C} screen including optimizing manual PrP^{C} screening protocols, establishing and validating automated programs relevant to manual protocols on the Biotek dispenser and the Labcyte platform.

3. The Initial screen

The aim of the initial round of screening was to assess the robustness of our procedure as well as identifying genes important for cell viability, genes important for regulating levels of PrP^{C} and genes involved in conversion of PrP^{Sc} or possibly its breakdown. We utilized three assays that have been developed by Bei Li to achieve this. The initial screen targeted 786 genes of the mouse endocytome with an average of four siRNAs per gene. These genes were chosen due to my supervisor's previous work with the endocytome.

3.1. The three assays

We initially developed three assays that would allow us to assess cell viability, levels of PrP^{C} and levels of PrP^{Sc}. Three assays are necessary to determine at which stage there is a modification of PrP levels. First we need to assess whether our siRNA is toxic to the cells by running a toxicity counter screen. We then wish to identify which siRNAs modulate endogenous levels of PrP^{C}. Lastly we are interested in siRNAs that alter the level of PrP^{Sc} present after prion infection of the cells.

3.1.1. Cell viability assay

The aim of this assay was to infer which genes are essential for cell viability. Loss of cell viability will lead to a non-specific decrease in protein concentration and is therefore not of interest to us. To interrogate cell viability we used a reagent that emits luminescence using ATP as a substrate (CellTiter-Glo® 2.0 Assay) (CT-Glo). If the siRNA in a specific well was toxic to the cells then there would be a decrease in cell number which results in a decrease in ATP and therefore a decrease in the luminescent signal (Promega (Madison, USA)). This cytotoxicity counter screen is often done to assess specificity of the observed phenotype.

3.1.2. $MPrP^{C}$-HPFRET assay

This murine homogenous-phase Förster resonance energy transfer (HPFRET) assay is designed to detect changes in the levels of murine PrP^{C}. It relies on a FRET signal emitted by Europium and APC bound to the PrP targeting antibodies POM 19 and POM 1 respectively. The donor fluorophore europium is stable in various aqueous buffers. It also has a large Stokes shift with its excitation peak at 335nm and its emission peak at 616nm. Due to its long fluorescence half-life of 1.02 ms it enables time-resolved FRET (trFRET). This greatly reduces background fluorescence and

enables a clearer signal (Nishioka et al. 2006). The acceptor fluorophore APC has an absorption maximum of 652 nm and absorbs light at 616 nm with 67% efficiency (lifetechnologies 2015). Using these two fluorophores enables us to detect in a semi quantitative manner the amount of PrP^{C}.

3.1.3. MPrP^{Sc}-HPFRET assay

This assay is designed to detect changes in levels of murine PrP^{Sc}. It also uses FRET based fluorescent detection but the cells are infected using rocky mountain laboratory 6 prions. Furthermore, proteinase K (PK) is added after cell lysis to digest PrP^{C}. PrP^{Sc} is partially resistant to PK digestion and therefore cells which are successfully infected with RML6 will have a high fluorescent signal. Genes that when knocked down decrease levels of PrP^{Sc} but leave levels of PrP^{C} intact may be involved in the conversion of PrP^{C} to PrP^{Sc}. An alternative scenario is that silencing a gene causes an increase in PrP^{Sc} clearance. Conversely wells with higher values will have either an increase in conversion or a decrease in clearance. Genes that affect PrP^{C} conversion are of great interest not only as therapeutic targets for prion diseases but because they may play a generalized role in amyloidogenic diseases.

3.2. Method and materials

3.2.1. Cell culture techniques

The main cell line used in this screen was CATH A differentiated (CAD2A2D5) subsequently referred to as just CAD 5. A stock for the screen was created using a single aliquot. The cells were stored in liquid nitrogen at -196C°. This cell line was used due to its high responsiveness to a variety of prion strains (Mahal et al. 2007). HPL Prnp -/- cells were used as a positive control cell line.

3.2.2. Defrosting cells and growing the cells.

Cells were stored in aliquots at -196C° in OFBS (88% Opti-mem reduced serum medium, no phenol red (Life Technologies, (Carlsbad, USA)) 1% glutamax (Life Technologies, (Carlsbad, USA)), 1% pen/strep (Life Technologies, (Carlsbad, USA)) and 10% fetal bovine serum(Life Technologies, (Carlsbad, USA))) with 10% Dimethyl sulfoxide DMSO. The aliquots were slowly warmed at room temperature until they defrosted. The cells were diluted in 20 ml of OFBS and cultured in T150 cell culture flasks (Techno plastic products, (Trasadingen, Switzerland)). Cells were stored in an incubator at 37°C, 95% humidity and 5% CO_2 during our experiments unless stated otherwise. Media was changed approximately every two days and the cell culture was expanded to yield a large amount of cells. Typically twelve T150 flasks of 90% confluent CAD 5 cells and four flasks of HPL cells were used for 30 assay plates.

3.2.3. Preparation of cells for transfection

The night before transfection media was changed to Pen/Strep free OFBS. On the day of transfection media was changed and cells were detached from flasks by repeated washing of OFBS over the surface of the flask. Cells were pelleted by centrifugation for four minutes at 185g and resuspended in Pen/Strep free OFBS. Cells were counted and an appropriate dilution is made to reach the desired concentration of approximately 3000 cells per well in a 384 well viewplate-384 (Perkin Elmer, (Waltham, USA) containing a pre-prepared mixture of of siRNA and lipofectamine (see 3.2.7).

3.2.4. Robot utilization

786 genes with approximately four different siRNAs targeting each gene and duplicates of each siRNA were used. Therefore, a total of 6288 pipetting steps were required for plating the siRNAs. Each well then required several more pipetting steps

until the assay read out (See figure 14). Performing such as large amount of work is not feasible without robotic automation. A PerkinElmer Janus automated work station (PerkinElmer, (Waltham, USA) was used for all pipetting steps. The work done by the robot can be sequentially described. First the siRNA plates were converted from a 96 well format into three 384 well format assay plate containing all the appropriate controls. The assay plates were labelled A, B or C with A corresponding to the PrP^{C} assay, the PrP^{Sc} assay corresponding to group B and the cell viability assay corresponding to group C (see figure 11 and 12). The robot has two liquid handling heads the modular dispense technology (MDT) 96 well head. To dispense liquid with this head we have to load either 20 or 50μL tips onto the head. The MDT head then aspirated liquid from a Nalgene robotic reservoir (ThermoFisher (Waltham, USA)) and dispenses it into the assay plate. The other liquid handling head is termed varispan arm which can dispense only into eight wells at a single time but is far more flexible into which wells it dispenses. The varispan arm utilized either 25 or 175μL conductive filter tips and aspirates the liquid from a reservoir from Perkin Elmer.

3.2.5. SiRNA printing (Day 0)

The siRNA library we received from Novartis (Basel, Switzerland) were reformatted from 96 well plates into 384 well plates which already contained the controls (Figure 12). The siRNA was prediluted to 600 nM in the 96 well plates by addition of RNAse/DNAse free water. 4μL of siRNA was then transferred to the wells in the assay plate (1.2 picomole of siRNA per well). 4μL of the controls were added to the assay plates and assay plates were frozen at -20°C.

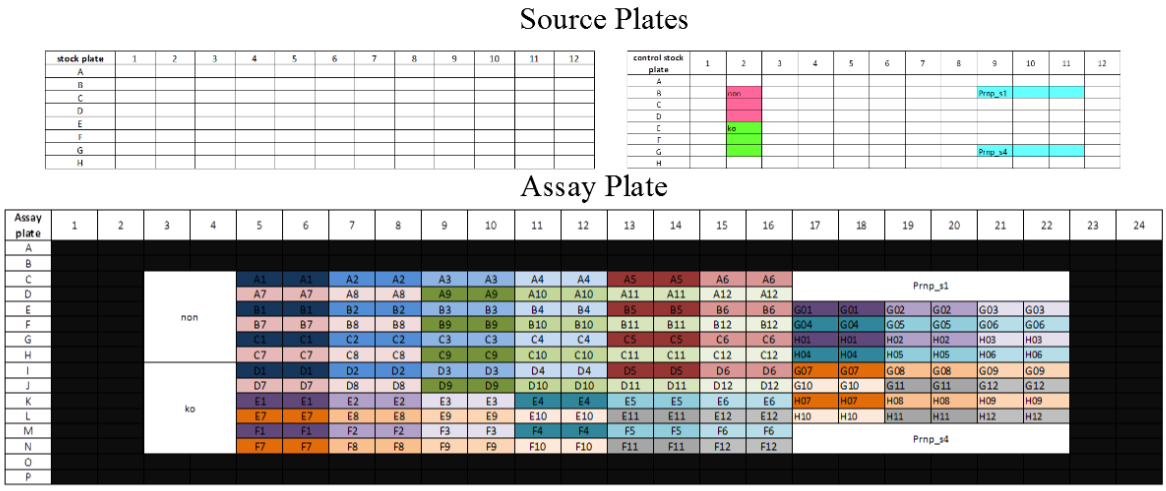

Figure 12. Schematic of plate layout used in the screen. The stock and control plate were reformatted to yield the assay plate. Each well on the assay plate contains 4 µL of siRNA diluted in sterile water to a concentration of 600 nM. Wells C3 to H4 contained normal CAD5 cells and nonsense siRNAs to ensure that the effect seen was due to specific siRNA treatment. Wells I3 to N4 contained HPL Prnp -/- cells. These wells were used for data normalization and as a positive control for the PrP assays. Prnp_S1 and Prnp_S4 (Ambion, Carlsbad, USA) are siRNAs targeting the Prnp mRNA and served as a control for transfection efficacy and possible toxic effects of transfection (See supplementary figure 4 for sequences). Wells C5 to N22 contained sample siRNAs with the same colour representing the four duplicate siRNAs targeting the same gene.

3.2.6. Lipofectamine addition and cell plating (Day 1)

3600 µl of lipofectamine RNAiMAX (Life Technologies, (Carlsbad, USA)) was diluted in 56400 µl of opti-mem, vortexed and poured into a disposable Nalgene robotic reservoir (ThermoFisher (Waltham, USA)). The 96 channel pipetting modular dispense technology (MDT) head using 20µL tips added 4µL of the diluted lipofectamine to the wells C3 to N22 in a 1:1 ratio. CAD 5 WT cells diluted in OFBS without pen/strep were vortexed and poured into a disposable Nalgene robotic reservoir (ThermoFisher (Waltham, USA)). The 96 tip head of the robot was then utilized with 20µL tips to transfer 12µL of OFBS containing cells into the wells as detailed in figure 12. An average of 3000 cells is dispensed per well. The final siRNA concentration is 120 nM at time of transfection. 12µL of OFBS containing HPL Prnp -/- cells are added to wells I3 to N4 using the varispan arm of the Perkin Elmer automated workstation. Empty side wells were filled with 40 µl OFBS. Plates were stored in an incubator overnight.

3.2.7. Prion infection and Media addition (Day 2)

Twenty-four hours after cell plating 20 µl of OFBS with antibiotics was added to the wells in the PrP^{C} and cell titre assays using the 96 tip MDT head. For the PrP^{Sc} assay 88 µl of brain homogenate from mice infected with Rocky mountain laboratories (RML-6) prions was dissolved in 79912 µl of OFBS and 20 µl of the mixture was added to each cell containing well using the 96 tip MDT head. Plates are then returned to the incubator. The PrP^{C} and cell titre assay are then left to incubate for about 72 hours (96 hours from cell seeding). The PrP^{Sc} assay was left to incubate for 96 hours (110 hours from cell seeding) to allow for prion replication.

3.2.8. Cell viability assay and mPrP^{C}-HPFRET assay (Day 4)

The incubator containing the plates was set to 0% CO_2 and shaking at 500 revolutions per minute. 40 µl of Cell-titre Glo 2.0 (Promega, (Madison, USA)) was added to all the wells of the C series. Plates were loaded into the Perkin Elmer envision plate reader (Waltham, USA) and luminescence was measured 15 minutes after adding of the reagent recording emission at 560 nM. For the PrP^{C} assay 10 µl of 5X lysis buffer (supplementary figure 5) was added to all the wells and the plates were incubated in a shaking incubator for one hour. Subsequently 6 µl of Eu Pom 19 and 6 µl of APC POM 1 were added to each well. Antibodies had an end concentration of 5 nM. Plates were left to incubate for one hour in the shaking incubator (see figure 14). Plates were then loaded into the Perkin Elmer envision plate reader. Excitation was at 340 nM by laser. Primary emission filter APC 665 and secondary emission filter Europium 615 were used to generate values for the APC and EU channels.

3.2.9. MPrP^{Sc}-HPFRET assay (Day 5)

10 µl of 5X digestion solution, consisting of 25 µg/ml Proteinase K (PK) and 5X lysis buffer (supplementary figure 5), was added to each well to digest PrP^{C} and simultaneously lyse the cells. PK digestion was halted by addition of 4 µl 30 mM phenylmethylsulfonyl fluoride (PMSF). 7 µl of 0.5M NaOH was added to each well to stop the PMSF reaction to prevent antibody denaturation 8 µl of 0.5M NaH_2PO_4 was added to neutralize the pH. Finally 6 µl of Eu Pom 19 was added to all the wells followed by 6 µl of APC POM 1. Antibodies had an end concentration of 5 nM in each well. The plate was left to incubate for one hour while shaking. Plates were then loaded into the Perkin Elmer envision plate reader (Waltham, USA) and the FRET

signal in each well is detected. Excitation at 340 nM by laser. Primary emission filter APC 665, secondary emission filter Europium 615 .

3.2.10. Data analysis of the cell viability assay

Luminescent values observed in wells containing no cells were subtracted from wells containing the samples. This allowed accurate relative estimation of the number of cells alive in each well. The standard deviation and the coefficient of variation were calculated.

3.2.11. Data analysis of $mPrP^{C}$-HPFRET and $mPrP^{Sc}$-HPFRET

Primarily FRET correction need to be performed for this data. This requires subtracting the spectral overlap from the Europium donor and the background auto fluorescence. Additionally, the APC fluorescence caused by direct excitation of APC from the excitation laser is also subtracted to obtain a true FRET value (Figure. 13). As Time resolved FRET was used spectral bleed through and auto fluorescence was minimized (Xia & Liu 2001). After obtaining corrected values the values were normalized by dividing them by the average of corrected FRET value of the Prnp KO cells and finally the average of the two standardized duplicates was taken.

$$P = \frac{APC\ channel\ I}{Eu\ channel\ I}$$

$$APC\ channel\ I = Mean\ (EU\ alone) - Mean\ (detection\ buffer)$$

$$Eu\ channel\ I = Mean\ (EU\ alone) - Mean\ (detection\ buffer)$$

$$Net\ FRET = APC\ channel\ II - P\ (Eu\ channel\ II)$$

$$APC\ channel\ II = Observed\ value - Mean\ (APC\ alone)$$

$$Eu\ channel\ II = Observed\ value - Mean\ (detection\ buffer)$$

Figure 13.Calculations performed to correct FRET values. While FRET is a very useful technique it requires overlap of the donors emission spectrum and the acceptors excitation spectrum this leads to two main problems primarily the emission spectrum of the donor often reaches into the detection channel of the acceptor. Furthermore the acceptor can also be excited by the excitation of the donor (spectral bleed through). The two fluorophores used here have a relatively large stokes shift minimizing this problem. Furthermore using time-resolved FRET minimizes background fluorescence. However these calculations are designed to remove the spectral bleed through and background fluorescence resulting in the true observed FRET effect.

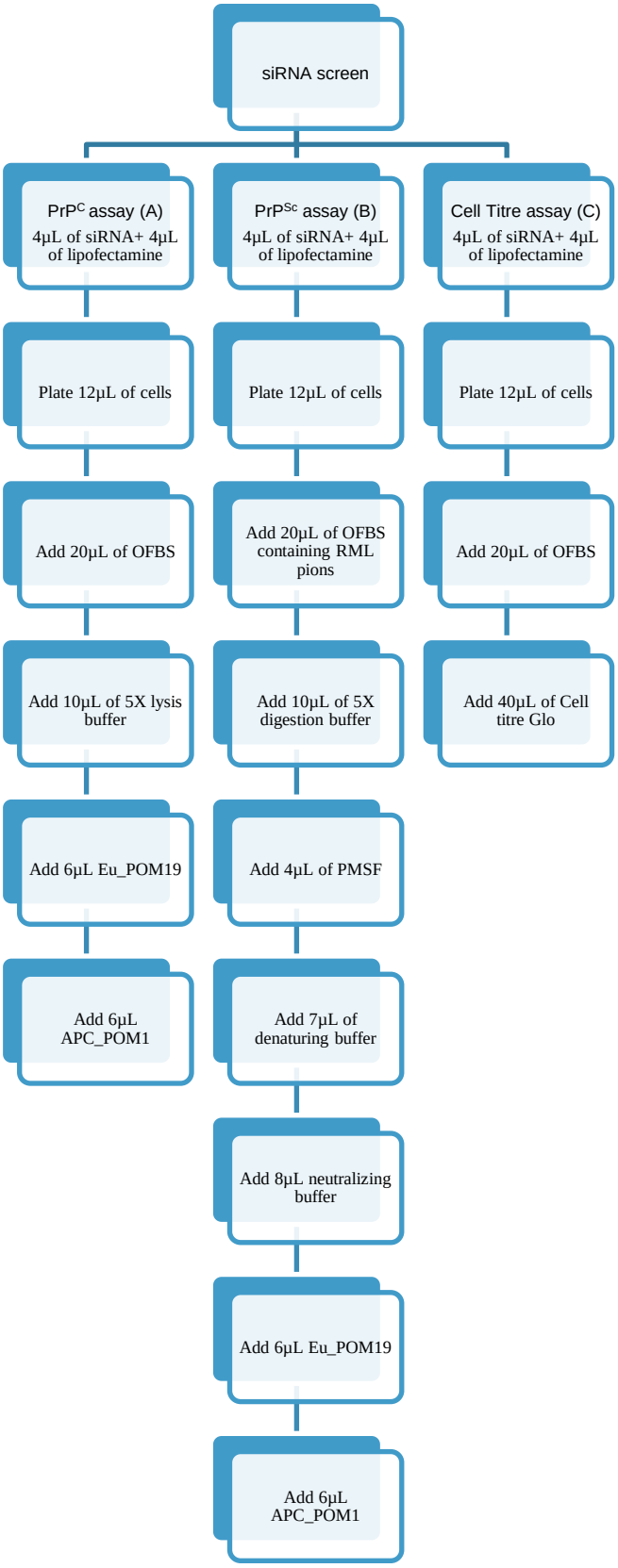

Figure 14. Workflow diagram for the three conducted assays. The assays have sequential steps that allows for addition of reagents in a cumulative manner without ever requiring liquid to be removed.

3.2.12. Generating target genes

A gene was considered essential to PrP^C synthesis if it increased or decreased the value of PrP^C by three standard deviations of the control cells. Genes were split into categories of importance depending on how many different siRNAs worked for the given gene. The same was done for PrP^{Sc} (Figure 15)

3.3. Results

3.3.1. Candidate genes identified by siRNA transfection followed by PrP^{C} or Prp^{Sc} FRET assay

Our aim was to determine genes involved in regulating levels of PrP in cells. Furthermore, we wanted to determine which genes facilitated conversion of normal innocuous PrP^{C} to pathogenic PrP^{Sc}. We started by looking at genes that are involved in the endocytosis pathway. We sequentially knocked down 786 genes in CAD5 cells. We used antibodies for two different epitopes present on PrP. The antibodies were labelled with fluorophores that produce a FRET signal when in close proximity. Relevant genes were determined as having a three standard deviations larger or smaller FRET intensity compared to controls (Figure 15).

