The role of RNA in prion aggregation and disease

PETAR STEFANOV KOVACHEV
As humanity evolved to witness an exceptionally high standard of living, Alzheimer’s, cancer, and diabetes gradually replaced infections as the main limiting factors in longevity. It is both disturbing and captivating that such degenerative conditions are caused by the most ubiquitous biomolecule – the protein. Indeed, proteins are not only the most functional, but also the least understood of the cellular biopolymers. It is then not surprising that many severe human ailments are associated with aberrant proteostasis. The key, causative mechanism of proteinopathy is protein aggregation. Naturally occurring and sometimes functional, aggregation is an auxiliary pathway in protein folding. In the context of a crowded cellular environment, folding and aggregation are the least and one of the least understood molecular processes, respectively. Unravelling one can help deconstruct the other and vice versa, but also can provide mechanistic insight on degenerative proteinopathies. A special class of proteins, which appear to propagate their own aggregation, occupy center-stage in the scientific field devoted to this goal. These proteins known as prions, can exist in at least two distinct forms. With the human prion, one of those is functional and benign and the other is infectious, aggregation prone, self-replicating and fatally pathogenic. As it happens, prion disease shares many of the descriptive features of other proteinaceous neuropathies. That, and the seductive idea that prions dwell in the twilight zone between folding and aggregation, have made the prion phenomenon a fixation for many molecular biologists. This thesis, although not the product of fixation, deals with one aspect of the prion process – the involvement of a molecular cofactor.

Of all plausible adjuvants, RNAs have been proposed as likely participants in the prion process. Their prominent secondary structures and attractive polyanionic surfaces allow RNAs to freely engage in interactions, at times transmitting conformational information through induced fit effects. The present work summarizes the influence of various RNAs on the aggregation profiles of three prionogenic model systems. The produced results indicate a generic role for RNA in the molecular processes prion propagation and aggregation. Altogether, this study illustrates a previously overlooked RNA function, of potential relevance for protein-based disease.

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“I would rather have questions that cannot be answered than answers that cannot be questioned.”

- Richard Feynman

To my parents who inspired me with curiosity and appreciation for the workings of all things natural!

In teaching or communicating science, passing this on has been my first and foremost prerogative.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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## Contents

Introduction ................................................................................................... 11

Proteins ..................................................................................................... 12

Protein folding and misfolding ............................................................ 13

Protein aggregation .............................................................................. 15

Functional aggregates .......................................................................... 16

Folding diseases and amyloidosis ........................................................ 17

Prions ........................................................................................................ 19

Prion disease ........................................................................................ 20

A note on functional prions ................................................................. 20

”Protein-only” hypothesis in prion propagation ................................. 21

Potential cofactors in prion propagation ............................................. 23

RNA ......................................................................................................... 24

RNA structure ...................................................................................... 25

The basis of RNA interactions ............................................................ 25

RNA in protein synthesis and folding ............................................... 26

Ribonucleoprotein granules in health and disease ......................... 27

Model systems in the present work ...................................................... 29

p53 – the tumor suppressor protein ................................................... 29

p53 function ......................................................................................... 30
List of abbreviations

CJD  Creutzfeldt-Jakob’s disease
CNS  central nervous system
CTD  C-terminal domain
DBD  DNA binding domain
DLS  dynamic light scattering
DNA  deoxyribonucleic acid
FL   full-length
GPI  glycophosphatidylinositol
IDR  intrinsically disordered region
LS   light scattering
NA   nucleic acid
NTD  N-terminal domain
MDM2 mouse double minute 2 homolog
mRNA messenger ribonucleic acid
mrPrP murine recombinant prion protein
OR   octapeptide repeat
p53  tumor suppressor protein
p53C central core domain of the tumor suppressor protein
PFAR protein folding activity of the ribosome
PFD  prion forming domain
PK   protease K
PMCA protein misfolding cyclic amplification
POH  protein only hypothesis
PrP  prion protein
PrPc cellular prion protein
PrPsc scrapie prion protein
PTC  peptidyl transferase center
RNA  ribonucleic acid
RNP  ribonucleoprotein
rRNA ribosomal ribonucleic acid
SN   supernatant
TEM  transmission electron microscopy
ThT  thioflavin T
tRNA transfer ribonucleic acid
TSE  transmissible spongiform encephalopathy
WT   wild-type
Introduction

The one categorical characteristic of life is the perpetual, active processing and replication of biological information. While inorganic matter could incidentally convert one form of energy to another, a living organism employs energy conversion as fuel for its main purpose – preservation, amplification and evolutionary modification of its genetic code. Encrypted within the nucleotide sequences of deoxy- and ribonucleic acids (DNA and RNA), the genetic code holds the instructions necessary for life to exist. This is the primary format biological information is found under and for it to become any functional relevance the genetic code first needs to be translated from a nucleotide to an amino acid sequence – a polypeptide chain. This process is governed by the principles outlined in the central dogma of molecular biology, which describes the stages in the unidirectional flow of biological information from DNA to RNA to polypeptide (figure 1).

![Figure 1](image)

**Figure 1.** The central dogma of molecular biology. The flow of biological information follows the consecutive steps of DNA transcription into RNA which is then translated into a polypeptide chain of amino acids. DNA, being the main repository of the genetic code, is amplified during cell replication.

Just like the term “information” incorporates the word “form”, so does the three-dimensional form of polypeptides carry biological information. Polypeptides are the materialization of the genetic code, but unlike DNA they impart information through both sequence and structure. Based on their unique amino acid sequence, polypeptides become functional proteins only after adopting a specific, spatial conformation. Because small alterations in the primary sequence can profoundly affect the final product of folding, protein sequence homology does not necessarily equate to functional similarity. Inability to adopt a native conformation can have detrimental effects on protein function and cell physiology. It is therefore obvious that function and structure are intimately related. Furthermore, in some cases the same polypeptide sequence can adopt several alternative conformations, of distinct biochemical properties. A prominent example of this type of polymorphism is observed in
a special class of proteins called prions, the existence of which has challenged our understanding of how biological information is transmitted.

Similarly, single-stranded RNA – a much more flexible and dynamic genetic code vessel than DNA, can obtain catalytic functions by folding into specific conformations. Even more curious is the potential ability of RNA to enforce conformational changes in proteins during interaction, in this way modulating their biochemical properties.

All in all, the principles which govern the transmission of biological information likely extend beyond canonical, sequential processing of the genetic code. Understanding how specific conformations are imparted on polymeric macromolecules can elucidate the structure-information relationship and help us understand fundamental, cellular processes such as protein folding and aggregation. Those complex, interaction driven events bear unparalleled significance for normal cellular function and pathophysiology.

This work was conceived to examine one aspect of the structure-information relationship, concerned with the causality of prion-like and disease associated aggregation of proteins. The innate propensity of the herein described model systems to self-organize in multi-protein complexes is studied in the presence of single-stranded RNA, testing the potential role of the nucleic acid as an adjuvant in this process. Prior to revealing the results of the included studies, the topics of protein folding and aggregation, the prion phenomenon and ribonucleic acids will be overviewed. This intends to provide a glimpse into an inherently complex subject matter, which involves many tightly knit, inter- and intramolecular events.

**Proteins**

Constituting for almost half of the dry mass of any cell, proteins are the most abundant type of biopolymer found in a living organism. They are also the most complex of all biological macromolecules. With a few exceptions, all proteins are built up from 20 standard, but varying in chemical property, blocks called amino acids [1]. During translation those are coupled by peptide bond formation on the ribosome to create linear polypeptide chains. The number of possible combinations between the amino acids, just along the length of the average eukaryotic protein, is unimaginable and also one of the reasons why proteins are the most versatile components of the cell. They carry out,
stimulate or control virtually every cellular process – from chemical catalysis to structural support, signalling and molecular transport.

Sequence diversity, however, is not the only reason why proteins are responsible for such a broad, almost unlimited, range of functions. A newly synthesized polypeptide chain is nothing more than a polymer with no particular benefit for the cell. To become functional, the nascent polypeptide must first take on a certain shape. The specific conformation a given polynucleotide sequence adopts is decisive of its function. The necessity of a functional conformation stems from the spatial requirements the living cell imposes on its components. At any given moment, thousands of chemical reactions happen within the nanometre scale of the cellular compartment. These interwoven reactions do not only need to be effectively isolated, but also perfectly coordinated, positioned and controlled to produce the desired outcome of the complex cellular processes. For each of the countless reactions life depends on, proteins provide custom-fit and secure platforms for operation. This ability of proteins to form and maintain unique three-dimensional structures is the main reason for their diversity and specificity. How proteins reach their functional, native conformation, in the context of a teeming cellular environment, is not only one of the biggest mysteries in molecular biology, but also one of the most crucial events in the cycle of the cell.

**Protein folding and misfolding**

As each of the 20 building blocks of a protein have distinctive chemical properties, the conformation of a polypeptide chain is the direct result of their individual orientation with respect to each other, and can therefore be attributed to sequence [2]. It is generally believed that the native state of a protein corresponds to the conformation which is most stable, while still soluble, under physiological conditions. However, for a polypeptide of 100 amino acids (three times shorter than the average eukaryotic protein) the number of possible conformations, fractionally less stable than the native, is unimaginable [3]. Without doubt, if a protein was to fold in a living cell, it is impractical to sequentially undergo all possible rearrangements on the search for a native state. Nevertheless, a polypeptide gains function only after sampling an extremely complex and heterogeneous free energy landscape, following one of many folding pathways. The graphical representation of all possible folding pathways for a given protein would be a funnel-shaped potential energy surface, where the largest number of conformational variations and the highest free energy lie at the very top (figure 2). Descending the energy landscape, a polypeptide samples one intermediate conformation after another as it approaches its most stable, native state, at the bottom of the funnel.
Protein folding is a spontaneous process, largely dependent on hydrophobicity, which minimizes the exposure of aliphatic residues to the aqueous environment. These forces drive the initial collapse and burial of non-polar side chains within the core of the protein and away from the solvent. Simultaneously, the formation of many, weak non-covalent interactions (van der Waals forces) occurs. This nucleation event results in an immediate and substantial gain in stability and a huge reduction in the number of meta-stable intermediates to be explored. The remainder of the side chains continue to sample the folding landscape, excluding more and more water molecules as they pack close to the hydrophobic pocket. The final state is achieved once all native-like interactions are established. Disulfide and salt bridges, along with the weak but numerous hydrogen bonds, are the final touches in the design, which give the native state stability, without making it rigid.

Figure 2. The free energy landscape of protein folding. Polypeptides move downward this funnel-shaped terrain sampling one conformation after the other until reaching the native state (green). Every depression (local minimum) in the rugged landscape represents a kinetically trapped intermediate conformation. Those are isolated by kinetic barriers which need to be overcome by returning to a higher free energy state before continuing the descend. When the kinetic barriers of local minima are high enough, extended conformational sampling occurs. This allows for adjacent, trapped intermediates to explore inter- instead of intramolecular interactions in attempt to minimize hydrophobicity. Such non-native interactions result in amorphous (orange) or amyloid (red) aggregates.
While reaching an explicit conformational state is crucial for a polypeptide’s gain of function, protein efficiency relies on a certain degree of flexibility. This is the reason why most proteins are only marginally stable in the context of their environment. This important feature has two important consequences for protein folding. First, this could lead to the unwanted accumulation of many non-native interactions as part of the folding process. The larger and more complex the native fold, the greater the risk of trapping the polypeptide in a partially folded state. Although such states can be very close to the native on the folding free energy landscape, the two are nevertheless separated by a significant kinetic barrier. Second, the marginal stability of the native state provides the opportunity for molecules of the same protein to fluctuate between several, more or less discrete conformations. Which of the states has greater occupancy will depend on the relative thermodynamic stability and rate of interconversion, under a given condition.

Both of the above scenarios will inevitably lead to the formation of misfolded or partially folded states. As they seek to reduce non-native interactions, such states are characterized by a slightly higher degree of conformational entropy, when compared to the native. This results in longer conformational sampling times, which in turn could cause temporary exposure of hydrophobic residues. When an increase in the local concentration of a misfolded state is also present, such species tend to minimize hydrophobicity through intermolecular interactions. In other words, incompletely folded states have a propensity to aggregate in a concentration dependent manner.

Protein aggregation

Protein aggregation can be perceived as an alternative pathway in the folding process (figure 3), where hydrophobic forces bury non-polar residues within the interface of two or more misfolded molecules, as opposed to the core of a single polypeptide. Because it involves more than one polypeptides, a certain threshold of incompletely folded species needs to be overcome before aggregation prevails over folding. Sudden changes in the local environment of folding proteins (temperature, pH, etc.) and/or destabilizing mutations can tip the equilibrium in favor of misfolding. When the proteostasis machinery of the cell is overburdened by the increasing number of misfolded species, the crowded environment of the cell, otherwise advantageous for functional interactions, becomes favorable for aggregation.

Two distinct types of aggregation can be described – amyloid and amorphous (figure 3), the latter being the more promiscuous form of aggregation. Unlike amorphous aggregates, amyloids are characterized by high order of organization, thermodynamic stability and a high free energy barrier for self-association [4]. Amyloid structures can be further divided into two classes, again
based on stability and morphology. Amyloid fibrils are elongated, linear self-assemblies of characteristic cross-$\beta$ structure. They feature the highest degree of organization and stability and their formation is usually accompanied by that of the less organized and much shorter oligomeric species. Certain biochemical properties, such as high $\beta$-sheet content, insensitivity to detergents and proteases and high affinity for specific dyes (Congo red and thioflavin T), can be attributed to amyloid but not amorphous aggregates [5]. Like regular protein folding, both types of aggregation are considered to be nucleation-driven events. This is quite obvious for amyloid fibrillation, where the initial lag phase of the process can be significantly shortened by addition of pre-formed “seeds” – the oligomeric precursors, which expose large, hydrophobic (sticky) surfaces.

**Figure 3.** The protein cycle – from synthesis to folding/misfolding, aggregation and degradation. Proteins play many complicated and essential roles, which cannot be exerted without them reaching their native state first. From the moment of their synthesis into a chain of amino acids with no fixed orientation, polypeptides have a small window of opportunity, in both time and space, to properly fold. If they fail, proteins face one of two possible outcomes – degradation or aggregation. Under the aggregation pathway proteins can form several structures of varying degree of organization. Those can either be functional or have an aberrant effect on their environment.

**Functional aggregates**

Although protein misfolding and aggregation connote abnormality, self-assembly into multimolecular structures of varying degree of organization is a
innate feature of all polypeptide sequences. When carried out under the conditions of a controlled environment aggregation can be of service to the cell (lower half of figure 3). Examples of functional aggregates can be found in prokaryotes, single cell and multicellular eukaryotes and we are just starting to realize the physiological significance of this process in humans [6].

In Escherichia Coli the Curli multiprotein assembly, allows for biofilm formation on inert surfaces and the chaplins group of amyloid proteins, secreted by different species of Streptomyces, aids colonization [7]. The yeast protein HETs is only functional in its amyloid form which regulates colony fusion [8]. Silkmoth cocoons and spiderwebs are examples of functional amyloids we are very much familiar with [7], [9]. By harnessing the unique properties of amyloid structures these unique materials provide an adaptive advantage to the animals they are naturally produced by. Interestingly, it has recently been reported that amyloids play a major role in promoting reproductive success in humans. As it turns out, seminal plasma is the only biological fluid in humans, where endogenous amyloid fibrils can be found [10]. Despite their enhancing effect on some sexually transmitted infections, such as HIV, the two classes of amyloids found in semen act as immobilizers of damaged sperm cells. Even more intriguing is the proposed prion-like, amyloidogenic mechanism of memory formation. The homologous pair of neuronal proteins CPEB and Orb2, found in Aplysia and Drosophila respectively, can exist in both monomeric and amyloid, oligomer states. Because inhibition of oligomerization for these proteins has been shown to be detrimental for memory persistence, their amyloidogenic conversion has been considered as a plausible biochemical switch in long-lasting memory formation [11].

It is worth mentioning that aggregation can also be harnessed by stressed eukaryotic cells as means of damage control. Under the conditions of excessive misfolding and overload of the protein degradation machinery, the cell responds by compartmentalizing the already labeled for degradation polypeptides, together with other cellular components like RNA, in non-membranous cytoplasmic inclusions called aggresomes [12]. Such structures are not entirely amorphous and exhibit a certain degree of organization. In this way the excess of misfolded, aggregation prone proteins are steered clear from non-specific interactions with functional cellular components.

Folding diseases and amyloidosis

Because of the relevance and ubiquity of proteins, failure to adopt and maintain a single and stable conformation can lead to overreaching, negative consequences for the cell and often the entire organism. As impairment in folding arises, proteostasis will attempt to eliminate peptides incapable of normal function. If that fails, and misfolded or partially degraded species reach critical
concentration, intra- or extracellular aggregation will inevitably ensue (upper half of figure 3). Much like abnormal enzymatic activity leads to metabolic defects, largescale misfolding inevitably cripples at least one molecular pathway, regardless of the occurrence of aggregation. Nowadays, it is increasingly evident that protein misfolding and aggregation are explicitly responsible for a wide range of often fatal conditions in humans, such as cancer, diabetes and neuropathology (table 1). Often referred to as protein folding or deposition disorders [13], [14], these conditions are considered to be among the most devastating, costly, socially disruptive and prevalent in the western world.

The largest and most notorious group of protein folding diseases is the one characterized by intra- or extracellular amyloid deposits. While incomplete folding is the undisputed cause of their origin, self-organization of peptides or full-length (FL) proteins into amyloid structures is the hallmark of their pathology [15]. It is still debatable which of the amyloid species (insoluble aggregate or soluble precursors) convey toxicity, but once established, their formation is perpetual, irreversible and fatal. In the cases where toxicity is associated with protein deposition, such as neurodegenerative disorders and diabetes, accumulation of large quantities of insoluble aggregates is disruptive of the normal tissues function. Alternatively, in certain types of cancer, the cause of the degenerative condition is inhibition of the tumour suppressor protein (p53) through abnormal sequestration.

Table 1. Examples of protein folding/deposition diseases, their causative agents and localization.

<table>
<thead>
<tr>
<th>Causative Agent</th>
<th>Disease</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prion protein (PrP)</td>
<td>Transmissible spongiform encephalopathy</td>
<td>Brain</td>
</tr>
<tr>
<td>Amyloid-β and fragments</td>
<td>Alzheimer's disease</td>
<td>Brain</td>
</tr>
<tr>
<td>Tau</td>
<td>Alzheimer's disease</td>
<td>Brain</td>
</tr>
<tr>
<td>Huntington</td>
<td>Huntington's disease</td>
<td>Brain</td>
</tr>
<tr>
<td>α-synuclein and fragments</td>
<td>Parkinson's disease</td>
<td>Brain</td>
</tr>
<tr>
<td>Lysozime</td>
<td>Hereditary systemic amyloidosis</td>
<td>Several organs and tissues</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>Senile systemic amyloidosis</td>
<td>Almost all organs and tissues</td>
</tr>
<tr>
<td>Insulin</td>
<td>Injection-localized amyloidosis</td>
<td>Skin, muscles</td>
</tr>
</tbody>
</table>
Protein folding disorders do not necessarily have to be associated with amyloid deposits. In fact, a pathological state can arise as a result from the reduction in the active concentration of a given protein through regular posttranslational mechanisms, before a concentration of the misfolded species, which promotes aggregation, has been reached. One such example is cystic fibrosis, an autosomal recessive condition, which results from the rapid degradation of a chloride channel protein upon mutation [16]. In addition, amorphous aggregation during protein folding is promiscuous enough to have its own pathogenic implications. A cataract-associated $\gamma$-crystallin mutant, for instance, displays interesting polymorphism of aggregation [17]. Under acidic conditions in vitro it readily forms amyloid fibrils. However, its aggregation is amorphous, when incubated at neutral pH, resembling the natural environment of the eye.

**Prions**

For decades a special and formidable class of infectious, amyloidogenic proteins, have captivated and bewildered biologists. With their singular ability to convey biological information through conformation, prions have become center-stage model systems for studying the governing principles of folding, aggregation and protein deposition disease. A set of proteins, no different in composition from any other polypeptide, prions stand out by their ability to adopt more than one, distinct and stable conformational states. In other words, prions can exist in at least two isoforms, one of which features increased propensity for amyloidogenic aggregation. More importantly, the amyloid state has the unique capacity to self-replicate in a dominant manner. Once present in the cell, the amyloid species draws in native molecules of the same protein, passing its conformational features onto them [18]. In this way the prion isoform is self-sustaining and transmissible over time and across cell divisions, much like a viral particle, but one devoid of a genetic vector, for unlike vi-
ruses, prions are encoded by chromosomal genes [14]. In some cases, and ultimately those which drew attention to the prion phenomenon, this conformation is also infectious and associated with the irreversible and fatal progression of a group of neurodegenerative disorders in mammals, commonly designated as transmissible spongiform encephalopathies (TSEs).