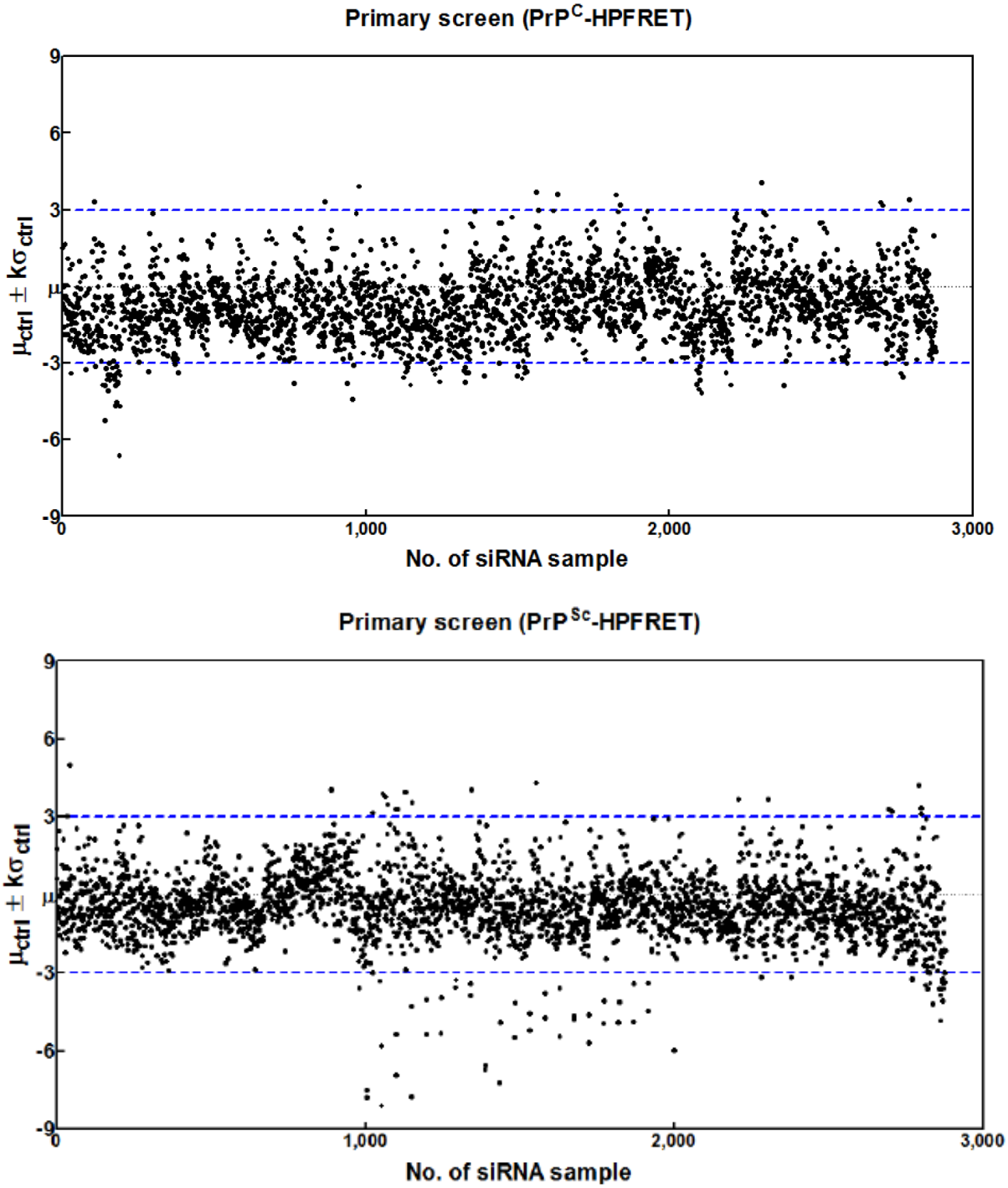

Figure 15. Scatter plot showing FRET intensity relative to the mean ± 3σ of the control for each siRNA sample. The average value of each siRNA duplicate was taken and the mean of the negative control was subtracted. The result was then divided by the standard deviation of the control. (Plots generated by Li Bei and Elke Schaper)

Genes involved in regulation of PrP^C	
Decrease in levels of PrP^C	**Increase In levels of PrP^C**
Atp6v1c1	Snap 29
Myo 1f	Srms
Snap23	Pnck
Rhoq	Mapkapk5
Dync1h1	Rab39
Ddr2	Cdk6
Tie1	Dyrk2
Nsf	Etnik1
Tgfbr2	
Map2k7	
Rhobtb3	
Sik1	
Rab37	
Phka2	
Ilk	
Lats2	
Aatk	
Rab30	
Wasf2	
Eif2ak	
Pak4	
Ror1	
Myo3a	
Rock2	
Riok2	
Vsp39	
Pgk2	
Plk4	
Nrbp2	
Pank1	
Ak1	
Nuak1	
Ripk1	
Map3k10	
Prkdc	
Pxk	
Flt4	

Figure 16. Genes that cause an up or down regulation of the levels of PrP^C

Genes involved in regulation of PrP^{Sc}	
Decrease in levels of PrP^{Sc}	**Increase in levels of PrP^{Sc}**
Arf5	Atp6v1c2
Itpk1	Mip
Grk6	Dapk1
Ptk7	Rhoa
Khk	Eef2k
Pmvk	Uckl1
Sphk1	Npr1
Ttbk2	Myo3a
Pfkb4	Grk5
Plk3c2b	Styk1
Mapk3	Mapk6
Ern1	Pck2
Prpf4b	Papss1
Insrr	Rab39
Epha2	Cdk6
Sphk2	Dyrk2
Stx1b	Etnik1
Nek8	
Hsob8	
Lrrk2	
Pacsin1	
Hk1	
Scyl2	
Gne	
Phkg2	
Ltpkc	
Mapkabpk3	
Stk16	
Rab35	
Cdc7	
Ret	
Map3k10	
Prkdc	
Pxk	
Flt4	

Figure 17. Genes that cause an up or down regulation of the levels of PrP^{Sc}

We determined 37 candidate genes in which levels of PrP^{C} were decreased. We found eight genes that when silenced resulted in an increase in the level of PrP^{C} (Figure 16). Additionally, we repeated this experiment but infected cells with RML6 prions followed by a proteinase K digestion step, ensuring that all PrP^{C} is digested only leaving PrP^{Sc}. From addition of these steps we hoped to be able to determine genes involved in conversion from PrP^{C} to PrP^{Sc}. We determined 35 genes that decreased levels of

PrP^{Sc} and 17 genes that increased levels of PrP^{Sc} (Fig. 17). However, system testing using untreated cells cast doubt on these results (Fig. 18 and 19).

3.3.2. System testing

To ensure that the data we observed was accurate we produced heat maps of the first 20 screening plates (excluding plate 6 due to experimental error) showing their relative PrP^{C} FRET signal.

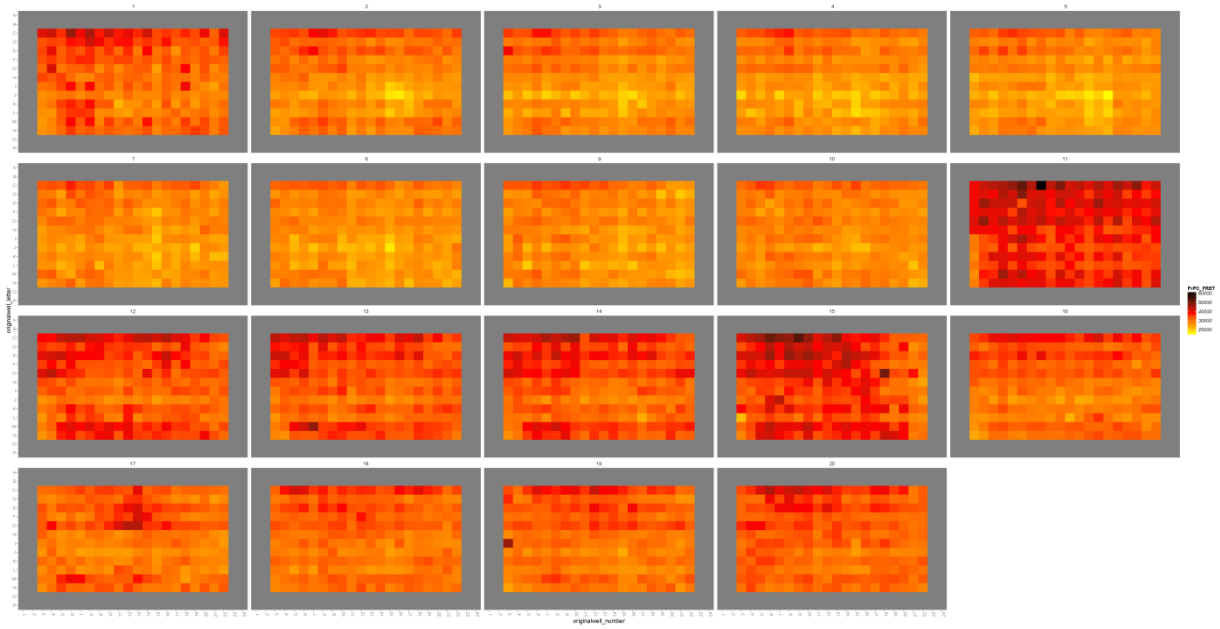

Figure 18. Heat maps of the 20 first screening plates showing relative obtained PrP^{C} signal. As can be determined from these plates there was wide inter and intraplate variance. Furthermore it is impossible to distinguish negative controls which are located at the bottom left hand side of the plates (see Figure 12.) (Data provided by Bei Li, Marc Emmenegger and Elke Schaper)

We are unable to determine potential hits from false positives in the screen as we cannot differentiate our WT and knockout cells or our positive siRNA control from the rest of the plate. Therefore, genes in figure 16 and 17 most likely represent false positives due to the large experimental noise observed in the plates. We wanted to determine whether the source of this large variance was caused by the addition of siRNAs or if this effect also occurs without addition of siRNAs. Therefore we ran the PrP^{C} protocol (see 3.2.8) without the addition of siRNAs.

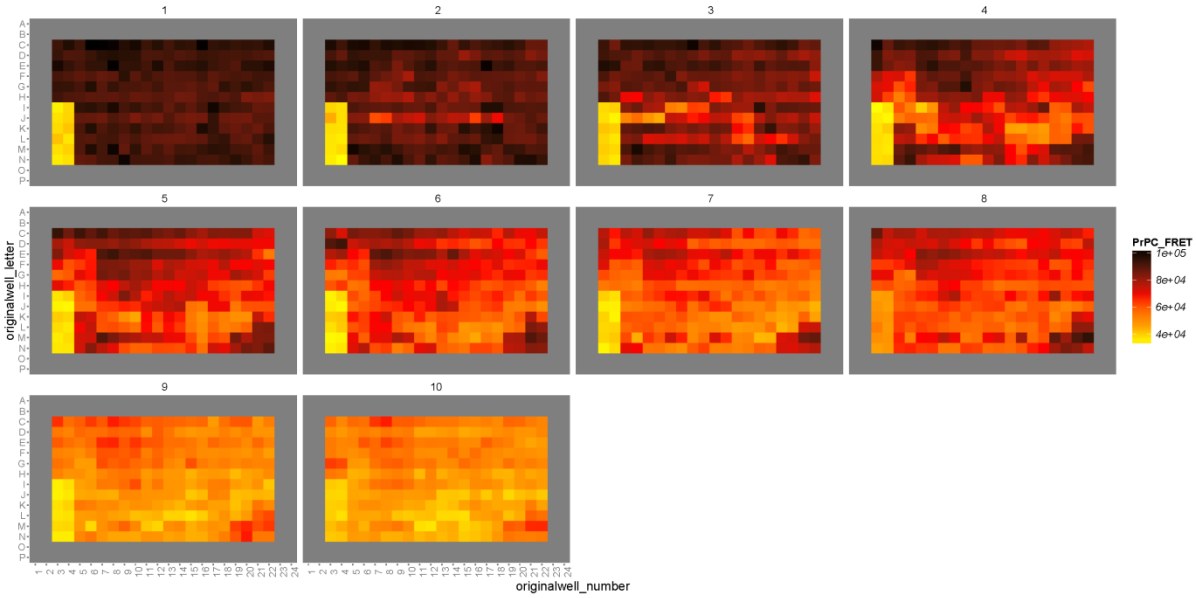

Figure 19. Relative PrP^C FRET over ten plates not containing siRNAs. The plate layout used was identical to the one used in the screen. (Data provided by Marc Emmenegger and Elke Schaper)

The first plate produced shows homogenous distribution and the negative control can clearly be distinguished from the positive control. The more plates are produced in sequence the worse the distribution gets. By the 10^{th} plate we observe the effect that the negative control is virtually indistinguishable from the other wells. Furthermore, a heterogeneous intensity can be observed in the top left corner and bottom right corner where there is uneven intensity of the signal.

3.4. Discussion of the initial screen

We performed a high-throughput siRNA screen using a HPFRET detection assay to determine genes important for regulation of levels of PrP^C and genes important for conversion and possibly clearance of PrP^{Sc}. We found a large number of genes that may be involved in these mechanisms. However, quality control of the data revealed that our results were indistinguishable from experimental noise. Furthermore, we would expect genes that result in a decrease of PrP^C to also result in a decrease in gene levels of PrP^{Sc}. This was not the case with only four genes that when decreased in the PrP^C assay also resulting in a significant decrease in the PrP^{Sc} assay, again indicating that our screen did not produce reliable results. Therefore, we did not perform any follow up studies to confirm primary hits as these can be assumed to be not significantly different from experimental noise.

The main question is why our screen generated such noisy data. Many fruitful discussions yielded various reasons for the noisy data. We will discuss how these problems arose and then attempt to resolve them in the section entitled screening optimization.

I. The seeding of the cells onto the plate resulted in a different number of cells in each well. Our assays are highly sensitive to cell number. Therefore by seeding different number of cells the value obtained by the FRET assay was highly heterogeneous and not necessarily representative of the level of PrP^C per cell.

As described in the methods we used a 96 well head to aspirate cells suspended in media and subsequently distribute them to the wells. We discovered that this is a major problem as cells quickly settled on the bottom and the side of the reservoir holding the cells. This resulted in a decreased cell number being aspirated as time went by. While we tried to counteract this by being very quick and mixing the cells during the procedure there seems to be an unavoidable heterogeneity in the dispensing of the cells (see figure 20).

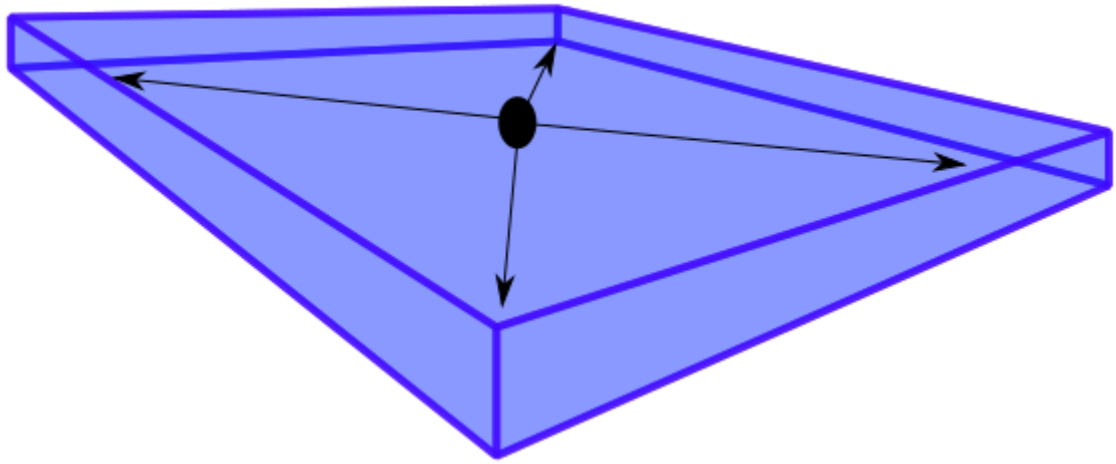

Figure 20 Cell dispersion in the reservoir. By utilizing a 96 well head cells which were in the reservoir quickly settled to the bottom and the repeated immersion of the pipettes caused them to disperse away from the pipettes. This led to slowly decreasing cell density and heterogeneity in cell plating.

II. The siRNA concentration used was too high. While seeding of cells was a major problem it can be seen that even the first plates seeded (1 and 11) in figure 17 have high heterogeneity and the experimental wells are indistinguishable from the positive control wells. The high siRNA concentration used may have contributed to this.

We used a siRNA concentration of 120 nM. Using such a high siRNA concentration frequently results in off-target effects. This is due to the competition of the exogenous siRNAs with the endogenous small hairpin and micro RNAs for nuclear export factors such as exportin-5 and competition for RISC complex binding (Campeau & Gobeil 2011). Furthermore, off target effects can be the result of the 5' region of the siRNA binding the 3' untranslated region of the mRNA in a semi-complimentary manner. This effect mirrors the ability of microRNAs to bind to multiple mRNA transcripts and cause wide gene silencing. Various studies have found that reducing siRNA concentration can potentially reduce off-target effects (Semizarov et al. 2003). However, other studies have found that siRNAs have off-target effects even the minimal efficient concentration (Jackson et al. 2003; Sigoillot et al. 2012). We partially compensate for off-target effects by using four different siRNAs targeting the same gene.

III. The cell number used for plating was too high. This may contribute as the cells stop in their linear growth phase resulting in an uneven cell number. CAD 5 cells may also alter their gene expression pattern once they reach confluence.

Cells may alter their expression profile when reaching confluence. Furthermore, confluence may lead to cellular stress and therefore alter their protein expression patterns. Heat shock of cells can lead to an upregulation of Prnp mRNA and PrP levels. Therefore, plating too many cells may add additional experimental noise by varying the level of PrP expression (Shyu et al. 2002)

IV. We were not able to assess cell numbers directly in the assay plate even though cell growth may be different in assay plate and the CT-Glo plate despite being treated the same.

Unfortunately CT-Glo interferes with the FRET signal. Therefore, assessing cell viability directly in the plates in which we performed the PrP^{C} and PrP^{Sc} FRET assays was not possible. Extrapolating cell viability from the cell titre assay plate and correlating it with the FRET signal in the other two plates did not really work as each plate is exposed to unique conditions such as temperature and air gradients as well as different processing times and different transfection efficiency of each siRNA.

Another difficulty encountered is that the CT-Glo assay is an endpoint assay meaning that only a single readout of cell viability is possible. It would be much more preferable to have an assay with multiple readouts as this would allow us to assess homogeneity of cell seeding as well as calculate toxicity kinetics by assessing cell growth rates rather than just using a single endpoint. We explored the use of CAD 5 cells that have stably integrated a YFP-GPI (yellow fluorescent protein – Glycosylphosphatidylinositol) fusion protein. In theory we assumed that the YFP signal could be used to predict cell number. However the fluorescent signal obtained from these cells was too low to estimate cell numbers.

V. Time is extremely critical to maintaining homogeneity. As more time passes gradients develop in the plate making observation of non-random effects more difficult. Furthermore, siRNA screens have a traditional measurement time point between 48 and 72 hours. We measured effects after 96 and 110 hours. It was shown that we had a reliable knockdown of PrP after 96 hours using siRNAs against Prnp. However, different siRNAs may have different efficacies and half-lives. Therefore, when performing the screen we may receive multiple false negatives because the siRNA against the gene in question was no longer effective or the knockdown was not efficient enough to detect an altered FRET signal.

Prion kinetics are very important to keep in mind when performing these experiments. Unfortunately no literature exists on the half-life of PrP in CAD5 cells. Furthermore, the two papers that look at PrP^{C} kinetics in N2A cells used anti-PrP antisera, as monoclonal antibodies were not yet readily available. Borchelt et al. (1990) put the half-life of PrP^{C} at 5.2 hours (Borchelt et al. 1990). The half-life of Prnp mRNA is approximately 7 hours (Pfeifer et al. 1993). Analysis of how mRNA degradation affects

level of PrP^{C} and PrP^{Sc} has been done (Minkel 2013a). If Prnp mRNA is knocked down the maximal PrP^{C} knockdown will be observed after about 72 hours. Therefore, readout of the PrP^{C} assay between 72 and 96 hours seems ideal to detect changes in level of PrP^{C}.

In the PrP^{Sc} assay the case is more complicated. Additional time to allow for prion replication is required. The kinetics of PrP^{Sc} are radically different as it is much slower to be degraded. Therefore after a 50% knockdown of Prnp mRNA the levels of PrP^{Sc} take around 110 hours to drop down to 55%. However, it also needs to be considered that running the assay for a long time can introduce errors into the plate by unequal evaporation due to temperature gradients and different gradients in cell growth. We will have to make a trade-off between optimal predicted level of PrP^{Sc} and errors introduced by the assay run time. Furthermore, it is not clear whether the siRNA is still effective after such a long time span and if we can determine whether the siRNA had an effect on prion replication. We can see that assay run time is extremely critical for achieving good results.