**Prion disease**

TSEs are, by definition, protein misfolding and deposition conditions and share most of the mechanistic and pathological features of the amyloidosis discussed above. The only difference lies in the undeniable infectivity of canonical prion disorders. For now, neither Alzheimer’s, Parkinson’s nor p53 associated cancers are considered to be absolutely transmissible. Nevertheless, the aggregated species behind their pathology have been shown to behave a lot like prions – triggering transcellular protein misfolding and aggregation [19]–[23]. As more evidence, in support of a common molecular mechanism, emerge [24], [25], the parallel between prion disease and other degenerative proteinopathies grows deeper.

The chronicles of prion disease, place the earliest official record of infection in sheep in 18th century Europe [8]. Chinese scriptures, thousands of years older, hint at a similar outbreak in China. Even so, the necessary and self-sufficient causative agent behind mammalian TSE was only identified in the early 1980s [26]. This turned out to be the self-templating, amyloidogenic state of a ubiquitous, cellular protein, simply designated the prion protein (PrP). The subsequent realization of the wide prevalence and prion-like mechanism of propagation of amyloidogenic conditions has fueled the rapid expansion of the prion field [27].

**A note on functional prions**

Even more interesting and perplexing is the apparent non-pathogenic role prions play in single cell fungi, where epigenetic, heritable, prion phenotypes appear to provide a selective advantage [8], [28]. Moreover, a large number of proteins, of wide phylogenetic distribution, capable of self-sustaining an amyloidogenic conformation has recently emerged [6]. Sustained protein aggregation associated with physiological activity is the descriptive feature of functional prions, on which many cellular processes, such as memory, have been proposed to depend. All this suggests that the prion phenomenon is not necessarily malignant, nor uncommon, but is likely an evolutionary conserved behavior of functional, aggregation prone sequences.
"Protein-only" hypothesis in prion propagation

Despite the significant progress, the nature of prions remains invariably enigmatic. The main reason for that is our limited understanding of the mechanism through which prion conformations emerge and propagate their structural and pathogenic features. In attempt to provide a unifying theory, the prion community has developed a tendency to place prion emergence, transmission, infection and toxicity on the same mechanistic platform, introducing a great deal of confusion in an already complex matter. While these are all properties of a true disease associated prion, not all prions are pathogenic and many functional amyloidogenic proteins are proposed to have a prion-like behavior. Even if these features share a mechanistic and causative relationship, it is important to remember that they are not equivalent. Since the ability to adopt alternative conformations lies in the center of the prion phenomenon, it is essential to first address the emergence of various prion conformations and then establish a causative relationship with other properties. The most popular, but nevertheless thought-provoking attempt to put forward a unifying principle for prion propagation is the “protein-only” hypothesis (POH). According to this conjecture, launched by Griffith half a century ago [29], a distinct conformational state is the sole constituent of a prion and is necessary and self-sufficient for the replication of all prion features. A turning point in the prion field, which affixed this idea as a bona fide molecular principle, was the isolation and biochemical characterization of infectious prions in the 1980s [26], [30].

The central premise of the POH presumes that, in the absence of a genetic factor, prion replication is conveyed by the amyloid, prion state either through induction of aggregation or conformational change of the native state (figure 4). If this is the case, then the existence of prion strains of different phenotypes must be in direct violation of the central dogma and the fundamental principle of protein folding, proposed by Anfinsen [2] and their reproduction from prion protein alone should be possible in an in vitro setting.
Figure 4. Two possible models of prion conversion under the “protein-only” hypothesis. A – the template-assisted model assumes a high, free energy barrier between the native and prion isoforms, the latter being more thermodynamically stable and exogenous. This barrier can rarely be crossed but when the two isoforms come in contact the native inherits the structural features of the prion state, through a molecular mechanism which is poorly understood. B – under the nucleation-polymerization model, the two isoforms are endogenous and in a reversible equilibrium. When two monomeric, prion conformations happen to form a highly ordered, seed-like homodimer, further amyloid aggregation ensues. This initial step is extremely slow, but potentially provides an explanation for the emergence of sporadic TSE in the absence of mutations.

Several cell-free methods, such as protein misfolding cyclic amplification (PMCA) and high-frequency shaking of brain homogenates, have been successful in demonstrating that the biochemical characteristics of prion strains are maintained during prion propagation in vitro [27]. Nevertheless, such approaches are neither close to free nor independent of the endogenous non-prion protein components available in the homogenates they employ. Although infectivity of WT animals is yet to be achieved from purified, native prion protein, infectious prion states have been generated in a minimal component system, in vitro [31]. All this is indicative of the importance of non-proteinaceous adjuvants in prion conversion and propagation.
Potential cofactors in prion propagation

Of all the molecules investigated to date (proteoglycans, glycosaminoglycans, lipids, copper ions, various proteins and nucleic acids) RNAs have emerged as likely cofactors in this process [32]–[34]. Reduction of prion conversion in cyclic amplification assays upon RNase treatment [35] and complex formation between the prion protein and RNA [36] are direct indications of the accessory role played by these polyanions. Moreover, the first ever de novo generation of a prion state in the absence of preexisting prion seeds has been demonstrated in a minimal component system supplemented with RNA [37]. It remains to be clarified whether RNA has a structural or informational contribution in the prion process. The facts that prion infectivity remains insensitive to photofragmentation of incorporated RNAs and the stimulating effect of polyanions on prion conversion is only size- but not sequence specific, exclude the possibility of a classical genetic information role [27]. Nevertheless, based on their chromosome encoded sequence, endogenous RNAs vary in secondary structures and therefore polyanionic surfaces. In this way different RNAs could play a hybrid role, exerting both structural and informational input on the emergence of distinct prion conformations. This coincides with the “cofactor variation” hypothesis in prion strain diversity [38], [39], which predicts that different cofactors would have variable effect on prion conversion, leading to the propagation of specific prion conformations and therefore phenotypes (figure 5).

![Figure 5](image.png)

**Figure 5.** The effect of cofactors on the conversion of prions into different states. Through direct interaction with the native prion conformation, different polyanions may decrease the free energy barrier of conversion to the prion conformation. Due to the variable nature of the interaction, different cofactors provide, it is presumed that this may lead to the generation of distinct prion conformations and phenotype (prion strains) – “cofactor variation” hypothesis. N stands for native, I – for intermediate and U – for unfolded conformations. Modified from reference [40].
Decades after its conception the protein only hypothesis remains neither refuted nor confirmed. To advance their rhetoric, supporters [41] and opponents [42] of the POH use the same arguments [31]. What has irrefutably transpired from this stalemate situation is the nomination of a putative cofactor in prion disease propagation – RNA. As discussed above, conformational polymorphism, which results in aggregation is not limited to prion disease, but is the hallmark of all protein misfolding and deposition disorders. Aberrant accumulation of misfolded protein, associated with pathogenicity, is another common feature of both groups of degenerative conditions. Even though TSEs are the only proteinopathies considered infectious, aggregation in protein misfolding disorders is a nucleation-driven process accelerated by the presence of preformed seeds [43]. With prions, this universal mechanism is crucial for conversion and propagation and is likely dependent on the participation of specific cofactors. It is therefore an effortless leap of the inquisitive mind to invite the question whether all misfolding disorders share a prion-like mechanism of propagation and infectivity. Not surprisingly, indications of this have already began to surface [25], [44].

**RNA**

Ribonucleic acid (RNA) is another of the macromolecules essential for life. Although not as versatile as proteins, RNA is a biopolymer involved in many physiological functions from encoding genetic information and structural support to regulation and catalysis. Occupying the midway post in the central dogma (figure 1), it is best known for its intermediary role in the transfer of biological information from genes to functional proteins. Messenger RNA (mRNA), produced during gene transcription, is the actual biological template, used by ribosomes for protein synthesis. As it so happens, along with constituting the bulk of the ribosomal particle (over 60% of the total mass is ribosomal RNA – rRNA), RNA is also its main functional component [45]. The peptidyl transferase center – the ribosomal catalytic site, which fuses amino acids together, during polypeptide synthesis, is entirely comprised of RNA. Transfer RNAs (tRNAs) are a sort of a dual-role, hybrid molecules, responsible for delivering amino acids to the ribosome. The molecular equivalent of a courier service tRNAs transport the right amino acid to the right spot, according to their codon nomenclature. Micro and small interfering RNAs cause up- or downregulation of various genes, while small nucleolar
RNAs modify RNA. Because of its ability to exert genetic, structural or catalytic functions, RNA has long been proposed to predate both DNA and proteins in the evolution of life [46].

**RNA structure**

Like DNA – the main repository of genetic information, RNA is a nucleic acid. Both nucleic acids nucleic acids are comprised of the same purine and pyrimidine nucleotide bases, with RNAs incorporating an uracil instead of a thymine. Another major difference between the two is the extra 2´-hydroxyl group on the pentose ring of RNA nucleotides. This feature is the main reason for the divergent structural and functional properties of DNA and RNA [47].

Unlike DNA, all RNA is single-stranded upon synthesis. Together with the 2´-hydroxyl group – the only hydrogen bond donor/acceptor on the phosphodiester backbone, this allows RNA to fold into a large variety of structures, which are reminiscent of both DNA and proteins. Expectedly, this process is quite akin to protein folding as the same driving forces apply – hydrophobic effects, hydrogen bonds formation, van der Waals interactions and solvation/neutralization of exposed charges. Single-stranded RNA, however, has a uniformly, negatively charged backbone, which is also much more flexible, due to the larger number of variable torsion angles. The combined stabilizing effect of backbone hydrogen bond interactions, non-Watson- and Watson-Crick interactions leads to the emergence of a set of stable, secondary structure elements, characteristic for RNA folding, such as bulges, turns, helices, and multi-way junctions. Those provide diverse topological surfaces, which can serve the purpose of catalytic and binding sites – the main reason for the functional versatility of RNAs.

**The basis of RNA interactions**

To accommodate their many functional roles, RNAs are prone to various macromolecular interactions. The forces which stabilize these noncovalent complexes are expectedly weak, but numerous. The practically polyanionic nature of NAs, and RNAs in particular, is attractive to proteins of net positive charge, and these electrostatic forces are predominant in NA-protein complexes. However, hydrophobic interactions are also quite common, usually when proteins bind non-canonical RNA secondary structures. Polar interactions, such as direct or water-mediated hydrogen bonds, are also quite frequent [45].

In terms of preferred protein binding sites, b-sheet surfaces and b-strand connective loops, seem to be favored by single stranded RNA. Inversely, double-stranded and loop RNA regions favor α-helices. An often-observed phenomenon during NA-protein complex formation is the induction of conformational
changes in the structures of one or both interaction partners, as a result of solvent displacement and reduction of conformational flexibility. This so-called induced fit effect undoubtedly plays an important role in normal physiological functions, such as catalysis and recognition. Besides, this curious scenario where the whole differs from the sum of the components could also have relevance for prion propagation. After all, RNAs have been proposed as conversional cofactors during this poorly understood process. Direct evidence of the induced fit between murine recombinant PrP and RNA, upon interaction, is provided in paper II of this work.

**RNA in protein synthesis and folding**

One of the major and most significant cellular functions of RNA, is protein synthesis. The largest molecular machine of the cell – the ribosome, catalyzes the successive peptide bond formation of amino acids by simultaneously decoding the genetic information mRNAs encrypt and orchestrating a sequence of auxiliary, molecular events, such as tRNA accommodation, proofreading, translocation etc. This remarkably complex and finetuned process is almost entirely dependent on RNA. At the heart of this molecular production line lies the peptidyl transferase center (PTC), located on the large ribosomal subunit, within domain V of its major rRNA – the 23S in prokaryotes and 28S in higher eukaryotes. The PTC features a high concentration of nucleotide elements, which are universally conserved in sequence through all domains of life [48] – figure 6.

![Figure 6. Schematic representation of the PTC of the large ribosomal subunit, based on the 23S rRNA of *E. coli*. Green regions correspond to nucleotide sequences, which are highly conserved among all domains of life [49]. Sequences identified to interact with denatured proteins during refolding are colored in red [50]. Virtually most of the PTC region of domain V on the 23S rRNA of bacteria is conserved – gray and green areas.](image-url)
Peptide bond formation has long been considered the singular function of ribosomes. A quarter of a century ago, this widely accepted notion was challenged by a series of *in vitro* experiments, which demonstrated the ability of ribosomes to aid refolding of denatured proteins [51]. This auxiliary activity of the protein synthesis machinery was again suspected to reside within domain V of the 23S rRNA [52]–[54]. Later, mutational and cross-linking studies mapped the exact nucleotides involved in interaction with denatured proteins, confirming this suspicion [50] – figure 6. Unlike peptide bond formation, the exact molecular mechanism, which governs the protein folding activity of the ribosome (PFAR) remains unclear. Nevertheless, the colocalization of these two, complementary ribosomal functions on one of the most conserved domains of rRNA, is a strong implication of their parallel coevolution. In paper III we revisit the topic of PFAR in attempt to disconnect this function from yet another, more general, role of RNA, concerned with protein aggregation.

Ribonucleoprotein granules in health and disease

In recent years it has become increasingly obvious that RNA has functions not only within, but also beyond our conventional understanding of the central dogma [45]. Clear evidence of this can be found in the assembly and regulation of ribonucleoprotein (RNP) granules.

Characteristic of eukaryotic cells RNPs are non-membrane-bound organelles, enriched in RNA and RNA-binding proteins. Cajal bodies, stress granules, the nucleolus and processing bodies are examples of such structures [55]. They feature higher protein concentrations than their surrounding environment – the cytoplasm or the nucleus, and assemble through protein-protein and protein-RNA interactions.

RNPs are an important class of organelles, directly involved in many physiological processes, such as stress response and regulation of the flow of genetic information. Regardless, their abnormal homeostasis has been proposed as contributing factor in protein deposition disease [56]. As previously discussed, intracellular aggregation is the hallmark of most degenerative proteinopathies and, obviously, the main argument in favor of this notion. Even more conspicuous is the demonstration that the majority of proteins recruited by RNPs contain intrinsically disordered regions (IDRs). Such low complexity regions have been directly implicated in the formation of pathogenic amyloids and are characteristic for canonical prions, such as [PSI+] and PrP. Dissecting the molecular mechanisms behind RNP assembly could, therefore, prove essential outlining the events which precede the onset of amyloidosis.
Instead of being a passive component of RNP granules, RNA appears to occupy an executive position in their formation and homeostasis. It has recently become evident, that RNP assembly is driven by liquid-liquid phase separation. This process is nucleated and controlled by RNA and is largely achieved through multivalent RNA-protein interactions, which increase the local concentration of bound proteins. As a result, hydrophobic contacts between the IDR(s) of the adjacent proteins ensue and contribute to RNP integrity [57]. RNA secondary structure, size and charge distribution, as well as the RNA-binding properties of the protein, appear decisive for RNA-protein interactions and, therefore, the phase transition process [55], [57]. Furthermore, different RNAs lead to the formation of RNPs with variable biophysical properties. Moreover, this also seems dependent on the molar ratio between the RNA and protein components. Finally, it has been demonstrated that maturation of RNPs containing IDR-fused proteins is associated with decrease in the dynamics and increase in the stability of granules, leading to progressive amyloid fibrillation [55]. Through protein and ribonuclear homeostasis, it is presumed that RNPs are regulated to meet the cellular requirements for macromolecular assemblies of varying structural and physicochemical properties. However, when regulation is compromised or aberrant mutations in the IDR(s) of associated proteins are present, this could promote formation of pathogenic aggregates [56].

To conclude, an auxiliary, non-conventional function for RNA has emerged from studying RNP granules and PFAR. In addition to its traditional role of a genetic element in the central dogma, RNA seems to provide structural information crucial for protein phase transitions and refolding. This ability of RNA to serve as template for protein aggregation could prove instrumental for the understanding of protein folding (in the crowded cellular environment), prion propagation and emergence of degenerative proteinopathies.
Model systems in the present work

Three unrelated proteins were chosen as model systems for studying the effects of RNA on the aggregation propensity of prionogenic systems. As a result of their normal function or as part of an aberrant process, all three have a natural tendency to self-organize in amyloid and/or amorphous aggregates. Those proteins are the central core domain (p53C) of the human tumor suppressor protein, the murine, recombinant prion protein (mrPrP), and the yeast prion [HET-s]. Although not a prion in the canonical sense, the prion-like behavior of the tumor suppressor and its core domain has previously been described. Unlike the full-length protein, however, p53C a much easier target for recombinant overexpression. Furthermore, 95% of all cancer associated related mutations of the tumor suppressor, are located within the DBD of p53C. All this has made p53C an attractive model system in cancer research. The murine prion protein, on the other hand, shares a high degree of homology with the human variant, but unlike it has not yet been shown to be infectious to humans. Furthermore, its recombinant expression in *E. coli* generates high yields of pure protein. For those reasons mrPrP is a widely used and safe model prionogenic system. Both PrP and p53 are disease associated proteins and studying their aggregation behavior is instrumental for understanding the molecular events which govern their pathology. [HET-s] on the other hand was the first ever prion with a proven physiological functional, making it a valuable model for exploring the evolutionary relevance of prions.

p53 – the tumor suppressor protein

In 1979, during immunoprecipitation of large T-antigen from SV40-induced tumors, several groups, independently discovered what was to become one of the most extensively studied proteins ever [58]–[61]. For a while various, unrelated laboratories kept documenting the isolation of that same protein in complex with cancer-promoting proteins, expressed by cells transformed by different, tumor-causing retroviruses [62]. The novel, 53 kDa protein gained
the reputation of an oncogenic factor, at first, due to its low abundance in normal tissue and high levels in cancer cells. This pretty much drove the frenzy for its gene isolation and cloning. It took years before consensus was reached to designate the new cell-cycle accelerator as p53, and several more before showing that in fact it was exactly the opposite – a tumor suppressor. The realization came after a series of genomic analyses which provided evidence that colorectal tumor cells regularly lost the gene for p53 (TP53) [63]. The wild-type (WT) protein also turned out to be a potent inhibitor of cell transformation by oncogenes [64], [65], making it the first tumor-suppressor to be identified.

**p53 function**

Even though the full picture of p53’s function is yet to be constructed, decades of research and thousands of studies have unequivocally shown that wild-type p53 is a potent tumor suppressor and mutations in its gene are likely the most prevalent cause of cancer in humans. Inversely, either through dominant-negative inactivation of WT p53 or through actual gain-of-function [66], p53 mutants are directly related to over half of all p53-associated tumors and therefore can be considered as oncogenes. In fact, TP53 somatic mutations can be detected in almost every form of cancer, with different rates of occurrence [67]. The one major distinction between WT p53 and essentially all of its cancer causing mutant forms is the ability to bind DNA in a sequence specific manner and transactivate nearby genes. It is for those reasons p53 research has been instrumental for our understanding of tumorigenesis. For reasons discussed further in this text, it has also been the source of great confusion and controversy.

**p53 – a cell cycle regulator**

A single nick in the double helix of DNA can activate p53, which binds to consensus responsive elements, upstream or within target genes, in a sequence specific manner [68]. It then coordinates a succession of molecular events meant to regulate the expression of those genes and bring about one of several possible outcomes – cell cycle arrest, senescence or apoptosis, being the three main and also best studied (figure 7) [69]. Although there is growing evidence that p53 can also induce cell death through non-transcriptional cytosolic interaction with apoptosis-regulatory proteins [70], its major function remains that of a sequence specific, transcriptional factor. The list of genes p53 regulates the expression of is continuously growing, and not all of those targets encode for proteins involved in genome maintenance and apoptosis [69], [71]. This widespread transactivation activity of p53 is directly dependent on its ability to first recognize specific DNA sequences and then recruit general or specialized activators or repressors of the transcriptional machinery. How and if p53
exerts its regulatory function on a given target gene is cell type and context specific event, which is the consequence of a fine-tuned regulatory process.