4. Optimization of screening procedures

We have encountered various difficulties in our initial screen. We therefore addressed some of the initial problems which were identified. At the same time we optimized data and experimental workflow by creating automated systems for data analysis and moving our existing screening protocols to new devices. Primarily, I was concerned with optimizing the cell seeding density and siRNA concentration levels. Furthermore our cell viability cytotoxicity counter screen was not effective for detecting toxic siRNAs. Cytotoxicity counter screens are typically done for small molecule inhibitor libraries where the majority of molecules are biologically inactive. SiRNAs however are all biologically active. Therefore, cell viability is strongly affected by factors such as transfection efficiency, specific cell seeding density and processing time of plates. We were unable to determine which siRNAs were toxic or cytostatic by an endpoint assay because of inherent noise in the signal which is introduced by our screening procedure. Therefore, measuring the viability of cells directly in the assay plates is desirable. However the reagent used in our previous screen interferes with the FRET signal making it not useable. I tested and implemented a new reagent that allows us to determine cell viability directly in the assay plate.

4.1. Method and materials

Methods such as cell culture and the three assays were not significantly altered. However, both manual protocols for siRNA forward and reverse transfection were used. Implementation of multiple novel machine and robotic systems also took place to replace the Perkin Elmer Janus robot.

4.1.1. Cell culture

CAD 5 cells for all experiments were cultured as described previously. Hpl Prnp -/- cells were replaced by CAD 5 Prnp -/- cells that were created using the CRISPR / CAS 9 system.

4.1.2. Bicinchoninic acid assay (BCA assay),

Cells were washed using cold sterile phosphate buffered saline (PBS) and then detached using trypsin 0.05% EDTA (Gibco (Carlsbad, USA)). Trypsin was neutralized by addition of OFBS. The cells were placed in a 15 mL falcon tube depending on volume and centrifuged at 180 G for 5 minutes. The supernatant was discarded and the pellet resuspended in 500 - 1500 µL of 5X lysis buffer followed by rigorous

vortexing for 1 minute (Supplementary figure 5). The tube was then left for 20 minutes on ice followed by another round of vortexing. Finally the tube was centrifuged at 16,500 G for 15 minutes. The supernatant was transferred into a new Eppendorf tube.

The standard for the BCA assay was created using 2 mg / mL bovine serum albumin (BSA) (Pierce, (Waltham, USA)) adding 25 µL per well. Depending on the amount of sample available it was diluted 1:10. 200 µL of reagent AB (Pierce, (Waltham, USA)) is added to all the wells. The assay plate was incubated for 20 minutes at 37C° and then read using a spectrophotometer to estimate protein concentration.

4.1.3. SiRNA transfection

Both forward and reverse transfections were used. In Forward transfection the desired number of cells was plated in a 384 well viewplate (Perkin Elmer, (Waltham, USA) in antibiotic free OFBS. The siRNA was diluted in Opti-mem to the desired concentration from a 10 µM aliquot. Lipofectamine RNAiMAX was diluted in opti-mem proportional to the siRNA concentration used (0.3µL of RNAiMAX per 1 picomole of siRNA). SiRNA and lipofectamine were mixed and left to incubate for 15 minutes at room temperature. The desired volume of siRNA / lipofectamine complex is added to the cells.

While forward transfection generally results in higher transfection efficiency and less cellular toxicity it is not practical in a high through-put format. For reverse transfection siRNA dissolved in Opti-mem was plated either by hand or using the Echo acoustic dispenser (Labcyte, Sunnyvale, USA). These plates were either processed directly or frozen for future use. Lipofectamine RNAiMAX was added either manually or utilizing the Biotek MultifloFX (Luzern, Switzerland) peristaltic pump using the 1 µL cassette. The plates were left to form siRNA lipid complexes for 15 minutes at room temperature. Cells were added either by hand or using a 5 µL cassette with the MultifloFX.

4.1.4. Addition and reading of Real Time Glo

Real time Glo was diluted in OFBS and added either manually or using a 5 µL cassette with the MultifloFX. Luminescence reading was conducted up to every twelve hours or every 24 hours- Recording of emission was at 560 nM using the Envision plate reader.

4.1.5. PrP^{C} and PrP^{Sc}-HPFRET assay

The FRET assays were performed manually as described in figure 14. Additionally the PrP^{C} assay was adapted for use with the Biotek MultiFloFX. A 5 µL cassette was used to dispense 10 µL of 5X lysis buffer. Plates were sealed and placed on thermomixers (Eppendorf (Hamburg, Germany)) at 750 RPM at room temperature for one hour at room temperature. Eu-Pom19 and APC-POM1 were added using two different 1 µL cassettes. Plates were sealed again and placed on the thermomixer at 750 RPM at room temperature for one hour and centrifuged at 2800 G for four minutes. Plates were then read as described previously.

4.1.6. Statistical analysis

Graphpad Prism 5 (California, USA) was used for generating all data and statistical tests. Significance is indicated as following.

ns $P > 0.05$

* $P \leq 0.05$

** $P \leq 0.01$

*** $P \leq 0.001$

**** $P \leq 0.0001$

4.2. Results

A variety of optimization tests were run. Each experiment is given its own section briefly giving an introduction to the experiment and summarizing the experimental protocol. This is followed by a description of the main findings and the significance of the findings.

4.2.1. Real-time Glo is a viable alternative to determine cell viability in screening plates

In the initial screen we used CT-Glo to determine cell viability and whether siRNAs exerted a toxic effect on cells or slowed their growth. This toxicity counter screen allows us to exclude siRNAs that reduce cell growth or cause cell death. However, we realized that extrapolating cell growth using a single end-point assay in a separate assay plate did not allow conclusive determination of the cytotoxic or cytostatic effects of a specific siRNA. We therefore tested the compatibility of real-time Glo (RT-Glo) (Promega (Madison, USA) with our FRET assays. RT-Glo consists of NanoLuc luciferase and a pro-NanoLuc substrate that is converted into the substrate for NanoLuc in living cells based on their reduction potential.

We performed a PrP^{C} FRET experiment adding 20 µL of 2X RT-Glo diluted in OFBS medium 24 hours after transfection of 3000 CAD 5 cells with 120 nM siRNA. Furthermore, we validated the use of RT-Glo when performing a PrP^{Sc} FRET. We plated 3000 CAD 5 WT and 3000 CAD 5 Prnp -/- cells and incubated them over night. The next day we added 20 µL of RT-Glo of OFBS containing brain homogenate from RML 6 infected mice diluted by a factor of 1:6000 to halve the wells and non RT-Glo containing OFBS to the other halve.

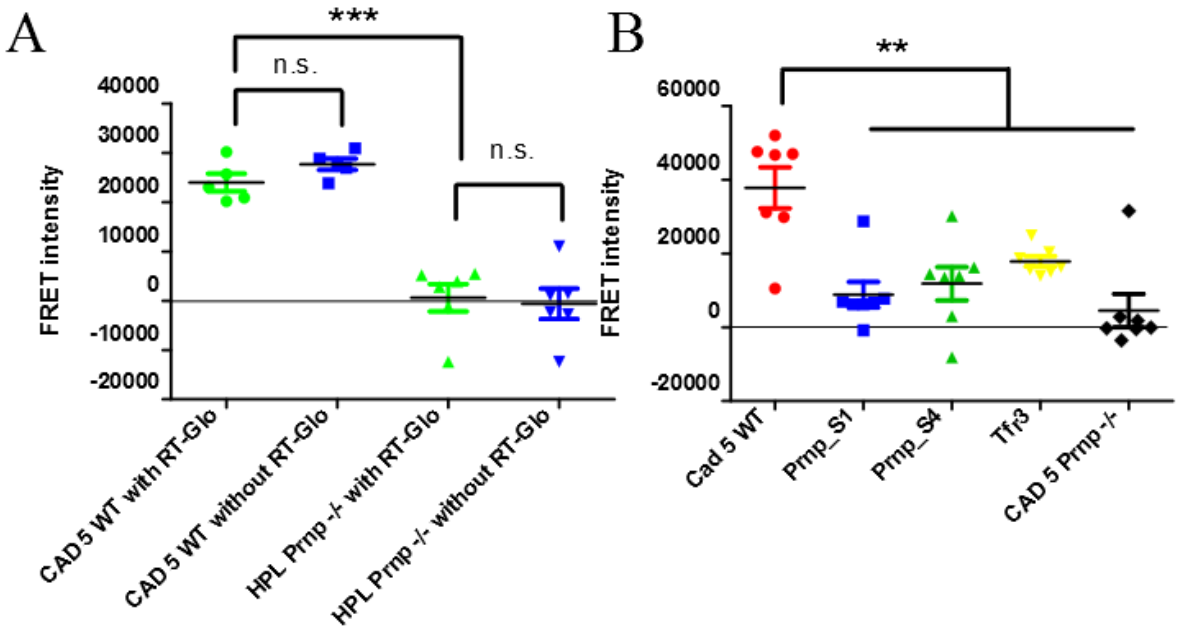

Figure 21 PrPC and PrP^{Sc} FRET performed with RT-Glo. A) PrP^{Sc} FRET in which RT-Glo containing media was added to half the wells and normal media to the other. B) PrP^{C} FRET with siRNA targeting Prnp and the transferrin receptor (TfR) where media containing RT-Glo was added.

It can be seen that there is no observable difference between adding RT-Glo and not adding RT-Glo for the CAD 5 WT and the HPL Prnp -/- cells when performing the PrP^{Sc} FRET experiment ($P>0.001$, unpaired t-test). We could also confirm that siRNA knockdown and our PrP^{C} FRET assay still functions correctly when using RT-Glo ($P<0.01$, One-way ANOVA with Dunnett's multiple comparison test). We establish that RT-Glo does not alter the observed FRET signal.

4.2.2. RT-Glo can model cell growth over prolonged periods of time

Our assay is very sensitive to variations in cell number. We established that RT-Glo can detect a change of between 750 and 1500 cells (supplementary figure 9). To further enhance our sensitivity we decided to measure each plate at regular intervals of approximately twelve hours during the screening procedure. From this data an automated line of best fit can be generated to model growth of the cells over time. We tested varying cell numbers with different concentrations of RT-Glo to establish what happens in our assay plates over time.

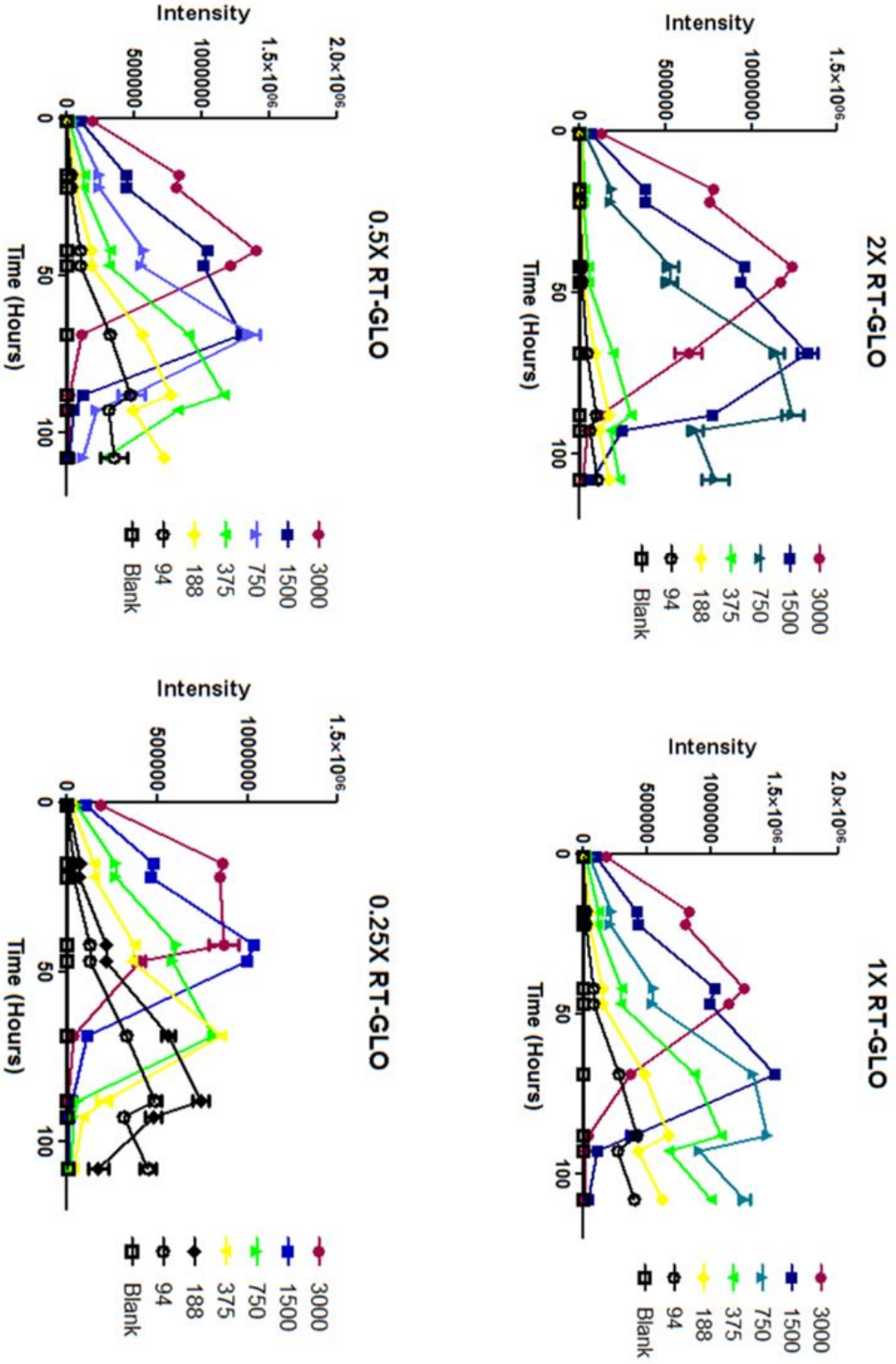

Figure 22 RT-Glo dilution grid probing various concentrations and cell numbers. To determine the optimal concentration of RT-GLO we tested four different concentrations of RT-Glo and seven different cell numbers. A linear increase in RT-Glo signal can be observed in the initial phase. This is followed by a rapid drop of the observed signal.

We can see that the signal obtained for 2X, 1X and 0.5X is virtually identical and therefore started performing all our experiments at 0.5X RT-Glo concentrations. Furthermore, we saw that when 3000 cells are plated and incubated for 96 hours that there is a drastic drop in the signal after about 50 hours. When only 1500 cells are plated the signal last much longer for over 72 hours, this effect was not dependent on the initial concentration of RT-Glo.

4.2.3. Optimizing cell number

We needed to reduce the cell number to prevent a decrease in the RT-Glo signal. However the maximal number of possible cells should be plated to prevent loss of signal to noise ratio. Therefore, we tested whether we could detect a suitable FRET signal plating only 1500 or 1000 cells followed by manual addition of 120nM siRNA targeting the Prnp or Tfr gene.

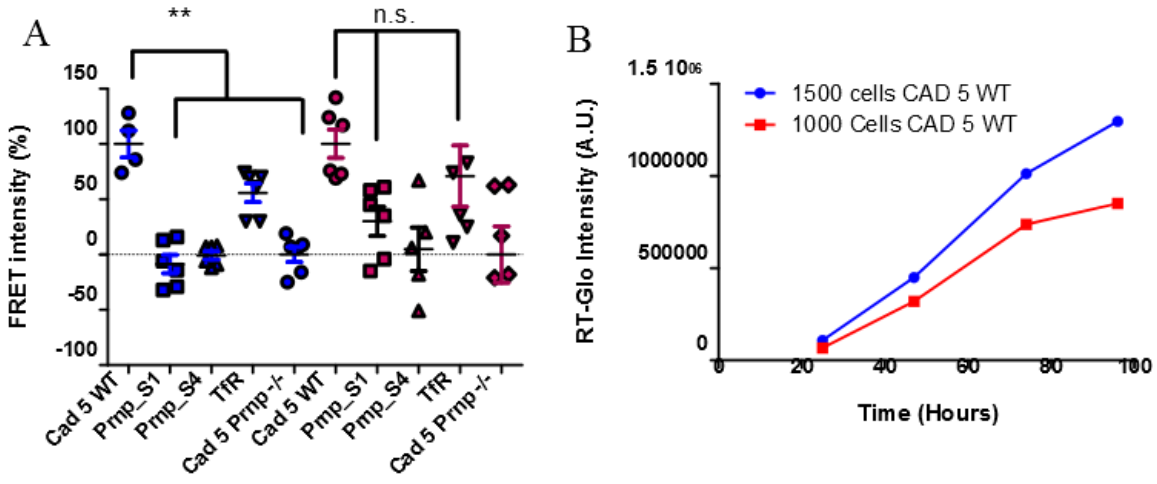

Figure 23. PrP^C FRET and RT-Glo values for 1500 and 1000 cells respectively. A) Relative PrP^C FRET intensity for 1500 (blue) and 1000 (red) cells after treatment with various siRNAs. B) Linear RT-Glo values confirming that we neither experience cell death or reagent depletion during our assay. (2 data points for the CAD 5 WT 1500 group were excluded that valued at -35 and -71 due to experimental error)

We found that plating 1500 cells gave us a robust FRET signal with a small coefficient of variation and showing a significantly decreased FRET intensity in the siRNA treated and CAD 5 Prnp -/- cells (One-way ANOVA with Dunnett's multiple comparison test, $p<0.01$) When only 1000 cells were plated there was an increase in the coefficient of variation leading to loss of significance in the Prnp_S1 and Tfr treated siRNA group. (One-way ANOVA with Dunnett's multiple comparison test, $p>0.05$). The growth rate as measured by RT-Glo was linear for both conditions a linear regression was calculated and the R^2 value for the respective lines were determined (R^2= 0.9903 for 1500 cells, 0.9687 for 1000 cells). We therefore obtained a robust FRET signal as well as linear signal for RT-Glo.

4.2.4. Optimizing siRNA concentration

While a high siRNA concentration can lead to efficient target knockdown it may also result in possible off-target effects and cytotoxic / cytostatic effects (Fedorov et al. 2006). Therefore, we determined the lowest siRNA concentration producing efficient knockdown. We plated four columns of CAD 5 WT cells in a 384 well plate with each well containing 1500 cells at the start. In three of those columns we added siRNAs complexed with lipofectamine RNAiMAX targeting Prnp and transferrin receptor mRNA. In the 5^{th} column we plated CAD 5 Prnp -/- cells to determine the efficiency of our siRNAs. We repeated this pattern to end up with a total of four blocks treated with 120 nM, 60 nM, 40 nM and 20 nM siRNA. We performed a PrP^C FRET to estimate the level of PrP in each well.

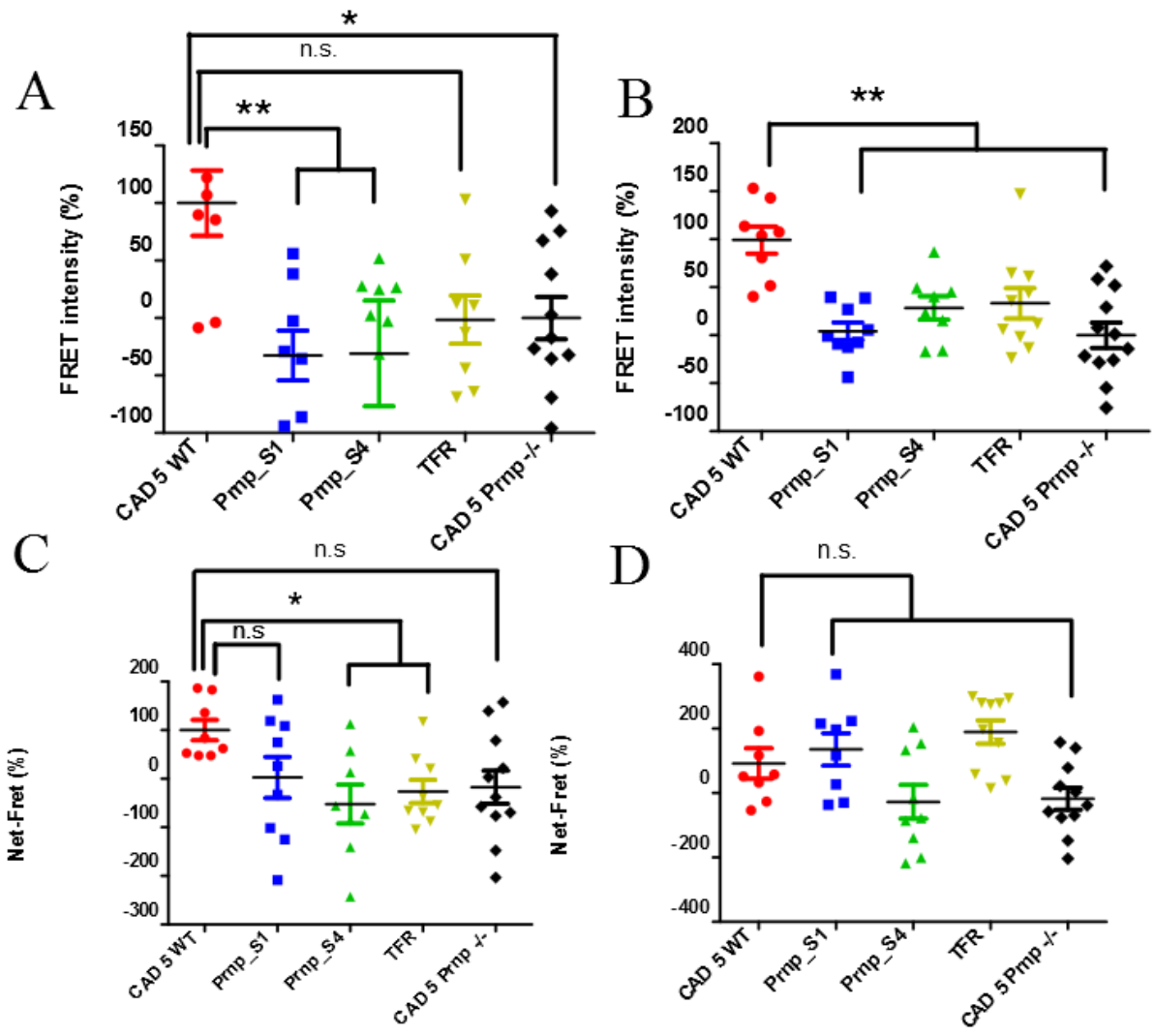

Figure 24. PrP^{C} FRET at varying siRNA concentrations. A) 120 nM, B) 60 nM, C) 40 nM, D) 20 nM.