Figure 7. p53 response with respect to stress levels. A prolific transcriptional activator, p53 can tailor the cell’s reaction to a specific stress factor. Under a no-stress situation for a non-embryonic cell, p53 activity is minimal, due to its fast degradation upon ubiquitination by MDM2 (discussed further below). Under low-stress conditions, the p53 pathway is responsible for decreasing oxidative stress and relieving sporadic DNA damage, which might be inflicted by external factors or encountered through normal cell growth and development. As the extent of sustained DNA damage increases, the response could lead to temporary arrest in cell development until DNA maintenance is complete. In extreme cases, where damage is beyond repair and oncogene activation is en route, the tumor suppressor sets in motion the programmed cell death machinery. p53 has also been proposed to be a major contributor in cellular senescence.

p53 activation

p53 activation is a stress response. Many intra- and extracellular factors, such as oncogene activation, DNA damage, ribosomal stress, nutrient deprivation, hypoxia [72], [73] and even everyday stress [69] can trigger increase in p53 levels or disrupt its tight regulation (figure 8). Each of these factors deploys a specific set of signaling molecules, designed to switch p53 on through a separate pathway. For instance, DNA damage is monitored by a set of highly conserved checkpoint kinases, which stabilize the tumor suppressor through covalent modification (discussed in more detail below) [71]. Oncogene induced aberrant growth, stimulates the expression of a small protein called ARF, which sequesters MDM2 [74] – a major negative control element in p53 regulation.
Figure 8. Simplified scheme of WT p53 activation, regulation and function. DNA damage and metabolic stress can activate WT p53 (center right) through the release of checkpoint kinases (blue box) which change its conformation through covalent modifications. Kinases either phosphorylate the transactivation domain (TAD) of p53, in this way protecting it from direct inhibition by MDM2, or modify its regulatory domain (RD), preventing it from blocking the DNA binding domain (DBD). Oncogene activation, leads to the synthesis of the ARF protein which impedes the interaction between MDM2 and p53. Under any of those scenarios, the negative regulation relieved and p53 is free to engage its target genes. This can result in cell-cycle arrest or apoptosis, but in all cases, leads to upregulation of MDM2 synthesis, as part of the negative feedback loop, which lies in the center of p53 regulation. Circles with the symbol “P” inside stand for phosphorylation sites; ellipses containing the symbol “Ac” mark acetylation sites and hexagons with the symbol “U” – ubiquitination sites.

*p53 regulation*

The p53 response to different genotoxic stimuli is multifaceted and regulation of the tumor suppressor is clearly an existential prerogative of the non-stressed cell, which maintains particularly low levels of the WT protein, estimated around 140 nM [75]. While transgenic mice carrying a null allele for *TP53* are viable they are quite susceptible to spontaneous and highly-penetrable tumorigenesis [76]. Vice versa, failure to maintain a low basal state of p53 leads to early embryonic lethality [77]. It is therefore obvious that p53 abundance and activity must be the subjects of an intricate regulatory mechanism. The
complexity of this network, negative in essence on many of its levels, is yet to be fully understood.

**MDM2 and the negative feedback loop**

Certainly the most significant and best studied regulatory pathway for the tumor suppressor lies within the negative feedback loop it forms with one of its many, protein interaction partners – the mouse double minute 2 homolog (MDM2) [62]. Often referred to as p53 gatekeeper, MDM2 is a ligase which labels p53 for ubiquitin-mediated proteolysis [78]. The gene for MDM2 happens to be a key transcriptional target of p53. Upon binding to its regulatory region p53 induces MDM2 expression, which in turn tags p53 for degradation. In this way a low basal level of p53 in the cell is maintained through degradation, rather than production. In addition MDM2 has been shown to impede the activity of p53 by direct interaction with its transactivation domain [79].

**Covalent modifications**

While the MDM2 feedback loop is an example of negative regulation, covalent modifications, which induce conformational changes in newly synthesized p53, activate its function or make it less susceptible to degradation [71]. Examples of such modifications include acetylation and/or phosphorylation of the C-terminus, which prevent it from folding back onto the DNA-binding domain of p53 and therefore enhancing its activity. Phosphorylation of the opposite end of p53 (the N-terminus), by checkpoint kinases, in turn modulates its affinity to MDM2.

**p53 isoforms and oligomerization**

In its functional state WT p53 is a homo-tetramer, which binds with high affinity to target responsive elements via its DNA binding domain. This functional requirement for the formation of a higher molecular complex provides even more opportunity for regulation. Aforementioned, post-translational modifications regularly alter the conformation of the monomeric subunit, exerting control over its ability to tetramerize and bind DNA. As discussed above, most TP53 mutations observed in human cancers not only lack the ability to specifically bind p53 responsive elements, but also apply dominant-negative pressure over the WT protein [66]. This activity exploits the functional requirement for oligomerization, effectively lowering the number of active p53 through hetero-tetramerization between mutant and WT protein. On top of that, p53 mutants implicated in cancer development, have decreased thermodynamic stability and higher propensity to self-associate or aberrantly co-aggregate with WT protein [80].

Through alternative splicing and variation of promoters and initiation of translation, the human TP53 gene can produce up to 12 different isoforms of varied
lengths and, presumably, transcriptional activity. Although a comprehensive grasp on the biological relevance of those isoforms is missing, it is clear that some ($\Delta 133p53\alpha$) affect the activity of WT p53 by direct interaction and hetero-oligomerization, and others ($\Delta 40p53\alpha$) enhance the dominant-negative effect of certain mutants [81]. On a side note, p53 isoforms seem to play a profound role in cancer progression and survival outcome.

It has long been speculated that p53 oligomerization and aggregation are key contributors to the complex regulatory network of this protein. All of the above examples not only reinforce this speculation, but also implicate these processes in tumorigenesis. Furthermore, the attribution of a prion-like characteristic to the aggregation process of p53, draws parallels between the molecular basis of cancer and some of the least understood and most devastating proteinopathies – the protein misfolding and deposition diseases [22], [23], [44], [82].

$p53C$

Up to this day, over 31 000 somatic p53 mutations have been identified in human tumor samples (p53.iarc.fr). 95% of those are located within the DBD and can either disrupt recognition and binding of DNA or compromise the stability of the entire protein [83]. This domain is contained within residues 94 – 312 of the tumor suppressor and an artificial construct based on this region was originally proposed by Alan Fersht as a suitable model for studying the effect of cancer associated mutations on the stability of the DBD [84]. This isolated region of p53 was designated the central core domain (p53C) and has gradually become a test platform for potential anti-cancer therapeutics. Subsequently, the ability of mutant and WT p53C to form amyloid structures in vitro and recruit soluble, WT protein in a prion-like fashion was demonstrated [80], [85]. Not long after, these key features of prion-like propagation were confirmed in an in vivo setting [23]. Both full-length and p53C exogenous aggregates were shown to penetrate cells and co-aggregate with endogenous p53.

The present work is the first of its kind, in-depth investigation into the in vitro aggregation of p53C in the presence of RNA. C-terminally truncated p53 variants, such as p53C, lack the regulatory domain, implicated in RNA binding. Nevertheless, it has been demonstrated that such constructs still participate in non-specific interaction with RNA [86]. This is quite clearly indicated by the findings of paper I in this thesis, which indicate a possible role of RNA in the prion-like behavior of p53C.
PrP – the mammalian prion protein

The prion protein (PrP) is found in all mammals and is highly conserved among all animal species – from fish to human [87]. Normally, it exists in a soluble, functional form – PrP<sub>C</sub> (C stands for cellular), but under rare and poorly understood circumstances it can also convert to an amyloidogenic and pathogenic conformation – PrP<sub>Sc</sub> (Sc stands for scrapie). This alternative isoform of PrP is associated with the irreversible progression of a group of fatal disorders in mammals called TSEs. Even though TSEs had been under vigorous scrutiny for decades, concrete evidence of the biochemical nature of PrP was only produced in 1982 as a result of the characterization of the infectious agent behind scrapie (TSE in sheep and goats) using a hamster model [26]. The infective properties of the isolated, small particle turned out to be resistant to procedures known to inactivate virulence through NA destruction, but remained sensitive to chemical denaturation and subsequent protease digestion. It was hence assumed that the scrapie agent carried a proteinaceous component and, because of its novel pathogenic properties, was the first of its kind to be assigned the term “prion” (word amalgam between pr-otein and infection) [30].

Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies are a group of neurodegenerative disorders, characterized by an extremely low frequency and a relentless progression to a fatal end (table 2). Unlike any other illness of a single cause, prion disease can have infectious, genetic or sporadic manifestation, the latter being the most prevalent (over 3/4 of all cases in human) [88]. The most common of the TSEs, which affect humans, is the Creutzfeldt-Jakob’s disease (CJD). It accounts for as much as 85% of all cases and its symptoms include myoclonus and dementia. Infectivity sets apart TSEs from other neurodegenerative disorders such as Alzheimer’s and Parkinson’s, which, so far, have shown no transmissibility from one host to another and between species. Despite the rich history of TSE outbreaks, often associated with dramatic accounts of cannibalism, for instance ritualistic among the Fore people of New Guinea and industrial – “mad cow disease”, the frequency of infectious prion disease is less than 1% [14].

Prion disease affects only the central nervous system (CNS) leading to neuronal loss and prominent spongiosis, followed by astrocyte gliosis and spongiform degeneration, akin to the histopathology of Alzheimer’s [28]. 10% of all CJD patients display amyloid deposits comprised of the scrapie prion isoform. The key molecular event, which marks the irreversible onset of prion
pathogenicity, is the conversion of soluble, cytosolic PrP to amyloidogenic PrP\(^{Sc}\) [89]. This is imminently followed by perpetual aggregation and, although several working hypotheses exist, it is not entirely clear how the various species produced during this process lead to neural degeneration [28]. As cell grafts overexpressing PrP\(^{C}\) remain the only infected regions in the brains of PrP-deficient mice exposed to scrapie protein it seems obvious that neural toxicity depends on the autonomous expression of PrP\(^{C}\) [90]. All familial, and some sporadic, forms of human TSEs are caused by dominant mutations in the gene of the human prion protein (PRNP).

Table 2. Examples of TSEs in mammals, their host and mechanism of contraction. Adapted from [14], [88].