The data generated has a general high coefficient of variation. Furthermore, we failed to detect significance in some cases even between the CAD 5 WT and CAD 5 KO cells (One-way ANOVA with Dunnett's multiple comparison test, $p>0.05$). This was due to various experimental problems. The siRNA addition was very slow leading to degradation of siRNA efficacy. Additionally, during addition of siRNA some cells were lost in certain wells due to mechanical abrasion of the pipette tips on the bottom of the well leading to uneven distribution of cells within some wells. The long storage of the plates led to temperature dependent plate gradients leading to variable cell growth rates. However, the experiment still indicated that a siRNA concentration around 40 nM would be the lowest concentration showing efficacy.

We repeated the Experiment with slightly varying siRNA concentrations. To confirm optimal working concentrations of siRNA we again performed a PrP^C FRET lysing cells 96 Hours after addition of the siRNAs.

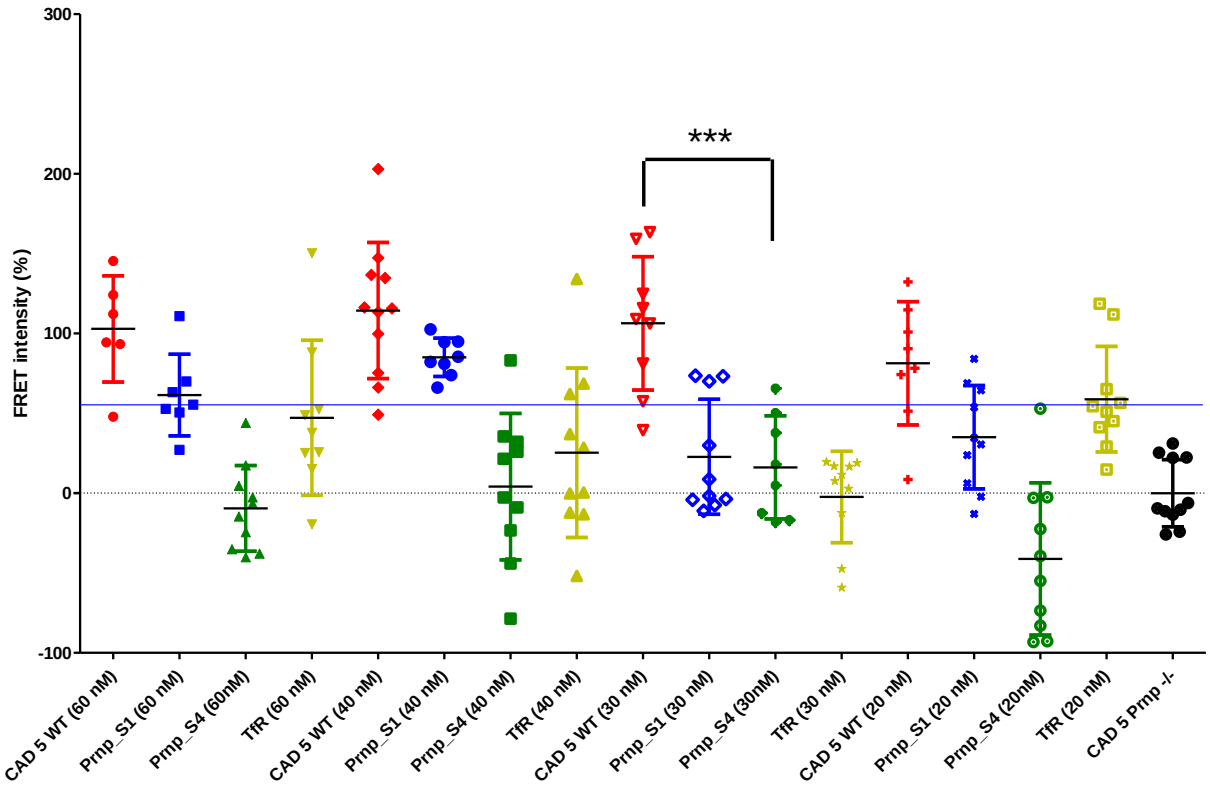

Figure 25. PrP^C FRET at varying siRNA concentrations. It can be observed that at 30 nM there is a significant decrease in FRET intensity for all three siRNAs ($p<0.001$, One-way ANOVA with Dunnett's multiple comparison test.)

Again we faced various problems such as plate temperature gradients. Furthermore we observed a large amount of cell death and decreased growth in certain wells. We posited that this may be not from off-target effects but due to toxicity of the transfection reagent itself. We decided to perform a toxicity vs transfection efficiency titration to increase the viability of our cells. While the variation in FRET intensity was very large we could still infer that 30 nM seemed like a good working concentration in which there was efficient knockdown of target mRNA.

4.2.5. Reducing the time scale

Optimal protein knockdown occurs 72-96 hours after siRNA addition. We attempted to adapt our protocol to work in a 72 hour timeframe to reduce plate gradients. We manually plated 1500 to 3000 cells to establish the optimal number of cells for this timeframe. We then proceeded to test the efficacy our 72 hour protocol using 30 nM siRNA concentration. The cell growth was monitored using RT-Glo.

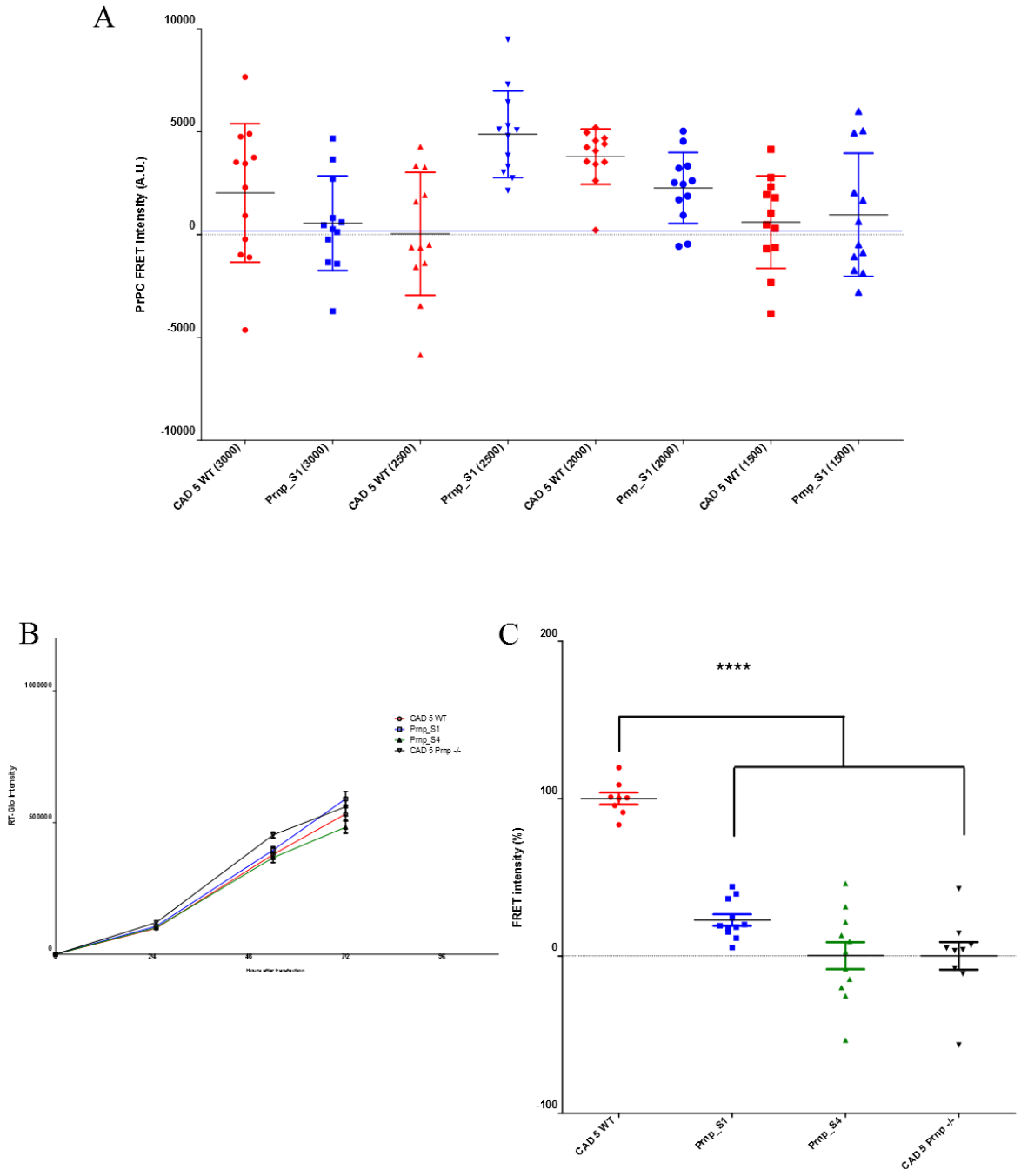

Figure 26 siRNA protocol reduced to a 72 hour run time. A) Determining optimal cell number for 72 hour format.(It is likely that the data points for the 2500 cells WT and Prnp_S1 treated are switched as the siRNA was added to the incorrect column) B) Cell growth curves showing linear cell growth when plating 2000 cells for all experimental conditions. C) FRET intensity for wild type CAD 5 cells compared to CAD 5 treated with Prnp_S1, Prnp_S4 and CAD 5 Prnp -/- cells.

We can establish that 72 hours is a good timeframe to conduct our experiments in. We expect to see less prominent effects of temperature gradients and have extremely nice RT-Glo growth curves in this timespan. Furthermore siRNA knockdown appears to be at almost 100% efficiency for this timeframe using 30 nM siRNA. We established around 2000 to 2500 cells to be optimal for the 72 hour timeframe. It has to be noted that we used a vastly inferior APC-POM 1 conjugate for this and the following experiments making interpretation of the results more difficult (See supplementary figure 3 for comparison of old and new APC-POM 1 efficacies).

4.2.6. Transfection reagent efficiency

Careful titration of transfection reagent needs to be done to obtain maximal transfection efficiency with minimal observed toxicity. Transfection reagents can lead to activation of cellular stress response pathways which prevents cell cycle progression leading to an arrest in cell growth. We tested three different concentrations of our transfection reagent lipofectamine RNAiMAX (Thermoscientific Fisher, Waltham, USA). Additionally, we performed siRNA titration to determine the lower limit of siRNA efficiency. 1X indicates the manufacturers recommended concentration of 0.3µL per picomole of siRNA. We waited for 72 hours before performing the FRET with an initial seeding concentration of 2000 cells.

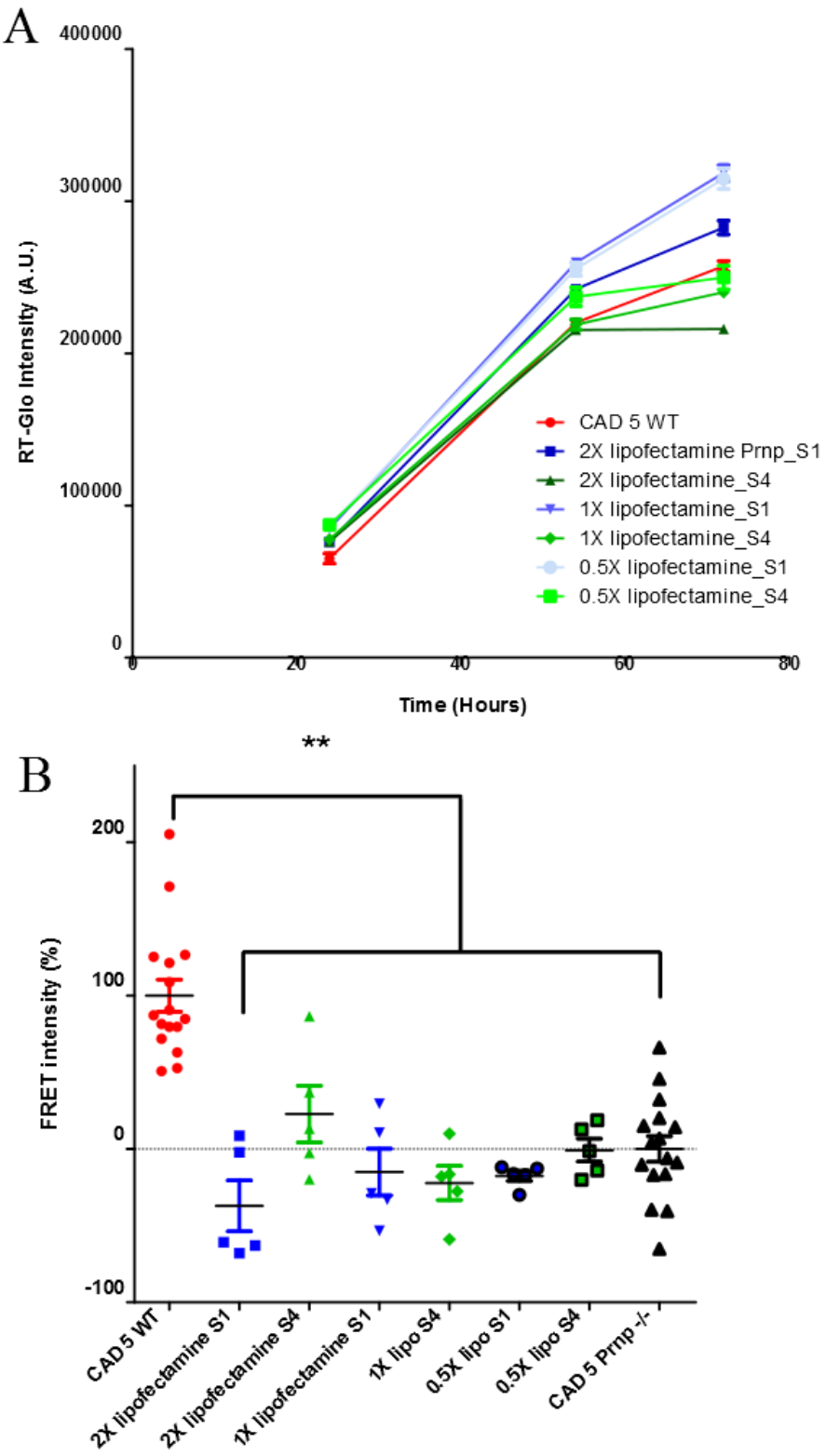

Figure 28 2000 cells plated for 72 hours at 3 different lipofectamine concentrations transfected with Prnp S1 and Prnp S4. A) Growth curves monitored using RT-Glo indicate that doubling the concentration of lipofectamine is toxic. Furthermore, it is clear that transfection with the siRNA Prnp_S4 reduces cell growth. B) The FRET intensity reveals that all transfections were successful at lower concentrations of lipofectamine the variability is reduced.

We could observe a significant decrease in FRET intensity for all test conditions ($P<0.01$, One way ANOVA with Bonferroni post-hoc test) (Supplementary figure 2) It is possible that halving the transfection reagent still produces very good transfection efficiency while decreasing toxicity. To verify this we decided to test transfection efficiency at various siRNA and transfection reagent concentrations. We repeated the previous experiment but added another transfection reagent lullaby (Ozbiosciences, Marseille, France) that was recommended by another siRNA screen publication (Shanks 2014). We followed the exact same procedure but instead diluted 50 µL of lullaby in 950 µL of Opti-mem to prepare the 2X reagent. We also performed serial dilutions on the siRNAs to determine the lowest possible effective concentration for Prnp_S1 and Prnp-S4.

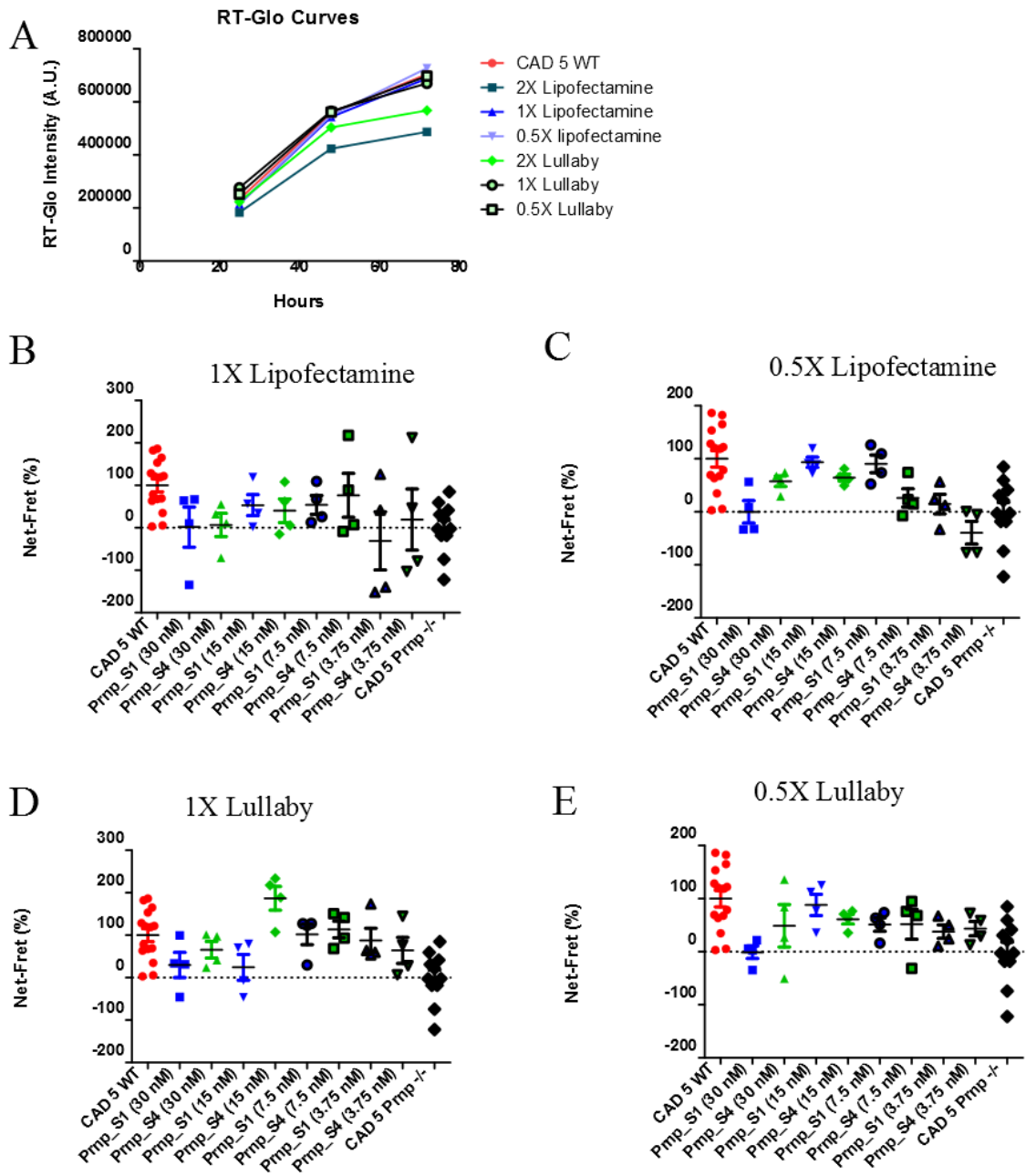

Figure 29. 2000 cells plated for 72 hours at 3 different lipofectamine / lullaby concentrations transfected with Prnp S1 and Prnp S4.A) Again there is a reduced cell growth for both products when the transfection reagent is doubled. Furthermore, it is clear that transfection with the siRNA Prnp_S4 reduces cell growth regardless of transfection reagent concentration. B, C, D, E) FRET observed at different concentration of transfection reagent.