<table>
<thead>
<tr>
<th>Disease</th>
<th>Host</th>
<th>Mechanism of contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJD</td>
<td>Humans</td>
<td>Infection through medical procedures and ingestion of contaminated animal products; Germ-line mutations in PRNP; Somatic mutations and spontaneous conversion of PrP.</td>
</tr>
<tr>
<td>Kuru</td>
<td>Fore people of New Guinea</td>
<td>Infection through ritualistic cannibalism.</td>
</tr>
<tr>
<td>Fatal familial insomnia</td>
<td>Humans</td>
<td>Germ-line mutations in PRNP.</td>
</tr>
<tr>
<td>Sporadic fatal insomnia</td>
<td>Humans</td>
<td>Somatic mutations and spontaneous conversion of PrP.</td>
</tr>
<tr>
<td>Scrapie</td>
<td>Sheep</td>
<td>Somatic mutations and spontaneous conversion of PrP.</td>
</tr>
<tr>
<td>Bovine spongiform encephalopathy</td>
<td>Cattle</td>
<td>Infection through industrial cannibalism.</td>
</tr>
<tr>
<td>Chronic wasting disease</td>
<td>Deer, elk</td>
<td>Unknown.</td>
</tr>
<tr>
<td>Feline spongiform encephalopathy</td>
<td>Cats</td>
<td>Infection through ingestion of contaminated animal products.</td>
</tr>
</tbody>
</table>

Mutations and polymorphism in PRNP

Over 40 different mutations in the PRNP gene have been associated to familial TSEs in humans [88]. These insertions, deletions or substitutions directly affect the primary sequence of PrP and therefore result in secondary and tertiary structure alterations, which in turn give rise to various PrP\(^{Sc}\) conformers [89].
As discussed below, not all mutations are deleterious. Deletion of one of the characteristic N-terminal octapeptide repeats of PrP has no pathogenic effect, while insertions of one to nine additional repeats have the opposite outcome. PRNP polymorphism has also been described. Unlike mutations, it does not directly lead to prion disease, but affects an individual’s susceptibility to the condition. For example, approximately 12% of the Japanese population carry a dominant negative allele encoding for a substitution at position 219, which has been shown to interfere with sporadic prion conversion of PrP<sub>C</sub> [14], [91]. Such dominant negative inhibition has been recorded for sheep as well, but at position 171.

**PrP structure**

The PRNP gene encodes a protein 253 amino acids in length prior to post-translational modifications [89]. At its N-terminus, the unprocessed PrP chain contains a signal peptide (residues 1 to 22), followed by a region of several octapeptide repeats, a hydrophobic region (113 to 135), which extends into a structured domain (up to residue 231), and finally a GPI (glycophosphatidylinositol)-anchor signal at the very end (figure 9). So far, only the structure of the C-terminal portion (residues 121-231) of recombinant PrP has been resolved by means of NMR. It consists of a small antiparallel β-sheet and three α-helices. The two asparagines within the structure (positions 181 and 197) are the sites of glycosylation and the single disulphide bond is between cysteines 179 and 214, connecting 2 of the helices. The hydrophobic region is believed to serve as a transmembrane domain and begins with a highly conserved, palindromic sequence (AGAAAAGA). This alanine-rich portion is conserved among mammals and its deletion inhibits PrP<sup>C</sup>-PrP<sup>Sc</sup> interaction in yeast models [92]. In contrast, insertional mutations introducing 1 to 9 additional ORs within the N-terminal octapeptide repeat region, have been shown to be pathogenic. This region is normally comprised of four octapeptide repeats preceded by one very similar nonapeptide. All five share the sequence motif: P(H/Q)GGG(-/G)WGQ, with the nonapeptide lacking the histidine and having an extra glycine.
Figure 9. Simplified scheme of the structural organization of the mammalian prion protein. At the very beginning of the N-terminus a signal peptide can be found, which is removed during synthesis in the rough endoplasmic reticulum. This is followed by the OR region (residues 51 to 90), a hydrophobic region (113 to 135) and a GPI-anchor signal at the C-terminus (residues 232-253). The OR region can vary in number of repeats, but normally contains 4 with the same sequence motif PHGGGWGQ. Those are preceded by a nonapeptide (PQGGGGWGQ) and mutations can either decrease their number (1 to 9 extra) or decrease it by one (unlike increase, decrease has no pathogenic effect). An alanine-rich, palindromic sequence lies at the beginning of the hydrophobic region (residues 113 to 121). Two asparagines (residues 181 and 197) serve as glycosylation sites (marked in orange) and two cysteines (residues 179 and 214) form a disulphide bridge (orange dotted line). When converted to its amyloidogenic form (PrPSc) the prion protein acquires partial resistance to proteinase K (PK). The PK resistant region is between residues 90 and 232. The N-terminal domain (NTD, from 22 to 121) is natively unstructured, while the C-terminal domain (CTD, 121 to 232) is structured.

PrP<sup>C</sup> function

The four histidine-containing ORs at the NTD of PrP display high affinity for Cu<sup>2+</sup>, suggesting a functional role of the native protein in copper metabolism. On the other hand, PrP seems to share a phylogenetic relationship to the ZIP family of zinc ion transporters [93] and indeed functional investigations have demonstrated that PrP<sup>C</sup> binds at least these two ions as part of a multicomponent protein complex. Nevertheless, little is known, but much is speculated, about the actual physiological function of PrP<sup>C</sup>. A role for the prion protein has been proposed in regulation of cognition and circadian rhythms, neuron and myelin development, protection of the nerve cells against oxidative and chemical stress, signaling, cell adhesion and differentiation [27], [94]. As promiscuous as PrP<sup>C</sup> might seem, its expression is dispensable for the normal development of PRNP knock out mice, a clear indication that prion pathology is unrelated to loss of native function. Strikingly, whatever the physiological role, cytosolic PrP has been identified as an accomplice in a number of non-prion degenerative conditions. Acting as a major receptor of Aβ oligomers, membrane bound PrP<sup>C</sup> was implicated as mediator of amyloidosis related neurotoxic effects during Alzheimer’s [95]. Finally, multiple studies have proposed a role for the prion protein in cancer development [27]. The upregulated expression of PrP in melanoma, breast, pancreatic, colorectal and gastric cancers, has been correlated with effects on apoptosis, invasiveness and drug resistance, all having a negative impact on survival probability.
**PrP$^C$ versus PrP$^{Sc}$**

In the lack of somatic or germ-line mutations, the native prion protein and its infectious, scrapie form are completely identical in sequence. However prion conversion leads to significant changes in the structural and biochemical properties of the protein (figure 10) [96]. First and foremost, PrP$^{Sc}$ displays an alleviated aggregation propensity, which makes full structural determination of isolated monomers challenging. Nevertheless, comparative studies of secondary structure reveal a significant decrease in $\alpha$-helicity and increase in $\beta$-sheet content with respect to the native conformation, indicating a refolding event during prion conversion. This is also indicated by the typical cross-$\beta$ sheet organization of the amyloid, fibrillar assemblies, PrP$^{Sc}$ takes part in, upon conversion. Such higher-order, quasi-crystalline complexes can be observed under electron microscopy and, due to their characteristic interaction with amyloid dyes, such as thioflavin T and Congo red, are detectable by spectroscopic techniques (birefringence and fluorescence). A final consequence of the increase in $\beta$-sheet content of the scrapie prion protein is the acquisition of partial resistance to protease digestion. Because PrP$^{Sc}$ is considered to be the major component of the infectious agent behind prion disease, conversion induced structural alterations seem to play a crucial role in pathogenicity. The fact that prion conversion can give rise to various PrP$^{Sc}$ conformations of distinct pathologies, is a strong indicator for this and raises the question of how biological information can be transmitted and inherited through protein structure rather than nucleotide sequence [14].

![Figure 10](image.png)

**Figure 10.** The difference between cytosolic and scrapie prion protein. Although identical in sequence, the two isoforms of PrP possess different structural and biochemical properties (listed below each), induced by prion conversion. It remains unclear what is the molecular mechanism behind this process and whether a non-proteinaceous cofactor is involved.
[HET-s] – the fungal prion protein

The amyloid, aggregated form of the HET-s protein is known as the [HET-s] prion of the filamentous fungus *Podospora anserina*. It shares the same mechanisms of self-propagation as the better known *Saccharomyces cerevisiae* prions [URE3] and [PSI+]. The first ever functional role for a prion was assigned to [HET-s], which is involved in a fungal discrimination process, known as heterokaryon incompatibility [8].

The physiological role of [HET-s]

Heterokaryon incompatibility is a process which is ubiquitous to all fungi and regularly causes the death of conjoined cells of individuals from separate strains, which have undergone vegetative hyphal fusion [97]. In attempt to distribute nutrients and signaling molecules between neighboring colonies, cells fuse to form somatic heterokaryons, which combine the cytoplasmic content of the involved individuals. More often than not, however, this attempt is unsuccessful and abruptly aborted. It is hypothesized that heterokaryon incompatibility is a fail-safe feature, designed to prevent parasite transmission between strains.

The *het-s* locus, which is part of the genes controlling heterokaryon incompatibility, contains two alleles encoding for the proteins HET-S and HET-s, the latter being the non-prion form of the [HET-s] phenotype. Two fungal strains, carrying one genotype or the other are only compatible when the HET-s protein has not been converted to its prion form in the respective strain. When [HET-s] is present, somatic fusion is impossible. Nevertheless, HET-S and [HET-s] strains are sexually compatible.

The [HET-s] prion has been shown to be almost 100% transmissible to uninfected cells [8] and influences the sexual reproduction of fungi. [HET-s] infection is sexually transmissible only when carried by the female gametes as they contain much more cytoplasm than the male. Upon reproduction which involves an infected female partner and a male carrying the HET-S phenotype, an interesting phenomenon emerges – either the resulting progeny is cured from the [HET-s] phenotype or spores containing the male genotype (*het-S*) are killed. Which scenario prevails, depends on environmental and developmental factors. In any case, this phenomenon can have a favorable effect on the evolutionary prevalence of the [HET-s] associated allele.
**HET-s versus [HET-s]**

Although identical in sequence, the prion and native isoforms of the HET-s protein are not quite the same. Similar to the human prion protein (PrP<sup>Sc</sup>) and its non-infectious counterpart (PrP<sup>C</sup>), the HET-s and [HET-s] conformers can be distinguished by their structural and biochemical properties (figure 11). While α-helices comprise approximately a third, β-sheets account for only 1/5 of the secondary structure of the native and perfectly soluble protein [98]. The situation is completely reversed in the case of the infectious prion isoform. Furthermore, [HET-s] is markedly aggregation prone, readily forming amyloids capable of seeding the *de novo, in vitro* aggregation of soluble HET-s. Finally, because of its enrichment in β-sheet secondary structure elements, the prion isoform is also much more resistant to protease digestion. Nevertheless, non-infected cells can acquire the [HET-s] phenotype by the spontaneous conversion of native HET-s.