There was marked toxicity if the concentration of the transfection reagent was doubled concurrent with our initial results. There was also no significant decrease in siRNA transfected cells FRET intensity when 2X concentration of the transfection reagents were used ($p<0.05$, One way ANOVA with Dunnett's multiple comparison test) (data not shown).

The results using 1X and 0.5X transfection reagent seem to confirm our previous assumption that 30 nM is the lowest siRNA concentration at which we can still see maximal efficacy. However statistical analysis failed to detect significant differences between the different siRNA concentrations in many cases ($p>0.05$ One way ANOVA with Bonferroni multiple comparison test) (supplementary Figure 1). While lack of significance limits strong conclusions there seems to be a trend for decreased siRNA efficacy after 30 nM. This experiment was still performed with the new batch of APC-POM1 which showed severely reduced efficacy explaining the lack of significance observed (Supplementary figure 3).

The curve in siRNA efficacy when transfected using lipofectamine can be explained by the fact that transfection reagents are administered relative to the siRNA concentration. 1X refers to the manufacturer recommended concentration for 30 nM siRNA. Therefore, we first see an increase in the FRET signal indicating reduced efficacy after 30 nM of siRNA. We then observe a decrease in the FRET signal as well as a large increase in the standard deviation of samples. This is caused not by siRNA knockdown but by toxicity from increased lipofectamine to siRNA ratio. Lastly, we can confirm that the manufacturer recommended concentration of lipofectamine RNAiMAX to siRNA leads to most efficient knockdown with no observable toxicity to cells while reducing the siRNA concentration or lipofectamine concentration any lower reduces the effect of the knockdown.

4.2.7. Dispensing FRET reagents on the Biotek MultifloFX dispenser

The greatly increased dispensing speed and accuracy, the reduced dead volume and no need for expensive replaceable tips made the Biotek MultifloFX an ideal tool for dispensing the cells required for the assays and all other FRET reagents. We tested whether it was possible to dispense all the reagents required for our assay using the MultifloFX. We plated 2000 CAD 5 WT and 2000 CAD 5 Prnp -/- cells in a checkerboard pattern using the MultifloFX 1µL cassette followed by a 72 hour

incubation period. We then dispensed 10 µL of 5X lysis buffer followed by shaking at 750 RPM at room temperature on a thermomixer (Eppendorf (Hamburg, Germany)) for one hour. 6 µL of APC-POM 1 and EU-POM19 were added resulting in an end concentration of 5 nM per well. FRET signal was measured using the envision plate reader as described previously.

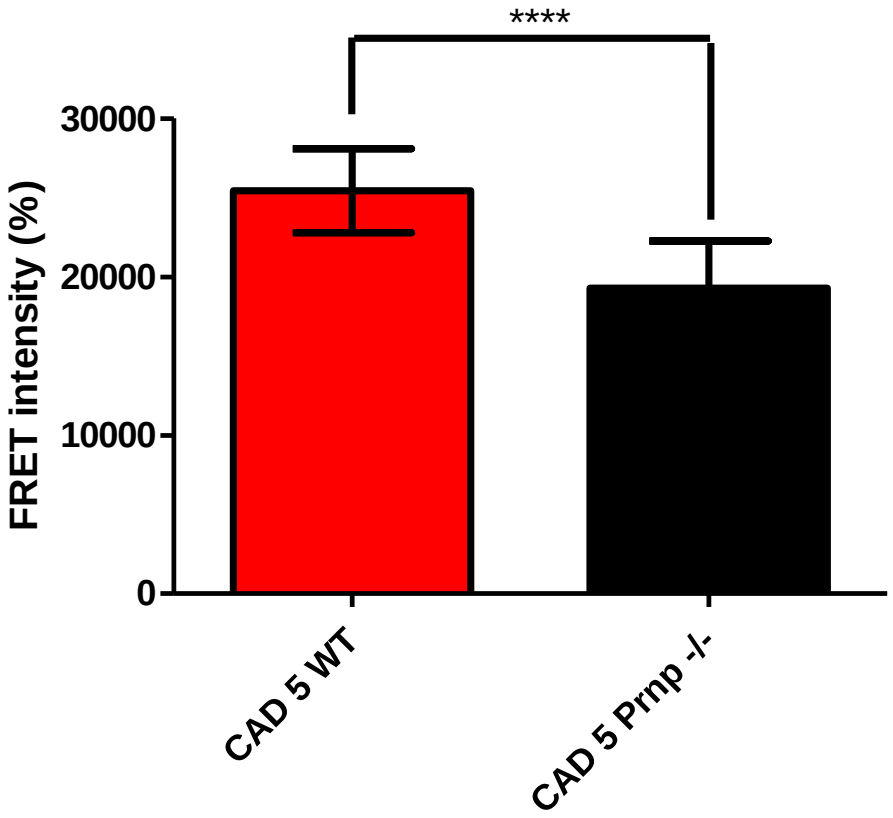

Figure 30 2000 CAD 5 Prnp -/-KO and CAD 5 WT cells were plated in a checkerboard pattern. We observed a highly significant difference between the FRET signal observed for the CAD 5 WT and CAD 5 Prnp -/-

A very significant difference can be observed between the CAD 5 WT and CAD 5 Prnp -/- cells in the FRET intensity they produce ($p<0.0001$, Two-tailed t-test). We can conclude that the Biotek MultiFlo FX is highly suitable for performing dispensing of all the reagents required in our FRET assays.

4.2.8. Testing of the new machine based assay

Having shown that the use of the Biotek MultiFlo FX is useful for conducting our FRET assays we now attempted to create a protocol that would use the Labcyte Access platform to dispense the siRNA samples required for our screen. Using the Echo acoustic dispenser allows for highly accurate dispensing of siRNA as well as allowing

for flexibility in the way we distribute our samples. This flexibility was not given with the Janus automated workstation.

We manually pre-plated 4 µL of Opti-mem into the assay plate with the exception of wells that will contain positive (Prnp_S1 (30 nM)) or negative control (Opti-Mem). We then used the Labcyte access workstation and the echo acoustic dispenser (Labcyte (Sunnyvale, USA)) to distribute either 1µL of siRNA (Dark red) or 1µL of opti-mem (lilac). The plate was then centrifuged at 1000G and sealed by the Labcyte access workstation. 1 µL of Lipofectamine RNAiMAX was added using the Biotek MultifloFX 1µL cassette and 15 µL of OFBS containing cells was added using the 5µL cassette. The plate was incubated overnight and the next day media containing RT-Glo was added. The plate was then incubated for 72 more hours and finally a PrP^C FRET was performed using the Biotek dispenser.

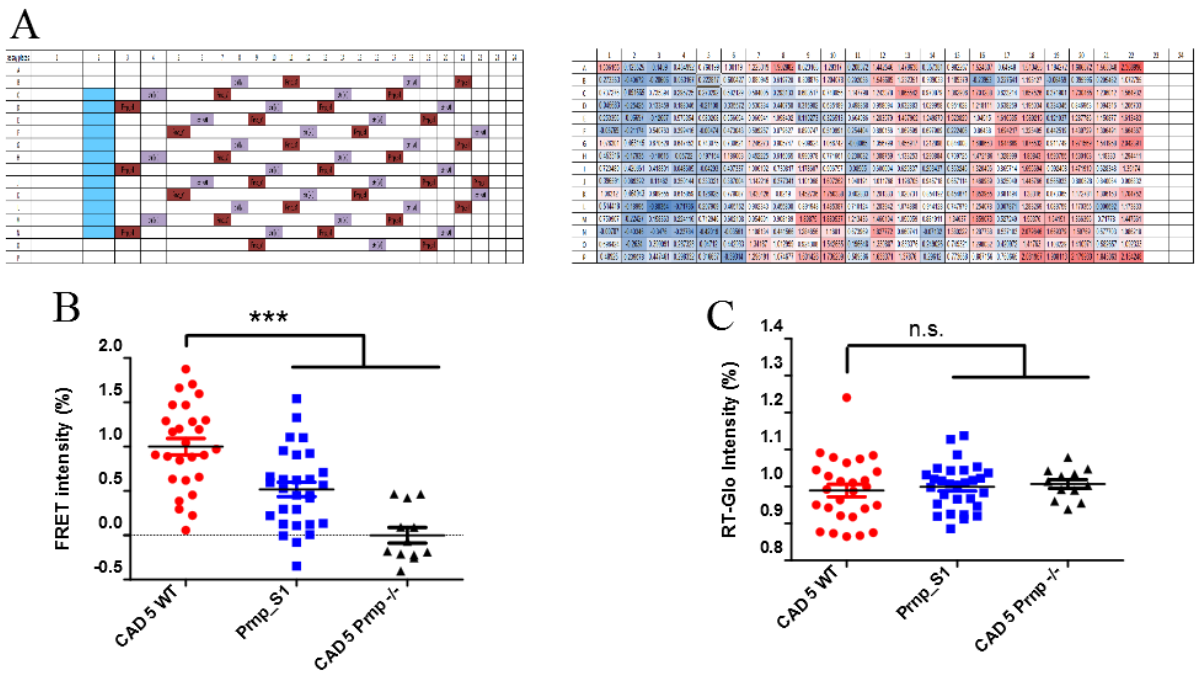

Figure 30 Dummy plate containing siRNA treated and mock treated CAD 5 WT cells A) We plated our positive and negative controls in such a way that the plate gradients would be spread evenly over all the samples. As can be seen from the heat map the top left part of the plate had significantly lower values than the bottom right part**.** B) We observed a highly significant difference between the FRET signal observed for the CAD 5 WT and the Prnp_S1 and KO cells. C) There was no observable difference in the RT-Glo signal between WT, Prnp_S1 and KO cells.

We can therefore conclude that the automated experimental workflow we have established theoretically works. However, due to having to pre-plate variable amounts of Optimem manually we introduced a large plate gradient. We circumvent this problem in future experiments by not pre-plating opti-mem but adding it after addition of the siRNA with the Biotek dispenser. Further repeats of the experimental procedure are necessary to truly confirm that this experimental set up is stable enough to perform a high throughput screen.

4.2.9. Maximizing the FRET signal

While reducing the cell number ensures that cells remain in a linear growth phase the signal to noise ratio is also dependent on the number of cells present. Having now established optimal transfection efficiency for CAD 5 cells and having robotized the workflow for increased accuracy we could now maximize the signal by varying the cells seeded per well using the Biotek dispenser. We performed our experimental protocol (Figure 31) manually plating 3000, 2800, 2600, 2400, 2200, 2000 and 1800 cells (supplementary figure 7). The end concentration of siRNA in this experiment is 30nM for all experimental conditions.

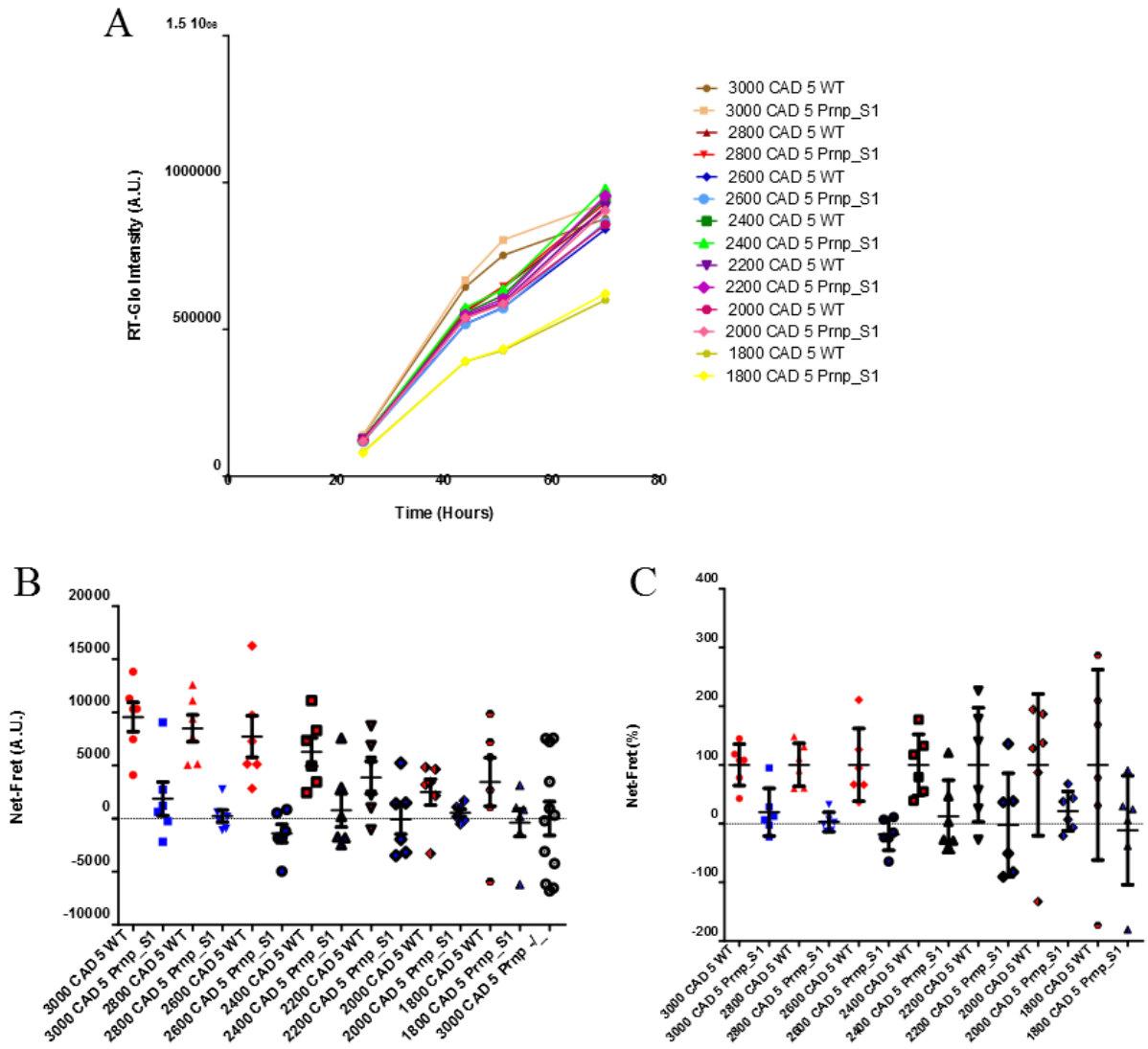

Figure 31. RT-Glo values and Net FRET signal of varying number of cells. A) It can be seen that after 72 hours nearly all cell concentrations were still in the linear growth range. As cell concentration decreased so did the difference between CAD 5 WT and Prnp_S1 treated cells. If the data is normalized we see that the difference between WT and S1 is strongest at 2800 cells (p=0.0001, unpaired t-test).

This finding is consistent with the initial attempt to adjust cell number to a linear RT-Glo gradient (See 4.3.3) where a large reduction in cell number lead to an increased standard deviation. Therefore, we have shown that the maximal signal can be obtained plating 2800 cells as it is right before there is a decrease in growth rate due to over confluence and the maximal difference can be seen at that cell concentration. However, care is needed in the interpretation of this experiment as we again faced the problem of pipetting a complex manual patterns leading to various plate gradients. I believe from this we can confirm the trend that larger cell numbers lead to a more

stable signal. We decided to use 2500 cells to have a stable FRET signal without running into the risk of accidentally reaching a non-linear RT-Glo growth curve.

4.2.10. Full system testing

We wanted to establish how much our optimizations have improved the screen. We used the Echo (Labcyte (Sunnyvale, USA) to create four assay plates with end concentrations of 60 nM Prnp_S1, 60 nM Prnp_S4, 30 nM Prnp_S1 or 30 nM Prnp_S4. The siRNA printed plates were kept on ice. Opti-mem and Lipofectamine were added using the Biotek dispenser to have a total volume of 5 μL. Plates were centrifuged at 700 G for one minute. 15 μL of Opti-mem containing cells was added to the plates with an average of 2500 cells per well. Plates were stored in an incubator and 20μL of OFBS was added the next day. The plates were incubated for 48 hours and subsequently the cells were lysed by addition of 5X Lysis buffer using the Biotek dispenser. Plates were sealed and placed on thermoshakers (Eppendorf, (Hamburg, Germany) at 750 RPM for one hour. APC-POM1 and EU-POM19 were added using the Biotek dispenser to reach an end concentration of 5nM (See figure 34). Plates were placed on thermomixers for one more hour at 750 RPM and then centrifuged at 2750 G for three minutes. Emission was read as described previously.

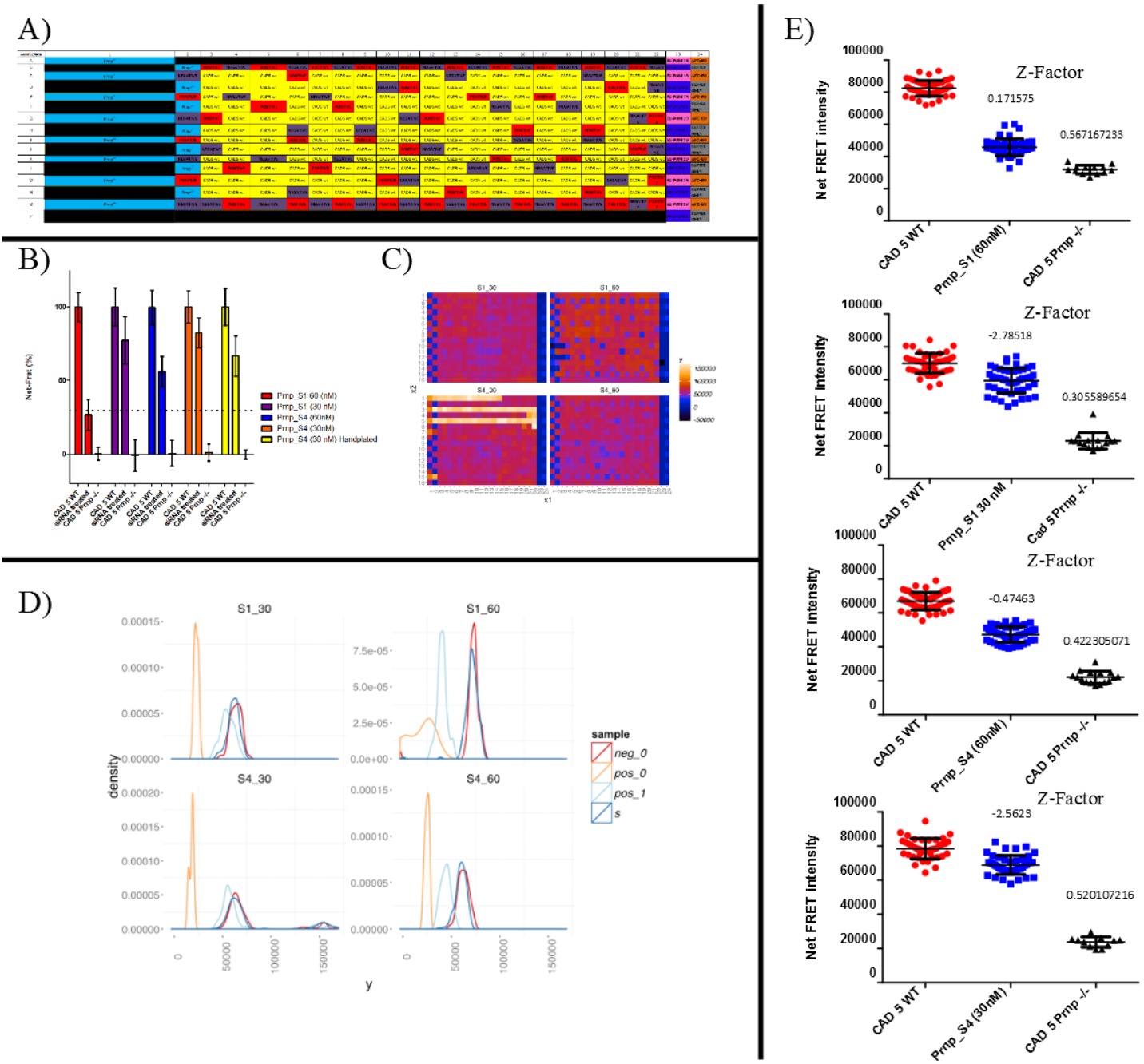

Figure 32. Summary of dummy plates ran using our final protocol. A) Summary of the plate Map used for printing of the siRNA Yellow represents sample locations which contain just Opti-mem; light blue is the location of the CAD Prnp -/- cells. Red indicates location of the positive control and purple of the negative control. Pink, dark blue, orange and grey are locations for the FRET controls needed for calculation of the net FRET signal. B) Summary of the findings each plate is represented by one colour (red= Prnp_S1 60 nM, Purple= Prnp_S1 30 nM; Blue=Prnp_S4 60 nM; Orange= Prnp_S4 30 nM and Yellow= Prnp_S4 30 nM where the plate was treated by hand after dispensing of the siRNA using the Labcyte. The Y-axis shows standardized Net-FRET for each plate. C) Heat map of the four plates that were done using our fully automated protocol (figure 34). D) Density distribution of Sample (S), Negative control (CAD 5 WT), positive control 1 (siRNA) and positive control 0 (Cad 5 Prnp -/-). E) Detailed look at the four assay plates comparing the distribution of CAD 5 WT (Negative control), siRNA (Positive control 1) and CAD 5 Prnp -/- (Positive control 0). The Z-factor compared to CAD 5 WT is indicated above each group. (Figures C and D created by automatic quality control software designed by Elke Schaper)

Overall an extremely good separation of CAD 5 WT and Cad 5 Prnp -/- can be seen. The Z-factor for separating these two groups is now consistently above 0 and tends towards 0.5. The Knock down observed by the siRNA was best seen at Prnp_S1 at a concentration of 60 nM. Only the 60 nM Prnp_S1 produced a positive Z-factor. Very little separation could be observed between the positive and negative control at 30 nM. It can be observed that our plates now show homogenous FRET signal and that we are now able to run the high-throughput screen with a high chance of success.

4.3. Discussion of optimization procedures

Many changes needed to be made to increase the accuracy and reliability of our assay. Most importantly we needed to achieve greater homogeneity within and between our assay plates. This was done by optimizing the assay run time, the cell numbers and the siRNA concentrations. Furthermore, we reduced the variation caused by different numbers of cells being seeded in different wells. We replaced the old pipetting robotic system with the Biotek MultifloFX for plating the cells and performing the FRET assay. Even with optimizing all the parameters it was still difficult to reach a z-factor that was conducive to a screen due to variations within the plate. Therefore, we also worked on creating a plate layout that allows separation of different siRNAs targeting the same gene (figure 33). We developed an algorithm that distributes siRNA replicates and the genes they target throughout the plate in a semi-random manner (Elke Schaper wrote the algorithm and finalized the plate layout under suggestion from the entire screen team). By having the controls distributed throughout the plate we should be able to asses local signal gradients within the plate to make a better estimation of the genes contribution to regulating the levels of PrP^{C}. Using the improved screening procedure we hope to have enabled a platform that will allow for reliable detection of PrP levels in the screening plates and thus determine the influence of various genes to regulating levels of PrP^{C}.

4.3.1. RT-Glo is compatible with FRET and shows cell growth dynamics better than an end-point assay

RT-Glo uses a pro-substrate that is reduced by metabolically active cells to yield a substrate for a luciferase enzyme. Therefore, the observed assay signal depends not only on cell number but also on the basal reducing potential of the used cells. We conclusively showed that there is no observable difference between the FRET signal observed in the PrP^{C} and PrP^{Sc} assay when RT-Glo is added. We then realized that there is a decrease in our RT-Glo signal approximately 50 hours after addition of RT-Glo when plating 3000 cells. Multiple scenarios could explain this drop. The most likely was that there was a depletion of the pro-substrate leading to a drop in signal. However, the drop in RT-Glo signal appeared at the same time regardless if 2X, 1X or 0.5X of RT-Glo was added suggesting that it was not depletion of the reagent that caused the drop in signal (See 4.2.2). Cell death is another potential explanation and has been shown to occur. Our initial seeding concentration of 3000 cells incubated for 96 hours leads to overgrowth of cells (Supplementary figure 6 and 7). Overgrowth

leads to at least a small amount of cell death. Additionally, there may be a marked shift in redox metabolism of the cells as it is known that reduction potential of cells varies greatly with the cell cycle phase and whether cells have differentiated or not (Schafer & Buettner 2001). It was essential to decrease the cell number for our testing experiments to ensure that we observe a linear RT-Glo signal so that we could be sure to sensitively detect cytotoxic and cytostatic siRNAs. Furthermore, keeping RT-Glo in the linear range is not only a good quality control for the number of cells but also for large changes in the metabolic state of the cell. Therefore, we established that 1500 cells was an adequate cell number to observe a linear RT-Glo gradient while still observing a strong FRET signal after 96 hours of incubation (Figure 23). We also established that reducing the cell number further weakened our FRET signal. We determined that 2800 cells allows for maximal PrP^C signal while retaining a linear growth phase of the cells in the 72 hour assay but decided to use 2500 cells per well to account for intrinsic variability in number of cells plated allowing for linear RT-Glo signal with maximal FRET signal.

4.3.2. Reduced siRNA concentration and cell number leads to robust signal with reduced off-target effects

We used Prnp_S1 and Prnp_S4 as representative examples of siRNAs that we know to lead to a decrease in prion protein expression and optimized knockdown efficiency in CAD 5 cells. We were able to reduce our siRNA concentration from 120nM to 30 nM while retaining the same or higher efficacy (figure 25). Reducing siRNA concentration for our control siRNAs below 30 nM led to a decrease in observed knockdown efficiency (Figure 29). Reducing siRNA concentration also results in a decrease of the transfection reagent needed as this is proportional to the amount of siRNA used. Optimal siRNA concentration depends on the cell line used, the transfection reagent, the transfection procedure and finally on the siRNA itself (Jarvis 2015). It is impossible to optimize siRNA concentration for every single siRNA used in the screen. Therefore, we made the assumption that different siRNAs have similar efficacy in CAD 5 cells as Prnp_S1 and Prnp_S4. It was found that we have reduced transfection efficiency or siRNA degradation in our final robotic protocol (see 4.2.10). The inability to separate positive and negative controls at the 30 nM concentration may indicate reduced transfection efficiency compared to the manual protocol or siRNA degradation in our robotic assay. A possible source of this may be pre-diluting the siRNA in Optimem

instead of DNAse/RNAse free water. The prolonged plate processing times may lead to reduced efficiency.

4.3.3. Reliable knockdown is observed in a 72 hour timeframe

The Z-factor is an indication of how robust a screen is. Z-factors observed in siRNA screens are typically lower than those observed in small-molecule screens (Birmingham et al. 2009). The major reason for this is the increased time span. SiRNA transfected cells need to be incubated for a minimum of 48 hours to observe an effect and maximal effect is typically seen at 72 hours. This increased time allows for differential cell growth and evaporation leading to plate gradients leading to different signal between theoretically identically treated wells. Reducing the assay run-time can improve the observed Z-factor by reducing the time for developing of plate gradients.

4.3.4. Optimizing the plate layout

We optimized data collection to yield the highest possible Z-factor with the lowest possible off target effects by maximizing the cell number, reducing assay runtime, reducing siRNA concentration, and using RT-Glo as quality control of cell growth within the assay plates. Furthermore, we attempted to avoid off-target effects by choosing a library in which there are on average four different siRNAs targeting each gene. It is unlikely that all four siRNAs lead to off-target effects resulting in a reduction of FRET signal.

Another major source of false positives we had stemmed from the plate gradients in the assay plates caused by long incubation times and the sensitivity of the FRET assay to volume variations. We designed a plate layout that distributes the different siRNAs and the replicates throughout the plate. The replicates are spread over two different plates and occupy a different position within the plate to again account for inter-plate and intra-plate variance. By using this intelligent distribution we hope to estimate the plate positional effects we see to obtain what is closer to a true average for the siRNA (Figure 33). Distribution of the positive and negative controls throughout and around the edges of the wells will also allow for normalizing the data to a local signal rather than estimating the signal across the entire plate. In geology a process known as kriging can be used to predict the value of the region given only a set of locations at the region. This is exactly what we wish to do with our plate. By modelling peaks and valleys in our plate introduced by temperature gradients we can predict how

the values of the samples around that area have been affected (Zhong et al. 2013). It can be imagined that a three dimensional map of the plate is created with the controls being used as sample points. Then from one control to the next a gradient is drawn and the values of the samples are adjusted based on that gradient. It has been shown that using Kriging can significantly increase the number of positive hits while keeping the false discovery rate at the same level (Zhong et al. 2013).

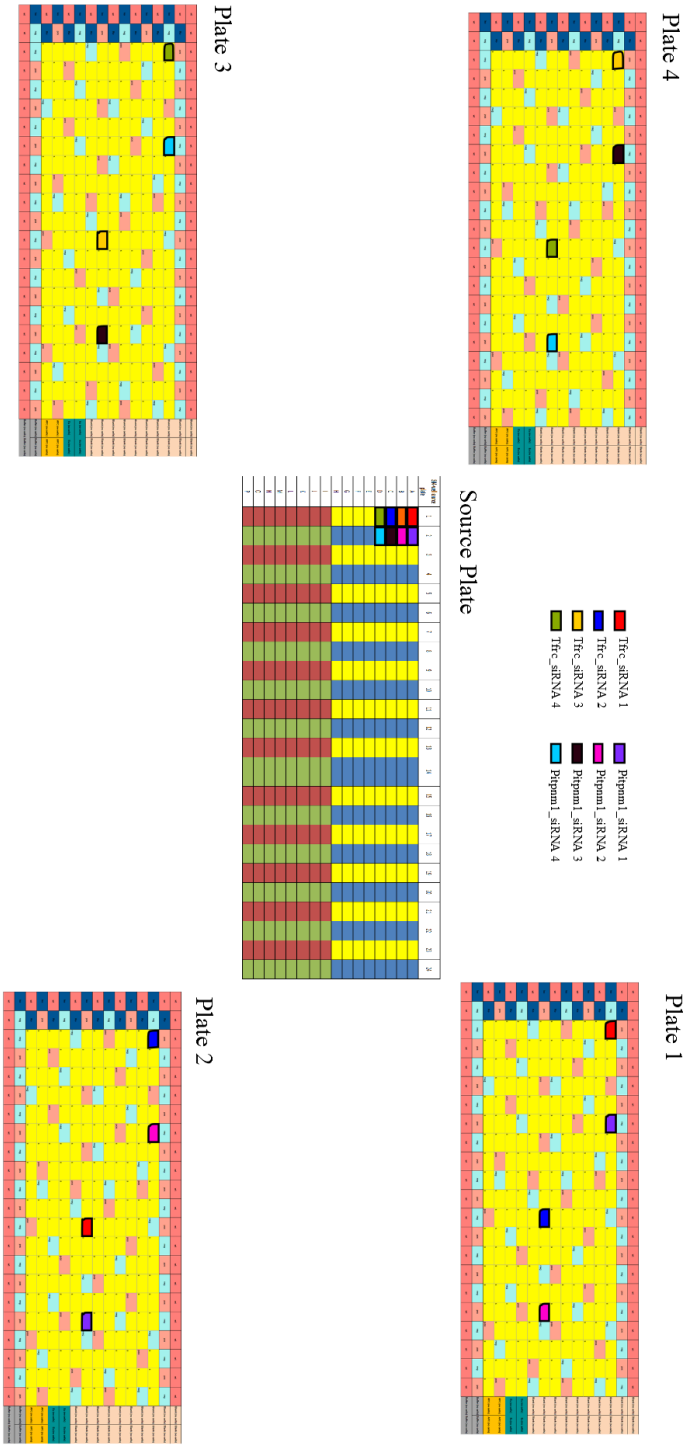

Figure 33. Distribution of samples and controls between and within plates. The four siRNAs targeting one gene are distributed in a semi randomized manner throughout and between the plates in such a way that the plate gradients observed should affect them all equally. (This was demonstrated using 8 random siRNAs targeting two genes). This pattern is possible thanks to the use of the Echo acoustic dispenser that allows for distribution of siRNAs by well to well transfer of siRNAs using a "picklist" (Full picklists for siRNA distribution available under: https://www.dropbox.com/sh/tghq1uqzo3vboh7/AADudm_MaV6D6inihvcqmh7ra?dl=0) Furthermore we decided to use Prnp_S1 as the positive control and untransfected CAD 5 WT as the negative control. We also spread these controls in a randomized manner throughout the assay plate.

4.3.5. The established workflow

We therefore established a new way to conduct the screen addressing all the majors problems faced in our initial screen (See 3.4). We now include dummy plates in every screen to recognize problems with our assay or our automated systems as they are being processed. The siRNAs are then printed using our Labcyte Access workstation. Lipofectamine RNAiMAX and cells are dispensed using the Biotek MultiFloFX to avoid unequal cell seeding. Furthermore, all the plates are barcoded which avoids experimental error and enables easier data analysis (figure 34). We perform the FRET assay using the Biotek MultiFloFX.

Figure 34. Newly established robotic workflow. We are now able to conduct the screen more effectively thanks to a new plate layout and better cell seeding. Also automated data processing enables faster analysis of results allowing us to see any potential problems with the screen while it is still being conducted.

4.3.6. Screening for factors involved in PrP^{Sc} replication

Prion replication in cellular systems is generally very fast taking only several days to reach a level detectable by the PrP^{Sc} FRET assay. However, we faced two major problems in the time-scale of the PrP^{Sc} screen. Primarily the level of PrP^{Sc} did not reach the desired optimum within the effective range of the siRNA treatment. Furthermore, longer assay times lead to increased levels of noise within the assay plates. One potential solution would be to create chronically infected cells that would constantly have high levels of PrP^{Sc} (Enari et al. 2001). Chronically infected cells can then be treated using our established optimal assay time of 72 hours or possibly after 96 hours to allow for clearance of produced PrP^{Sc} (Pfeifer et al. 1993). Cells that are chronically infected maybe less viable during transfection or have more variable transfection efficiency. However, it is hard to imagine an alternative in which a stable knockout would have to be generated. The CRISPR/ CAS 9 system would enable creation of such a stable knockout. However, this system is typically used in a pooled format rather than having an arrayed screen. To enable such an arrayed screen we would need to introduce a selecting condition along with the single guide RNA (sgRNA). A feasible method of doing this is to create stably expressing CAS9 CAD 5 cells. The library could then consist of lentiviruses that integrate into the host genome and expresses both an antibiotic resistance gene and the sgRNA. Once transfection has occurred addition of antibiotics could be used to select for stably expressing cell lines.

Using stable knockout cells would have the advantage of allowing for longer prion incubation times. Furthermore, the phenotypes observed are stronger in CRISPR screens due to complete Knockout. Therefore, transfecting cells in a 384 well format then selecting transfected cells with antibiotics and subsequently infecting them with prions may offer a viable alternative to chronically infected cells. However, CRISPR screens also have several pitfalls. Knock-out of essential genes will lead to cell death. During Knockdown these genes retain some function. Additionally, the caused indel mutation could also be in frame and not cause loss of function or alternatively cause toxic gain of function. So overall, both approaches have benefits and downsides. However, the implementation of creating chronically infected cells is easier and more likely to yield comparable results and is therefore the recommended approach.

4.3.7. Addressing the problems of siRNA screens

siRNA screens typically suffer from low reproducibility (Bhinder & Djaballah 2012; Bhinder & Djaballah 2013; Bushman et al. 2009).Different screens trying to identify the same targets very often show little to no overlap of genes they discover (Zhou et al. 2008; König et al. 2008; Brass et al. 2008). There are multiple reasons why this could be the case. The differences in hits are often caused by off-target effects designated as a positive hit. SiRNA screens also show significant overlap of negative and positive hits due to the long incubation period needed for siRNAs to work. Small Z-factors typically observed in siRNA screens as well as the problematic off-target effects lead to a high false discovery rate. Furthermore, standardized analysis methods for RNAi screening data does not exist leading to what has been designated a “magic funnel” where the several hundred hits are turned into a single promising candidate (Mohr et al. 2010; Bhinder & Djaballah 2012).

SiRNA screens can be divided into two critical stages. The data collection stage in which off-target effects, overlap of positive and negative controls and plate gradients are major factors for false positives and negatives, we have attempted to reduce the impact of these issues as much as possible by the use of multiple siRNAs meaning that off-target effects are less likely to be detected as they are often siRNA specific, unless the different siRNA sequences share significant sequence similarity. Using multiple siRNAs also simplifies post-screen deconvolution of off target effects. Furthermore, we reduced the siRNA concentration as much as possible while still seeing maximal target knock-down. After data collection data analysis is also critical as now the inevitable false positives have to be disentangled from the true hits. First a cut-off point needs to be created to designate hit or no hit. There are many ways to do this the most common is using the mean or median ± Kσ. Other methods include multiple t-tests, quartile based analysis, strictly standardized mean difference, redundant siRNA activity algorithm or a rank product algorithm (Birmingham et al. 2009).

However these methods typically have a high false positive rate. This is because they frequently fail to account for off-target effects caused by the siRNAs. It appears that observed phenotype is often siRNA specific rather than being specific to the actual knockdown of the gene of interest (Jackson et al. 2003). Reduction of siRNA

concentration did not seem to decrease the off-target effects and it was found that they appeared to be mediated by sequence similarity in the 3' UTR of the off-target gene transcript indicating that siRNAs can exhibit miRNA like function targeting up to hundreds of other mRNAs as well as the intended target (Schmich et al. 2015). Multiple ways have been proposed to counter these off-target effects.

After the screen various off-target filter methods can be utilized to correct for off-target effects. GespeR calculates the gene specific phenotype from the reagent specific phenotype. This method has shown to be effective at identifying genes involved in disease pathogenesis of various bacteria. Various screens showed substantial overlap of genes using different libraries after deconvolution but not before (Schmich et al. 2015). Another method is to create a filter that not only takes into account how "strong" the hit is but also the actual likelihood of the siRNA to produce off-target effects. A seven nucleotide sequence in the siRNA that can act as a seed sequence is used to predict potential miRNA like activity (Bhinder & Djaballah 2012). Heptamer sequences likely to promote off-target effects are often enriched in screening data and filtering out hits that produce only a small phenotype but have high predicted off-target effects may improve screen reliability. However, the efficacy of the method is unclear. applying this method to available data sets resulted in filtering of known true hits (Bhinder & Djaballah 2012).

The approach of predicting off-target binding by sequence similarity in the 3' UTR of mRNA has also been used to develop the Genome-wide Enrichment of Seed Sequence matches (GESS) algorithm. This technique looks for enrichment of siRNAs sequences that could bind to a certain 3' UTR sequence. This approach has actually yielded novel targets by identifying MAD2 as an important gene for the spindle assembly checkpoint. Therefore, off-target effects are not only be identified but if multiple off-target effects are likely to converge on a certain gene it also allows for prediction of target genes (Sigoillot et al. 2012).

After selecting hits and correcting for off-target effects, relevant pathways they act in need to be identified. Disruption of a biological pathways important for prion protein processing are likely to produce a similar phenotype regardless of what level the pathway is disrupted leading to more reliable prediction of true hits.

Even with all these measures there is still the possibility of false positives due to off target effects and variability in the efficacy of transfection. The only sure way to avoid false positives and reduce the number of false negatives is to repeat the screen preferentially with another library. Follow up studies focusing only on the hits can also be done repeating the screen with a smaller selection of targets using more replicates to allow for better statistical significance. A follow up screen in which decreasing concentrations of each potential hit siRNA is used could easily be done utilizing the Labcyte system. Having a dose-response curve for our siRNAs would be excellent prove that they are a "real hit". Other follow up studies can be very diverse (Mohr et al. 2010).While careful experimental procedure and good statistical analysis can avoid certain problems associated with siRNA high throughput screenings all discovered hits have to undergo a secondary round of testing. And it is in this second round of testing where most true candidates are likely to be discovered.