![Figure 11](image1.png)

**Figure 11.** The difference between HET-s and [HET-s]. While identical in sequence, the two isoforms of PrP are significantly different in structure and biochemical properties.

The protease resistant core of [HET-s] has been located on the CTD of the protein, between residues 218 and 289. This region, designated the prion forming domain (PFD), appears to be completely sufficient and necessary for prion conversion and propagation [98], [99]. In the native protein the prion forming domain is flexible and unstructured. Upon conversion, however, it transitions to an amyloid fold, therefore contributing to the increase in overall β-sheet content. The conversion process of HET-s is therefore different from that of PrP, where refolding of α-helices drives conformational transition [100]. Interestingly, PFD conversional reorganization follows a regular protein folding pathway of burying hydrophobic residues within the interior and exposing polar ones at the exterior. The [HET-s] conformation is further stabilized by dynamic salt bridges, which can be formed intra- or intermolecularly. Finally, the strain variation phenomenon appears to be atypical for the [HET-s] prion.

In paper III, the effect of RNA on the amyloid aggregation of HET-s is described. The results of this work provide yet another indication of the potential role of RNA in the prion conversion process.
Experimental procedures in the present work

The immediate objective of the papers, included in this thesis, was to investigate the effects of RNA on the aggregation of different prion-like proteins. Generally, protein aggregation is a convoluted experimental problem, the comprehensive study of which requires the application of a wide range of biochemical and biophysical methods. The situation becomes even more complicated when a macromolecule, different in nature from a polypeptide, is present in the system. Altogether, the present study called for the design of experimental assays, harnessing the strengths of one technique or another in the most optimal way.

Prior to all experimental procedures the model, aggregation prone proteins were recombinantly expressed in *E. coli*. After their purification to a folded, monomeric and soluble state, both p53C and mrPrP were directly used in the experimental assays. An important distinction in that respect must be made for HET-s, which was also recombinantly expressed, but purified under denaturing conditions. The aggregation propensity of HET-s was studied while the protein was refolding in the presence of various RNAs.

Alternatively, most of the RNAs used in this work were *in vitro* transcribed, from PCR amplified DNA templates. This allowed for the controlled, high-yield production of non-modified, uniform, highly-pure, single-stranded RNAs of various origin and cellular function. Those could be divided in two groups – ribosomal and messenger RNA, and varied in length and nucleotide composition. The RNA pool was further supplemented with bulk tRNA extracted from *E. coli* MRE600 cells (all papers) and total RNA from cultured neuroblastoma (N2a) cells (paper II). This provided a wide range of RNA sequences of diverse origin, secondary structure and function.

The following subsections include brief descriptions of the experimental assays and their respective methods, implemented in this work for studying the aggregation of the model systems in the presence of RNA.
**Assays**

*Equilibrium assays*

One of the principal approaches in this work, equilibrium assays were used for the fast and accurate generation of experimental data at the end of a fixed and uninterrupted period of aggregation. A set of experimental conditions (temperature, pH, buffer composition, incubation time, etc.) was standardized and kept constant for the aggregation of each of the model systems. The only experimental variable was the molar ratio between the protein and RNA components. After establishing a standard aggregation protocol the sample components are mixed, and the reaction is incubated to allow for the aggregation process to saturate. Once the fixed incubation period is complete, the end state of aggregation was analyzed. This approach was particularly useful when the concentration of one RNA was varied with respect to the protein and vice versa. Under this condition a straightforward correlation between component molar ratio and RNA effect on the outcome of aggregation is possible.

*Time-resolved and seeding assays*

The kinetics of protein aggregation in the presence and absence of RNA were studied by time-resolved assays. Unlike the equilibrium approach, such assays follow the gradual change of a single parameter of aggregation for the duration of the entire aggregation process. This allows for the real-time observation of the different phases in the aggregation process. The subsequent comparative analysis of kinetics, produced under alternative conditions, provides information on how RNA modulates the aggregation of a particular model system.

Seeding assays are a special subset of time-resolved experiments, which utilize the end product of equilibrium reactions as inoculum for *de novo* aggregation. This type of experiments is particularly popular in amyloid- and prion-like studies of protein aggregation. They are designed to probe the likelihood of a specific aggregation condition to result in the formation of soluble, amyloid oligomers. Such species are much more aggregation prone than amorphous aggregates or mature fibrils and serve as seeds, nucleating the aggregation of monomeric protein. Seeding has been proposed as an essential molecular mechanism in prion propagation.

*Sedimentation assays*

Designed to characterize the component distribution between the soluble and insoluble phases of an equilibrated aggregation reaction, sedimentation assays proved to be a simple, but powerful, quantitative tool in studying the effect of
RNA on protein aggregation. Furthermore, the combination of protein- and NA-based sedimentation, utilized in paper II, provided comprehensive evidence for the direct interaction between the prion protein and RNA, during aggregation. In these experiments, the soluble and insoluble phases of an aggregation reaction are separated, post equilibration. The protein or RNA composition of each phase is then analyzed by gel electrophoresis. This allows for a straightforward visualization of how protein and RNA distribute between the phases as a result of a specific aggregation condition.

**Methods**

The successful execution of the above assays relied on several analytical methods. A summary of the principal techniques is provided below.

**Light scattering**

One of the more popular methods in studying protein-protein interactions takes advantage of the optical phenomenon responsible for the blue color of the sky – Rayleigh scattering. When light travels through a transparent milieu the charges of particles on its path start vibrating with the same frequency as the oscillating light wave. This turns particles into radiating dipoles, which scatter light of the same wavelength as the incident in all directions. The intensity of emitted light is directly proportional to particle concentration and average size (molecular weight) and therefore a good probe for protein aggregation [101], [102]. For the purposes of this study, the intensity of scattered light was used as a direct measure of the extent of aggregation. A standard fluorometer, functioning as a right-angle photometer was the standard instrument of choice. Although insufficient for more in-depth, quantitative measurements, this static light scattering set up was perfectly adequate for evaluating the overall aggregation of protein solutions. Static light scattering was used as relative measure of the extent of aggregation at endpoint or as a function of time in equilibrium and time-resolved assays, respectively.

For more precise measurements on the size distribution profile of aggregated samples, dynamic light scattering (DLS) was employed. Although both dynamic and static light scattering rely on the detection of Rayleigh scattered light, the static method only measures the time-averaged amplitude of light intensity [103]. Alternatively, dynamic light scattering measures the intensity
fluctuations over time to extrapolate diffusion coefficients for the various particles in solution. In this way the hydrodynamic radii of particles can correlated to their diffusive behavior [104].

**Transmission electron microscopy**

Another popular technique used for studying protein aggregation is transmission electron microscopy (TEM). This well-established microscopic method allows for the qualitative characterization of aggregates. Since it was one of the first techniques to provide high-resolution, visual data on pathogenic aggregate plaques, TEM is the preferred tool for differentiation of aggregate morphology [103]. This two-dimensional, analytical technique is much more powerful than conventional light microscopy as it relies on the transmission of a focused electron beam through an ultrathin sample suspension on a grid. As electrons pass through the sample, some will interact with the particles on their path and scatter away. The remaining electrons will hit a fluorescent screen, resulting in a shadow-like imprint of the particle. The denser the particle, the more electrons are deflected and therefore the darker the image. Because the electron beam is of significantly lower wavelength than visible light, it interacts with particles on atomic level. This results in extremely high-resolution imagery. TEM was exclusively used as a method for visual characterization of aggregates produced by equilibrium assays.

**2D gel electrophoresis**

Gel electrophoresis is one of the simplest and most integral techniques in molecular biology. Nevertheless, it deserves a special mention within the primary methods employed in this study. Electrophoresis is used on every day basis for the fast analysis of macromolecular mixtures [105], [106]. Under the force of an electric field, proteins and NAs are sorted according to molecular weight. These charged molecules pass through an electro-conductive, porous gel, driven by the directional momentum provided by the electric current. When the charge-to-mass ratio is uniform, molecules will travel through the gel with different speeds. Larger molecules will take longer to traverse the length of the gel and smaller ones will move faster. In paper II, agarose and polyacrylamide gel electrophoreses were used to separate RNA and protein, respectively, at the analytical stage of equilibrium sedimentation assays. This allowed for both quantitative and qualitative characterization of aggregate species formed at different protein to RNA ratios. In combination with limited proteolysis and RNase digestion, it also provided direct evidence for the interaction between the two macromolecules, during aggregation.
Fluorescence spectroscopy and Thioflavin T binding

Fluorescence spectroscopy is another optical technique of wide application in protein research, as a whole, and protein aggregation studies, in general. It employs the ability of certain molecules (fluorophores) to leave their ground state and enter an excited one upon absorption of electromagnetic radiation of a specific wavelength (excitation wavelength) [107]. Upon their rapid returning to ground state fluorophores emit photons of a wavelength longer than that of excitation. The spectrum of emission can easily be recorded with a fluorometer, making fluorophores affordable and adequate spectroscopic probes. Those can be intrinsic or extrinsic, the former occurring naturally in biopolymers. In proteins, aromatic amino acids are a classic example of intrinsic fluorophores. One particularly useful property of fluorophores is their unique sensitivity to their immediate chemical environment. The indole group of tryptophan is an example of a solvent-sensitive fluorophore, the emission of which varies with changes in the environment. Tryptophans buried within the hydrophobic core of a protein will emit at shorter wavelengths than exposed ones. Being the predominant intrinsic fluorophore in proteins, tryptophan has become a standard reporter group for conformational change. Unlike light scattering, fluorescence is insensitive to particle size and only depends on the total number of fluorophores and their solvent exposure. This fundamental difference between the two methods was exploited in paper I to further elucidate the mode of aggregation of p53C in the presence of RNA.

Extrinsic fluorophores were also utilized. In papers I and III, the aggregation of the model systems was characterized through the use of thioflavin T (ThT), which is relatively specific to amyloid structures [108]. Interaction results in characteristic emission, which is often used as a reliable reporter of amyloid formation [109].