4.3.8. Proposed screen data workflow

So to identify true hits we recommend a thorough data analysis. First, data should be processed and normalized. Data not meeting the quality controls should be excluded (Figure 35). Data normalization can be done using either the z-score or the b-score. These measurements do not require the use of controls in the plate and are based on the assumption that most of the samples will be inactive (Malo et al. 2006). However, these methods are not suited to correct siRNA screens because of the large intraplate variance we observe as well as a very high number of samples with expected biological activity due to off-target or toxic effects. Therefore, we have included Prnp_S1 as a positive control and CAD 5 WT as a negative control. The Data can then be normalized in the following manner.

$$X\% = \frac{(Sample - \overline{S1})}{(\overline{WT} - \overline{S1})}$$

The samples are now given as a percentage where 0% indicates the same knockdown efficiency as Prnp_S1 and 100% indicates the same PrP level as CAD 5 WT. Now the median absolute deviation is calculated for each sample. An increase or decrease in three median absolute deviations from the median can be designated as a hit. After hit

designation we have to correct for off-target effects using gespeR to estimate the gene dependent phenotype. Lastly functional pathway analysis should be done to identify multiple genes involved in a single pathway (Figure 35). Hopefully this complex experimental setup allows us to disentangle the actual genes involved in regulation of levels of PrP^{C} in the cell and in the future enable us to determine genes involved in conversion from PrP^{C} to PrP^{Sc}.

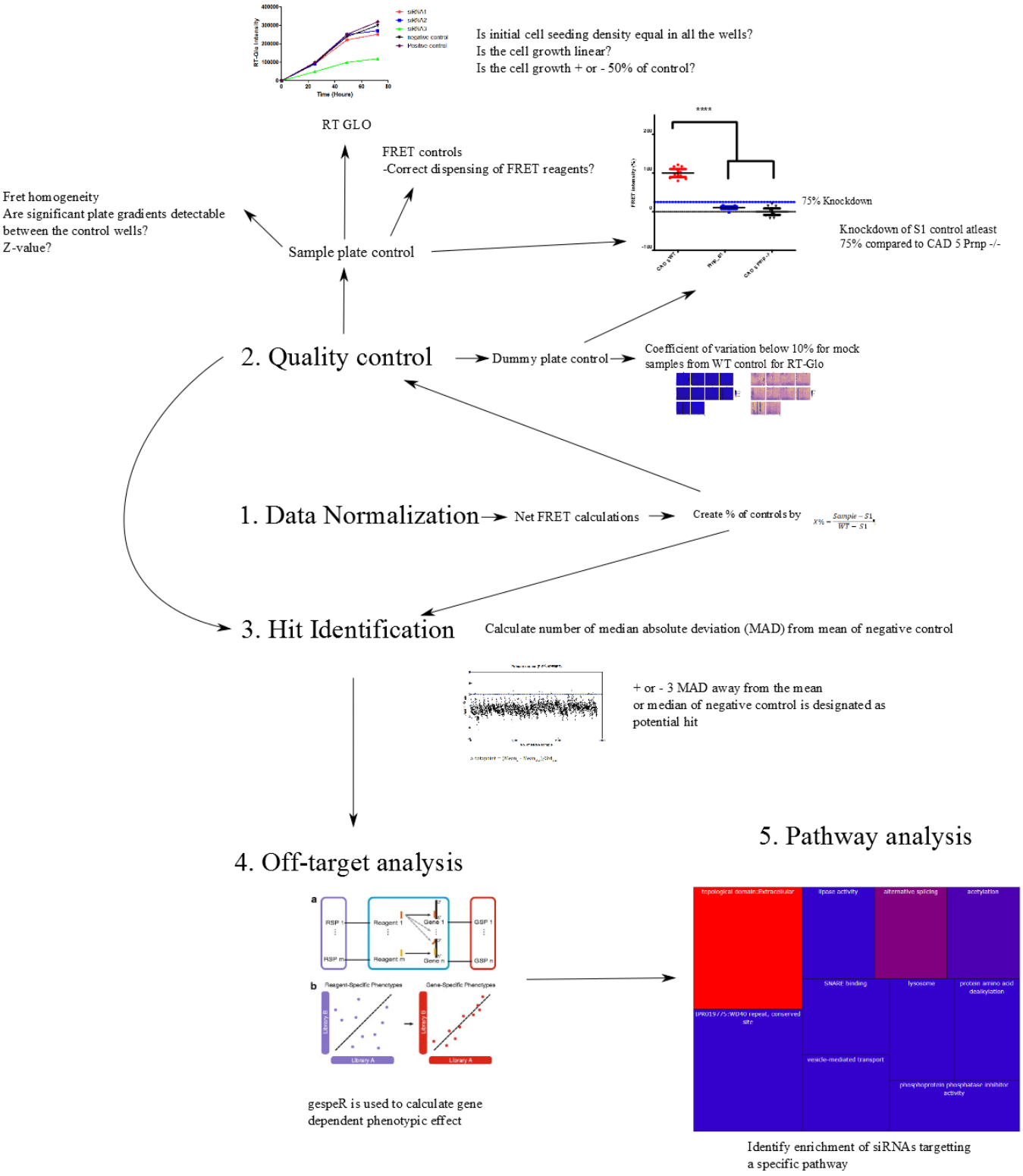

Figure 35. Proposed data workflow for screen. First data normalization by calculation of net-fret is done. Then every plate is normalized to the negative and positive controls present on the plate. (Example figures provided from data by Bei Li, Marc Emmenegger and Elke Scharper) as well as 4. Off-target analysis (Schmich et al. 2015) and 5. Pathway analysis (Diaz et al. 2014)-

A stringent test of quality controls is administered. The dummy plates in each run have to have a homogenous distribution of cells as measured by RT-Glo and the negative and positive control need to be separated by a Z-factor of over 0. In the actual assay plates the positive control should show at least a 70% reduction of FRET signal compared to the CAD 5 Prnp -/- cells. Incorrect dispensing of the FRET reagents will be visible by a change in the signal obtained from the europium channel. Furthermore, single wells are evaluated looking at whether cells were distributed homogenously between the wells and whether the siRNA inside the well is exerting a toxic effect. Furthermore plate gradients in FRET signal will be assessed and potentially data can even be normalized based on local plate control values rather than global plate control values allowing for a more accurate estimation of the actual values. Wells, Plates or even entire Runs that do not pass these stringent quality controls are discarded and repeated.

Hits are then identified by calculating the median absolute deviation and hits are identified by having a FRET signal that is outside ±3σ. Using gespeR we then calculate for enrichment of seed sequences in the discovered hits and correct for gene independent phenotype. Lastly we perform pathway analysis grouping siRNAs related to a specific pathway into clusters. (Diaz et al. 2014; Schmich et al. 2015)

In conclusion we have demonstrated a high throughput siRNA screen to detect changes in the level of prion protein. We identified and remedied many of the problems that we faced in our initial screen. We showed that achieving a Z-factor between 0 and 0.5 is possible using siRNA treated cells in a 384 well plate. We developed and implemented new methodologies using the Biotek MultiFlo FX and Labcyte Access platform to allow for greater dispensing accuracy and reduction of plate gradients. We showed that cell viability can be measured in our plate in parallel to the FRET assay using RT-Glo. We can therefore be hopeful that the results obtained by this round of screening will contain true hits that eventually lead to discovery of genes and pathways involved in prion disease. This will allow for development of new therapeutic strategies to cure or at least treat people afflicted with this terrible class of diseases.

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6. Appendix

Table Analysed	Standardized Lipofectamine 2X
One-way analysis of variance	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
Number of groups	10
F	5.120
R square	0.4898

ANOVA Table	SS	df	MS		
Treatment (between columns)	145481	9	16165		
Residual (within columns)	151537	48	3157		
Total	297018	57			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CAD 5 WT vs Prnp_S1 (30 nM)	-14.83	0.4691	No	ns	-103.1 to 73.42
CAD 5 WT vs Prnp_S4 (30 nM)	-10.61	0.3356	No	ns	-98.86 to 77.64
CAD 5 WT vs Prnp_S1 (15 nM)	-38.44	1.216	No	ns	-126.7 to 49.81
CAD 5 WT vs Prnp_S4 (15 nM)	32.03	1.013	No	ns	-56.22 to 120.3
CAD 5 WT vs Prnp_S1 (7.5 nM)	20.92	0.6615	No	ns	-67.33 to 109.2
CAD 5 WT vs Prnp_S4 (7.5 nM)	-29.50	0.9330	No	ns	-117.7 to 58.75
CAD 5 WT vs Prnp_S1 (3.75 nM)	2.738	0.08660	No	ns	-85.51 to 90.99
CAD 5 WT vs Prnp_S4 (3.75 nM)	-80.07	2.532	No	ns	-168.3 to 8.184
CAD 5 WT vs CAD 5 Prnp -/-	100.0	4.485	Yes	***	37.78 to 162.3

Table Analyzed	Standardized Lipofectamine 1X

One-way analysis of variance	

P value	0.0590				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	10				
F	2.007				
R square	0.2734				
ANOVA Table	SS	df	MS		
Treatment (between columns)	110594	9	12288		
Residual (within columns)	293891	48	6123		
Total	404485	57			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CAD 5 WT vs Prnp_S1 (30 nM)	98.36	2.234	No	ns	-24.54 to 221.3
CAD 5 WT vs Prnp_S4 (30 nM)	93.43	2.122	No	ns	-29.46 to 216.3
CAD 5 WT vs Prnp_S1 (15 nM)	46.72	1.061	No	ns	-76.17 to 169.6
CAD 5 WT vs Prnp_S4 (15 nM)	59.90	1.360	No	ns	-63.00 to 182.8
CAD 5 WT vs Prnp_S1 (7.5 nM)	46.08	1.046	No	ns	-76.82 to 169.0
CAD 5 WT vs Prnp_S4 (7.5 nM)	23.67	0.5375	No	ns	-99.23 to 146.6
CAD 5 WT vs Prnp_S1 (3.75 nM)	131.1	2.976	Yes	*	8.162 to 254.0
CAD 5 WT vs Prnp_S4 (3.75 nM)	80.71	1.833	No	ns	-42.19 to 203.6

CAD 5 WT vs CAD 5 Prnp -/-	100.0	3.220	Yes	*	13.33 to 186.7

Table Analyzed	Standardized Lipofectamine 0.5X
One-way analysis of variance	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
Number of groups	10
F	6.272
R square	0.5405

ANOVA Table	SS	df	MS
Treatment (between columns)	128952	9	14328
Residual (within columns)	109647	48	2284
Total	238598	57	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CAD 5 WT vs Prnp_S1 (30 nM)	100.0	3.720	Yes	**	24.97 to 175.1
CAD 5 WT vs Prnp_S4 (30 nM)	42.40	1.576	No	ns	-32.67 to 117.5
CAD 5 WT vs Prnp_S1 (15 nM)	6.491	0.2414	No	ns	-68.58 to 81.56
CAD 5 WT vs Prnp_S4 (15 nM)	35.37	1.315	No	ns	-39.70 to 110.4
CAD 5 WT vs Prnp_S1 (7.5 nM)	9.508	0.3535	No	ns	-65.56 to 84.58
CAD 5 WT vs Prnp_S4 (7.5 nM)	73.71	2.740	No	ns	-1.360 to 148.8
CAD 5 WT vs Prnp_S1 (3.75 nM)	85.25	3.170	Yes	*	10.18 to 160.3
CAD 5 WT vs Prnp_S4 (3.75 nM)	139.4	5.183	Yes	***	64.32 to 214.5
CAD 5 WT vs CAD 5	100.0	5.272	Yes	***	47.07

Prnp -/-	to 153.0

Table Analyzed	**Standardized Lullaby 1X**				
One-way analysis of variance					
P value	0.0720				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	10				
F	1.887				
R square	0.2265				
ANOVA Table	SS	df	MS		
Treatment (between columns)	7.367e+008	9	8.185e+007		
Residual (within columns)	2.516e+009	58	4.337e+007		
Total	3.252e+009	67			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CAD 5 WT vs Prnp_S1 lipofectaine 30 nM	622.5	0.1750	No	ns	-9238 to 10483
CAD 5 WT vs Prnp_S4 lipofectaine 30 nM	-2500	0.7030	No	ns	-12360 to 7360
CAD 5 WT vs Prnp_S1 lipofectaine 15 nM	-1912	0.5375	No	ns	-11772 to 7949
CAD 5 WT vs Prnp_S4 lipofectaine 15 nM	-4206	1.183	No	ns	-14067 to 5654
CAD 5 WT vs Prnp_S1 lipofectaine 7.5 nM	48.74	0.01370	No	ns	-9811 to 9909
CAD 5 WT vs Prnp_S4 lipofectaine 7.5 nM	-1219	0.3428	No	ns	-11079 to 8641
CAD 5 WT vs Prnp_S1 lipofectaine 3.75 nM	-2070	0.5819	No	ns	-11930 to 7790
CAD 5 WT vs Prnp_S4 lipofectaine 3.75 nM	-1519	0.4272	No	ns	-11380 to 8341
CAD 5 WT vs CAD 5 Prnp -/-	7129	3.062	Yes	*	674.3 to 13584
Table Analyzed	Standardized Lullaby 1X				

One-way analysis of variance	
P value	< 0.0001
P value summary	****

Are means signif. different? (P < 0.05)	Yes				
Number of groups	10				
F	5.209				
R square	0.4941				
ANOVA Table	SS	df	MS		
Treatment (between columns)	149665	9	16629		
Residual (within columns)	153226	48	3192		
Total	302890	57			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CAD 5 WT vs Prnp_S1 (30 nM)	70.27	2.210	No	ns	-18.47 to 159.0
CAD 5 WT vs Prnp_S4 (30 nM)	34.32	1.079	No	ns	-54.42 to 123.1
CAD 5 WT vs Prnp_S1 (15 nM)	75.70	2.381	No	ns	-13.04 to 164.4
CAD 5 WT vs Prnp_S4 (15 nM)	-86.85	2.732	No	ns	-175.6 to 1.889
CAD 5 WT vs Prnp_S1 (7.5 nM)	-1.934	0.06083	No	ns	-90.67 to 86.81
CAD 5 WT vs Prnp_S4 (7.5 nM)	-13.41	0.4216	No	ns	-102.1 to 75.33
CAD 5 WT vs Prnp_S1 (3.75 nM)	12.31	0.3871	No	ns	-76.43 to 101.0
CAD 5 WT vs Prnp_S4 (3.75 nM)	35.84	1.127	No	ns	-52.89 to 124.6
CAD 5 WT vs CAD 5 Prnp -/-	100.0	4.460	Yes	***	37.43 to 162.6
Table Analyzed	Standardized Lullaby 0.5X				
One-way analysis of variance					
P value	0.0024				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	10				
F	3.452				
R square	0.3929				
ANOVA Table	SS	df	MS		
Treatment (between columns)	82298	9	9144		
Residual (within columns)	127142	48	2649		

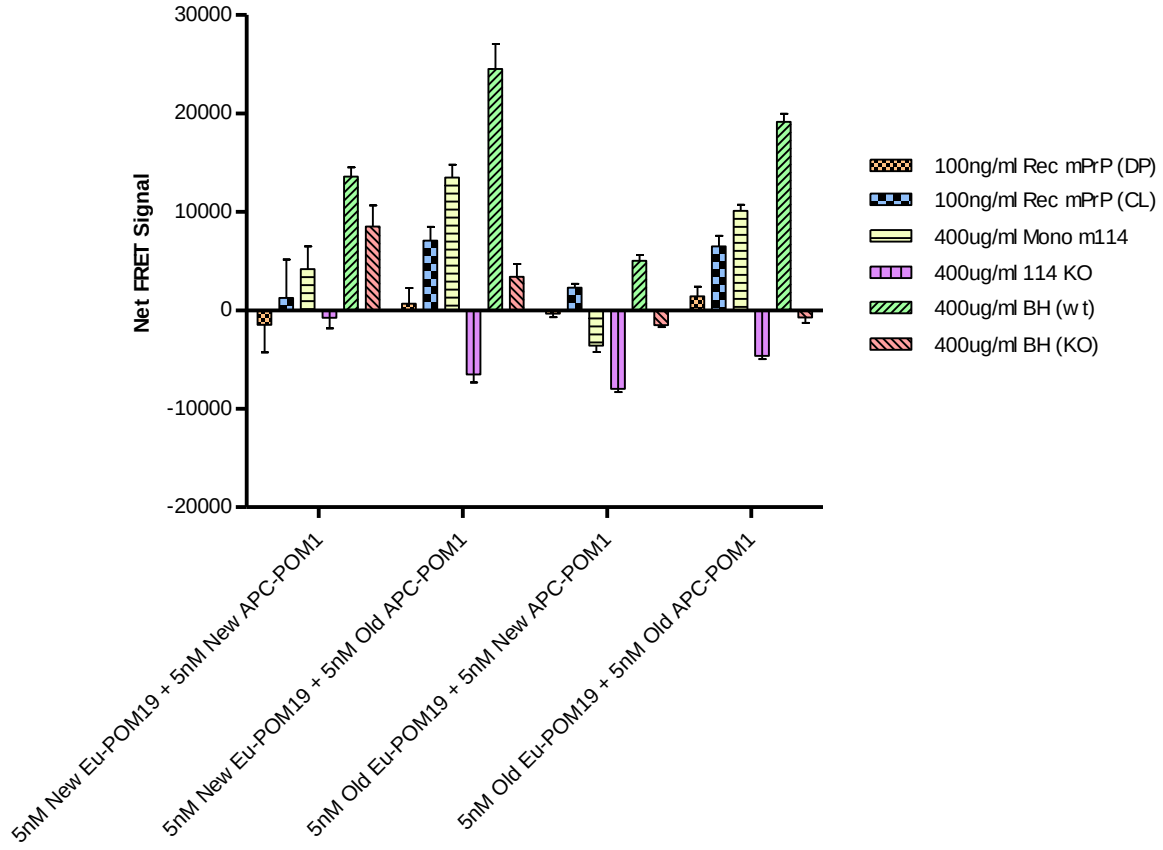
Total	209440	57			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CAD 5 WT vs Prnp_S1 (30 nM)	101.2	3.494	Yes	**	20.35 to 182.0
CAD 5 WT vs Prnp_S4 (30 nM)	51.11	1.765	No	ns	-29.73 to 131.9
CAD 5 WT vs Prnp_S1 (15 nM)	12.01	0.4146	No	ns	-68.83 to 92.84
CAD 5 WT vs Prnp_S4 (15 nM)	38.76	1.338	No	ns	-42.07 to 119.6
CAD 5 WT vs Prnp_S1 (7.5 nM)	48.84	1.686	No	ns	-32.00 to 129.7
CAD 5 WT vs Prnp_S4 (7.5 nM)	48.18	1.664	No	ns	-32.66 to 129.0
CAD 5 WT vs Prnp_S1 (3.75 nM)	62.06	2.143	No	ns	-18.78 to 142.9
CAD 5 WT vs Prnp_S4 (3.75 nM)	56.59	1.954	No	ns	-24.25 to 137.4
CAD 5 WT vs CAD 5 Prnp -/-	100.0	4.896	Yes	***	43.01 to 157.0

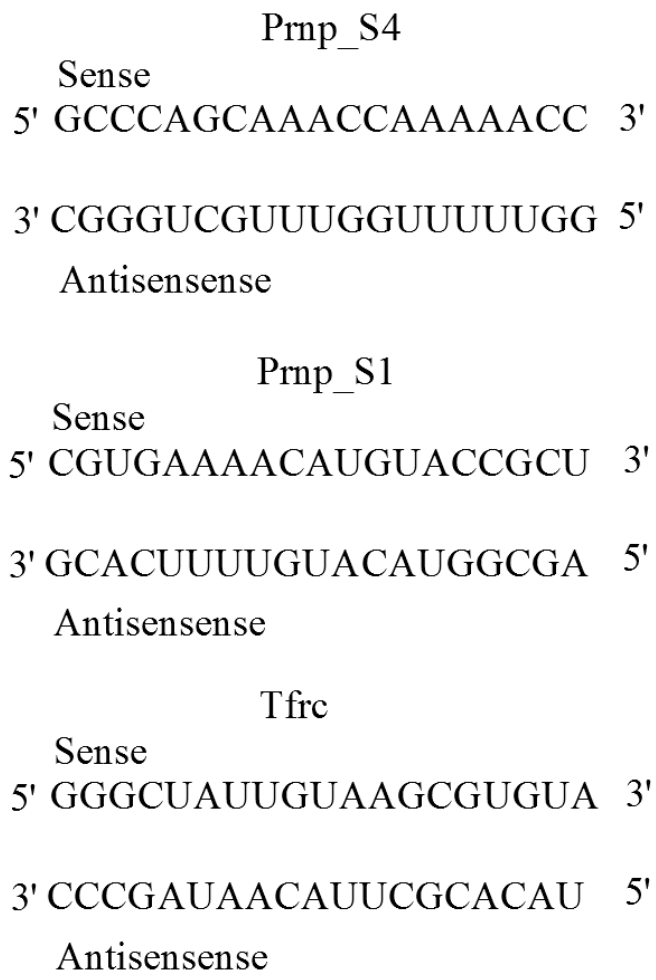
Supplementary Figure 1 One way ANOVA of various lipofectamine RNAiMAX and Lullaby dilutions at various siRNA concentrations using Dunnett's multiple comparison test.

Table Analyzed	FRET normalized					
One-way analysis of variance						
P value	< 0.0001					
P value summary	****					
Are means signif. different? (P < 0.05)	Yes					
Number of groups	8					
F	18.06					
R square	0.7006					
Bartlett's test for equal variances						
Bartlett's statistic (corrected)	12.84					
P value	0.0760					
P value summary	ns					
Do the variances differ signif. (P < 0.05)	No					
ANOVA Table	SS	df	MS			
Treatment (between columns)	149402	7	21343			
Residual (within columns)	63833	54	1182			
Total	213234	61				
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% of diff	CI
CAD 5 WT vs 2X lipofectamine S1	137.1	7.783	Yes	****	79.20	to 195.0
CAD 5 WT vs 2X lipofectamine S4	77.22	4.384	Yes	**	19.33	to 135.1
CAD 5 WT vs 1X lipofectamine S1	115.0	6.531	Yes	****	57.15	to 172.9
CAD 5 WT vs 1X lipo S4	122.2	6.938	Yes	****	64.33	to 180.1
CAD 5 WT vs 0.5X lipo S1	117.5	6.673	Yes	****	59.65	to 175.4
CAD 5 WT vs 0.5X lipo S4	100.8	5.722	Yes	****	42.90	to 158.7
CAD 5 WT vs CAD 5 Prnp -/-	100.0	8.227	Yes	****	60.05	to 140.0

Supplementary Figure 2 One way ANOVA of various lipofectamine RNAiMAX dilutions at 30 nM siRNA concentrations with Bonferroni correction



Supplementary Figure 3 FRET performed with 2 different Europium-POM 19 and 2 different APC-POM1 on recPrP, WT and Prnp -/- brain homogenate and monoclonal SH-SY5Y cells WT and Prnp -/-. It can be observed that the old stock of APC-POM1 produced a much more reliable FRET signal. (Data generated by Daniel Pease)



Supplementary Figure 4 SiRNA sequences and complementary sequences of siRNA used

OFBS		filter
	Optimem-	440 ml
	FBS	50 ml
	G-MAX	5 ml
	P/S	5 ml

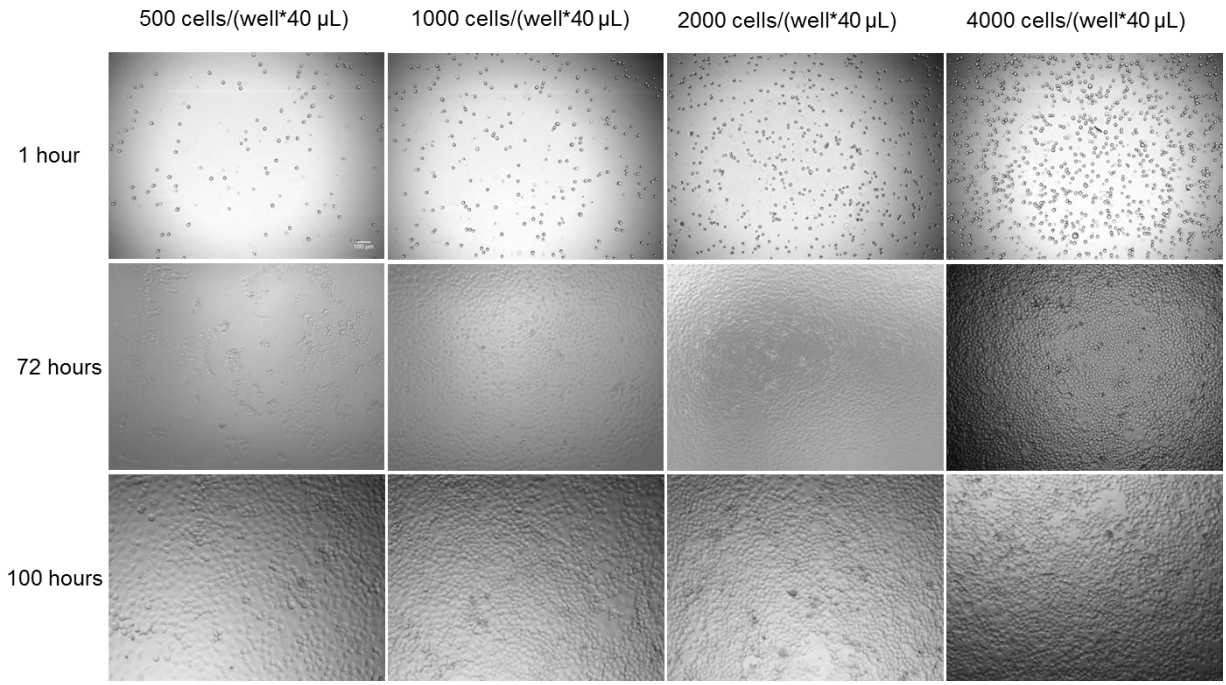
2mM EDTA/ PBS		filter
	EDTA (m.w. 372.2g/mol)	372.2 mg
	500ml PBS (red-cap	to 500 mL

5x Standard lysis buffer		Stock solution	filter
	250 mM Tris-HCl	1 M Tris.HCl (pH 8, adjust by 5M	125
	750 mM NaCl	5 M NaCl (m.w. 58.4 g/mol,	75
	2.5% Na deoxycholate	5% Na deoxycholate (Sigma,	250
	2.5% Triton X-100	100% Triton X-100 (Sigma)	12.5
	dH2O		to

1x Standard lysis buffer	keep at RT	Stock solution	filter
	50 mM Tris-HCl pH8	1 M Tris.HCl (pH 8, adjust by 5M	25
	150 mM NaCl	5 M NaCl (m.w. 58.4 g/mol,	15
	0.5% Na deoxycholate	5% Na deoxycholate (Sigma,	50
	0.5% Triton X-100	100% Triton X-100 (Sigma)	2.5
	dH2O		to

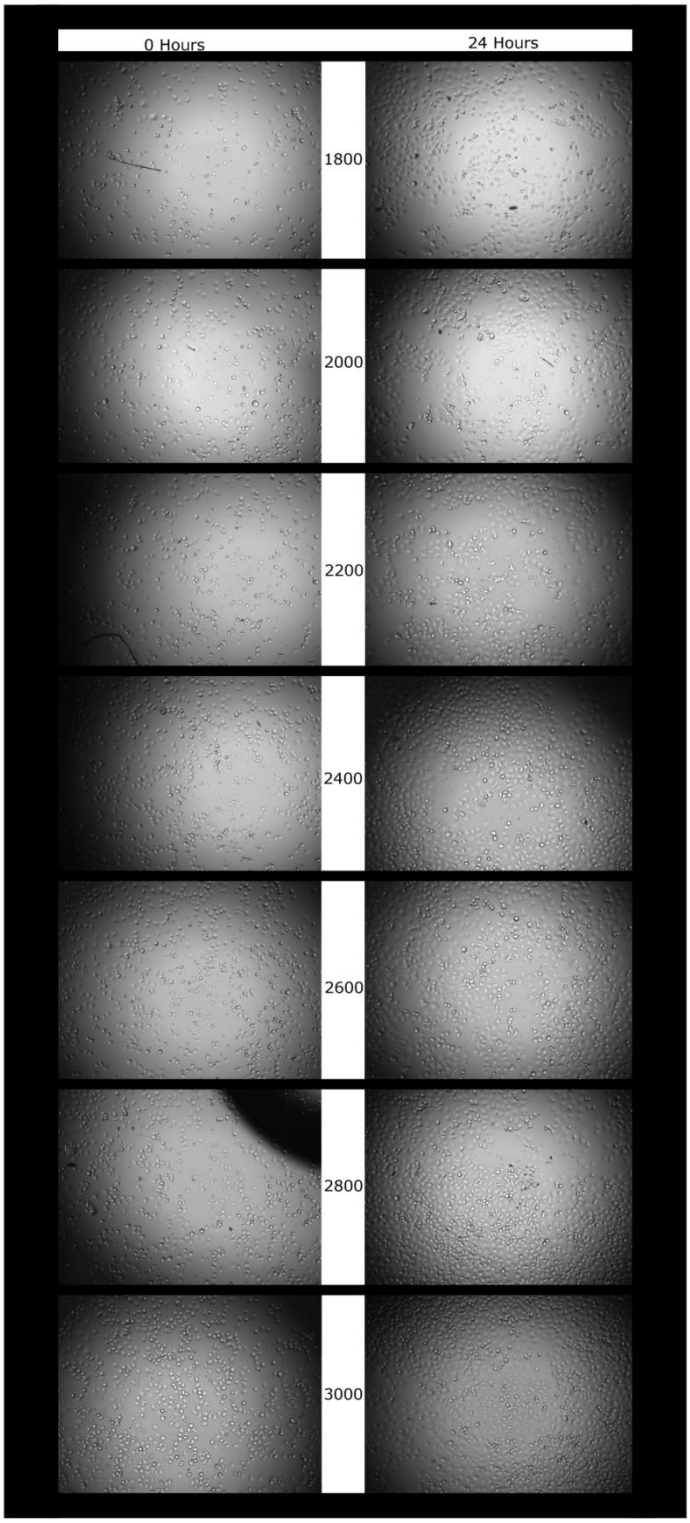
30 mM PMSF	keep at -20°C	filter
	PMSF m.w. 174 g/mol,	2.61 g
	Isopropanol (Sigma)	to 500
Denaturing buffer (0.5M	keep at RT	filter
	NaOH (m.w. 40 g/mol,	10 g
	dH2O	to 500 ml
Neutralizing (0.5M	keep at RT	filter
	NaH2PO4•H2O m.w. 137.99	34.5 g
	dH2O	to 500 ml
Proteinase		
	PK Stock, 22 mg/ml	

Supplementary Figure 5 Composition of buffers used for screen

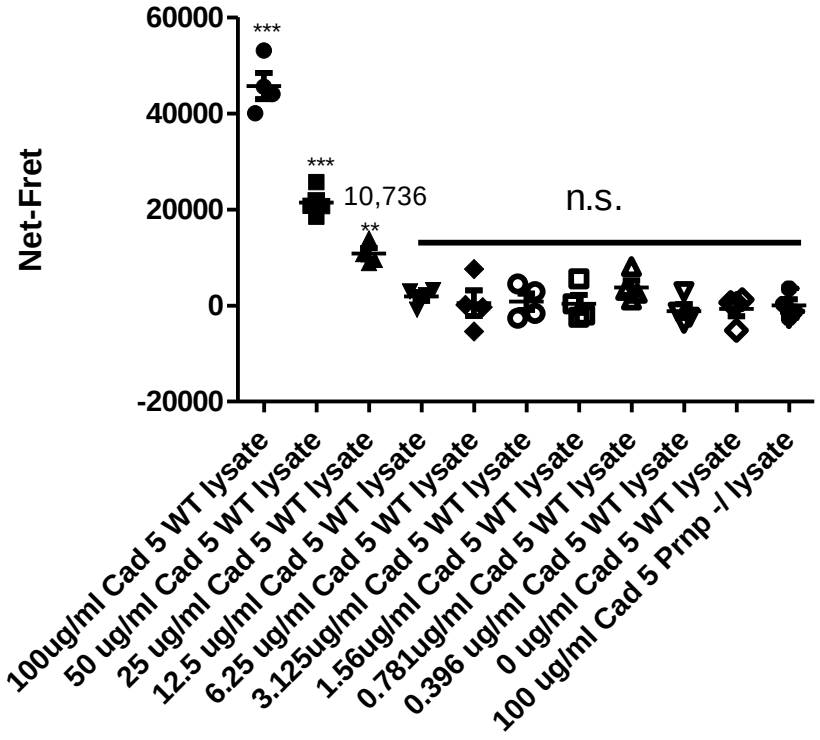


Supplementary Figure 6 cell density at various initial cell concentrations and time points. (Figure created by Marc Emmenegger)

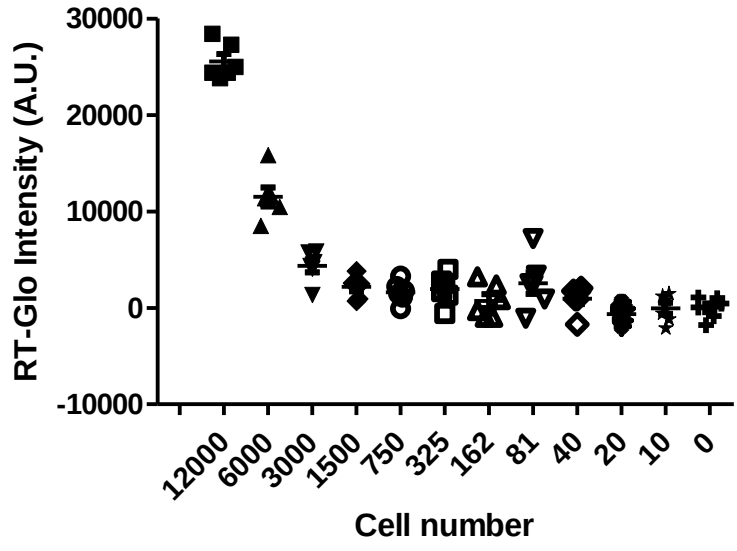
"Morphological assessment of cell proliferation over time with Zeiss Axiovert 200 M inverted microscope. Succeeding a very homogenous cell dispensing using MultiFloFX FX (morphologically, wells containing same amount of cells could not be kept apart), cells were grown and imaged several times. Both conditions, the initial amount of 2000 cells/well and 4000 cells/well, reached confluence at 72 hours. At 105 hours, they already started overgrowing. 1000 cells/well are near confluence at the 72 hours time point and fully confluent after 105 hours. 500 cells/well do not reach confluence at 105 hours incubation. Scale bar = 100 µm. "



Supplementary Figure 7 cell density of Prnp_S1 treated cells using reverse transfection. 4 µL of Opti-mem was added to each well. Some Opti-mem also contained 30 nM Prnp_S1. 1 µL of Lipofectamine RNAiMAX and the plate was incubated for 15 minutes. 15 µL of CAD 5 WT or Prnp-/- cells were added to the wells at various concentrations. Growth and distribution of cells seemed homogenous among all the wells and no difference was visible between siRNA treated and non-treated wells. An example of each cell concentration was imaged using a Zeiss Axiovert 200 M inverted microscope with Axiocam MRM. It appears that 3000 cells reach near confluence after only 24 hours even when exposed to reverse transfection.



Supplementary Figure 8 quality control of new APC-POM1. Previous work indicated that the detection sensitivity of the FRET assay is at 25 µG of CAD 5 WT lysate achieving a net-fret signal difference of about 10,000 versus the knockout control.



Supplementary Figure 9 Titration curve of RT-Glo intensity after 200 minutes of addition to CAD 5 cells. We established that we could reliably detect changes of 1500 cells ((P<0.01, One-way ANOVA with Dunnett's multiple comparison test). Even as little as 750 cells may be distinguishable but no longer show significance when correcting for multiple comparisons.

7. Statement of Authorship

I declare that I have used no other sources and aids other than those indicated. All passages quoted from publications or paraphrased from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Place: _____

Date: _____

Signature: _____

8. Curriculum Vitae

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name	Zurbrügg, Mark
Date of Birth	23rd November 1990
Citizenship	Swiss / German
Address	Bündackerstrasse 158 CH-3047 Bremgarten bei Bern
E-Mail address	m.zurbruegg1990@gmail.com
Degree	B.Sc. in Neuroscience with honours of the first class M.Sc. in Neuroscience (January, 2016)

SCHOOLING

1996 – 1999	Grundschule am Windmühlenberg (Berlin)
1999 – 2009	Berlin British School graduated with International Baccalaureate Diploma

MILITARY SERVICE

2009 – 2010	Infantry squad leader (Inf RS 3/3 2010)

UNIVERSITY EDUCATION

2010 – 2014 **B.Sc. in Neuroscience at the University of Glasgow**

2011 – 2012 **Study Abroad Program at McGill University**

Major Subject: Neuroscience
Specialized courses: advanced neuroanatomy, CNS neurotransmitters and drug development, the autonomic nervous system and bio-imaging in the life sciences

Student Research Project: "Glycine is enriched in a neurochemically distinct subset of laminae I-II inhibitory interneurons in the spinal cord of rodents" Supervising Professor: Andrew Todd

Undergraduate dissertation: "Excitotoxicity in Alzheimer's: a novel target for therapeutic approaches"

2014 – 2016 **M.Sc. in Neuroscience at the University of Zurich**

Major Subject: Neuroscience
Specialized courses: Neurobiology, Molecular and Cellular Neuroscience, Structure, Plasticity and Repair of the Nervous System and a Course in Scientific Writing

Master thesis: “RNAi screen of the mouse genome reveals genes involved in prion protein biosynthesis and conversion to its pathogenic misfolded conformational variant PrP^{Sc}" Supervising Professor: Adriano Aguzzi

WORK EXPERIENCE

Oct 2014 – Dec 2015 **Universitätsspital Zürich Institut für Neuropathologie, Master Student**

- Programming and operating of various robotic work systems
- Cell culture
- siRNA transfection
- Prion infection of cells
- FRET reading and data analysis
- Western blots and sandwich ELISA
- Immunohistochemistry

Mar 2015 – Apr 2015 **University of Zurich Institute of Molecular Life Sciences, Master Course Internship**

- Drosophila dissection and preparation
- Sharp electrode intracellular recordings
- Data analysis of excitatory post synaptic potentials
- Plasmid design and bacterial transformation
- Immunohistochemistry and confocal microscopy

Feb 2015 – Mar 2015 **University of Zurich Institute of Molecular Life Sciences, Master Course Internship**

- Oocyte culture
- Injection of mRNA into oocytes
- Voltage clamping of oocytes
- Data analysis of voltage clamp recordings

Aug 2013 – Jan 2014 **University of Glasgow Institute of Neuroscience and Psychology, Student Researcher**

- Preparing animal sections for immunocytochemistry
- Performing immunocytochemistry and confocal microscopy
- Computer analysis of confocal images
- Preparation of project report including statistical analysis and image processing using Photoshop

Jun 2013 – Jul 2013 **InselSpital Bern Universitätsinstitut für Klinische Chemie, Research Internship**

- Extracting DNA from patient blood
- Primer design and in silico testing
- DNA amplification of patient DNA using primers
- Sequencing of PCR amplified fragments

Jun 2009 – May 2010 **Swiss army, infantry squad leader**

- Following instructions precisely to complete a set task
- Team communication skills
- Structured thought processes to solve a posed problem

EXTRACURRICULAR ACTIVITIES

2010 – 2011	**Science correspondent** Glasgow Guardian (University of Glasgow newspaper) **Charity worker** for Venture Scotland a charity that works with underprivileged children and children with a history of substance abuse
2011 – 2012	**Member** of the McGill Outdoors club
2013 – 2014	**Member** of the Glasgow University mountaineering club
	Charity worker fundraising for Childreach International a UK based charity that works with communities in various countries to improve child welfare
2015 – 2016	**Member** of Reatch (research and technology in Switzerland)

SPECIAL HONOURS

2009	Bilingual International Baccalaureate awarded
2010 – 2014	Elected student representative for neuroscience in teacher student committee in year 1, 3 and 4
2010 – 2011	Deans Honour List for bright and upcoming students in biology
2011 – 2012	Selected for study abroad at McGill University on a highly selective basis
2013 – 2014	Selected as student research assistant; awarded on a competitive basis
2015	Participation in Roche continents a seminar on science and arts, selected as one of the top 100 students in Europe aged between 20-29

PUBLICATIONS

2015	Li, B., Emmenegger, M., Schaper, E., Zurbrügg, M., Zhu, C., Gribbon, P., Guex, N., Hornemann, S., Xenarios, I. and Aguzzi, A. (2015). A genome-wide murine siRNA screen to investigate the molecular machinery governing PrPc biosynthesis and PrPsc propagation. (Poster) SystemsX.ch day 2015 Bern

INFORMATION TECHNOLOGY SKILLS

Basic Knowledge	Mircrosoft Excel, Powerpoint and Word
Statistical analysis	Graphpad Prism 6, Microsoft excel
Image programs	Neurolucida, Inkscape, Adobe photoshop, Zeiss Zen
Automated systems	Labcyte access platform, Echo acoustic dispenser, Perkin Elmer Janus robot, Biotek multiflo FX, Envision plate reader.