Supplementary methods

Several other techniques were used to reinforce or elaborate on the data gathered with the above methods.

In paper I the application of amyloid specific antibodies was necessary for the characterization of p53C aggregates formed in the presence of RNA.

In paper II, circular dichroism – another spectroscopic approach, was used to detect secondary structure changes in PrP-RNA complexes. In addition, an attempt to quantify the interaction between PrP and RNA was made by means of isothermal titration calorimetry. This is an extremely sensitive method for
the direct determination of binding parameters without any preliminary modification of the interaction partners. Finally, sequencing analysis of co-aggregated N2aRNA was also performed.

Finally, a primer extension assay, based on reverse RNA transcription of UV cross-linked domain V rRNA in complex with denatured HET-s, was used to map out the protein interaction sites, in paper III.
Key results of the present work

The hereby summarized results of the present study illustrate the pronounced effect of RNA on the aggregation of the featured, model prionogenic systems. As demonstrated by various experimental assays, every aspect of the aggregation process of the model proteins is affected by the presence of the single-stranded polyanion. Without exception, each of the proteins appear to directly interact with RNA. This leads to significant changes in the kinetics and overall extent of their aggregation, and alters the morphology of the resulting aggregates and their ability to function as seeds. All this reinforces the previously hypothesized role of RNA as cofactor in the prion phenomenon and indicates the potential involvement of the nucleic acid in disease associated protein aggregation.

Effect of RNA on the extent of aggregation

All RNAs, tested in the equilibrium assays, significantly affected the aggregation of both p53C and full-length prion protein – FL mPrP, (papers I and II, respectively) in a concentration dependent manner. While RNAs of different sequence composition, origin and length stimulated the otherwise minimal aggregation of the prion protein, the same nucleotide sequences had a peculiar, bimodal impact on the extent of aggregation of the natively unstable and aggregation prone at physiological conditions p53C protein [84], [85] (figure 12A). A high protein to RNA ratio (50:1) produced an almost 2-fold increase in light scattering (LS) signal, indicating either more aggregation, larger aggregate formation or both. Vice versa, ratios lower than 50:1, resulted in significant decrease of overall aggregation, with respect to p53C alone.
Figure 12. Equilibrium aggregation of p53C – A, and FL mrPrP – B, at various protein to RNA ratios. Extent of aggregation was evaluated by measuring LS signal in a steady-state, right-angle fluorometer, after a fixed incubation period.

In contrast to p53C, and in line with previous reports [110], [111], FL mrPrP barely aggregated under physiological conditions. This process was, however, markedly stimulated at any protein-RNA proportion (figure 12B). A further reinforcement of this observation was produced by equilibrium sedimentation assays. Those demonstrated that only 20% of available protein aggregated, while 80% was still soluble, in the absence of RNA. The distribution between soluble and insoluble fractions, however, completely reversed at a 5:1 protein to RNA ratio. Even when the RNA was in a 2-fold excess (protein to RNA ratio of 1:2), the pellet contained almost half of all protein.

The aggregation of either of the two proteins remained completely insensitive to free ribonucleotides. RNase A treatment prior to incubation with p53C completely abolished the previously observed effect of RNA. Furthermore, judging from the results obtained with tRNAs – the shortest RNAs tested in both systems, an apparent correlation with size emerges. The shorter the RNA, the higher the concentration needed for achieving maximal impact on aggregation. These observations underline the significance of a polynucleotide surface in the modulatory role played by RNA in the aggregation of p53C and FL mrPrP.
Interestingly, of all RNAs tested in paper III, only ribosomal RNA affected the aggregation of HET-s. While messenger RNAs had neither stimulating nor inhibitory effect, various domains of the 23S rRNA significantly suppressed the aggregation of the fungal prion. Of those, domain V rRNA had the most pronounced effect, exerted in a concentration dependent manner. Human, mitochondrial, bacterial and yeast domain V rRNAs inhibited the aggregation of HET-s to a comparable extent. At a protein to RNA ratio of 5:1, overall aggregation was reduced to almost a third of the protein alone level. This result is notably different from the situation observed with p53C and FL mrPrP. Most likely, this is due to the fact that the starting material for equilibrium aggregation of HET-s was unfolded rather than soluble, native protein. The apparent inhibitory effect of rRNAs, and more specifically domain V rRNA, could then be an artifact of the protein folding activity of the ribosome (PFAR). As discussed above, this auxiliary ribosomal function is located within domain V of the 23S rRNA.

**RNA modulates the kinetics of aggregation**

On the next step, time-resolved assays complemented the study of the aggregation process of p53C and FL mrPrP in the presence of RNA. For both systems time-resolved LS experiments were in complete agreement with equilibrium measurements (figure 13A and C). In paper I, higher protein to RNA ratios seemed to promote the aggregation of p53C and resulted in kinetics with shorter lag phase and increased amplitude (figure 13A). Lower ratios had exactly the opposite effect and produced aggregation kinetics of a final amplitude only 16% of that for p53C incubated without RNA.

In paper II, on the other hand, aggregation of FL mrPrP alone was found to proceed at a negligible rate \( \left( k_{\text{obs}} = 0.007 \, \text{s}^{-1} \right) \) – figure 13C and D. Addition of RNA, however, produced the first ever report on the fast kinetics of prion protein aggregation. The rate of mrPrP aggregation peaked at the 20:1 protein to RNA ratio, reaching values 2000 times higher \( \left( k_{\text{obs}} = 13 \, \text{s}^{-1} \right) \) than those for protein aggregation in the absence of RNA. Further increase in RNA concentration gradually decelerated the aggregation kinetics. Still, a protein to RNA ratio of 2:1 resulted in aggregation 20 times faster than that for mrPrP alone \( \left( k_{\text{obs}} = 0.14 \, \text{s}^{-1} \right) \).
Figure 13. Kinetics of aggregation of p53C and FL mrPrP at various protein to DHFR mRNA ratios. Time-resolved aggregation of p53C was followed by LS – A, and fluorescence at 340 nm – B, in a steady state right-angle fluorometer. The fast kinetics of FL mrPrP aggregation – C, were recorded at a stopped-flow instrument. The kinetic rates were extracted by fitting the curves with single-term exponential function – D.

A peculiar feature of p53C allowed for even deeper examination of its aggregation kinetics in the presence of RNA. On account of the solvation/desolvation of the eight tyrosine and single tryptophan residues in p53C, during conformational change, native, unfolded and aggregated p53C species display distinct fluorescence emission spectra [84]. This motivated the application of intrinsic fluorescence as an alternative probe for p53C aggregation. By following the emission at 340 nm over time a complementary set of aggregation kinetics of p53C in the presence of RNA was produced (figure 13B). Once again, the suppressive effect of higher RNA concentrations on p53C aggregation was confirmed by this approach. However, unlike LS, fluorescence kinetics with lower RNA concentrations were only marginally faster and with a similar amplitude to those for protein alone. This discrepancy could be explained with the fact that LS depends on both particle size and concentration, while fluorescence accounts only for fluorophore concentration. With both data sets at hand it could be concluded that lower RNA concentrations do not necessarily affect the total amount of aggregated protein, but likely promote the formation of larger p53C aggregates.
**Effect of RNA on aggregate morphology**

Several different approaches were employed in studying aggregate morphology for each of the model systems in the presence of RNA. Transmission electron microscopy was a reoccurring technique in all papers, which provided on hand, visual information. While p53C self-associated in large amorphous structures as a result of equilibrium aggregation, FL mrPrP alone, produced few, small globular aggregates, (papers I and II, respectively). Protein to RNA ratios of 50:1 lead to the formation of large, amorphous aggregates (several hundred nanometers in size) in both systems. For p53C, those were comparable in size and structure to the no RNA scenario. An increase in RNA concentration with respect to mrPrP (2:1, protein to RNA) decreased the overall size of aggregated species in paper II. Still, the particles remained amorphous in structure even though they appeared to be denser. A slightly higher ratio (8:1) in the case of p53C, produced no detectable aggregates, which was in perfect agreement with LS data from paper I. In all cases, no fibrils were observed under the selected experimental conditions.

To validate the apparent absence of amyloid fibrils, the time-resolved incorporation of ThT during p53C aggregation was investigated in paper I. The protein readily incorporated increasing amounts of the amyloid specific dye, in a concentration dependent manner (figure 14A). This clearly indicated a propensity to form some sort of amyloid species, the nature of which remained unclear. At the time, due to the affinity of purine oligoribonucleotides to ThT [112], this experiment turned unreliable in the presence of RNA. For that reason, and because no fibrils were detected by TEM, a different approach was adopted. By means of an immunoassay specific to amyloid oligomers, but insensitive to mature fibrils or monomeric protein, the moderate propensity of p53C towards formation of amyloid-like oligomers was illustrated (figure 14B). More importantly, increased concentrations of RNA appeared to stimulate this type of aggregation. In combination with the LS, fluorescence and TEM data, this result provided an explanation for the bimodal effect of RNA on p53C aggregation. At low concentrations (high protein to RNA ratios) RNA promoted the formation of large, amorphous aggregates. Increase in RNA, however, stimulated amyloid oligomerization.
The morphology of HET-s aggregates was also studied by electron microscopy, ThT fluorescence and amyloid oligomer specific immunoassay (paper III). TEM revealed that the protein formed large, clustered aggregates, from which long, amyloid fibrils emerged in the absence of RNA. When domain V rRNA was present, the aggregate clusters reduced in density, but more importantly fibrils were replaced by short, branched-out structures. Interestingly, other, non-ribosomal RNAs also affected aggregate morphology. Although fibrillar structures were also predominant in the mRNA containing samples, those were heavily coated with RNAs. The effect on morphology exerted by rRNA was further reinforced by ThT equilibrium fluorescence, where domain V rRNA significantly reduced the signal from the amyloid specific dye. Both TEM and ThT experiments perfectly support the original observation on the inhibitory effect of rRNA on the overall aggregation of the fungal protein. Finally, aggregated HET-s interacted with antibodies specific to amyloid oligomers in a concentration dependent manner. This interaction was even more pronounced in the presence of RNA, indicating the stimulating effect of the nucleic acid on the oligomerization of the protein, in place of fibrillation.
RNA and seeding

The detected increase in amyloid oligomerization in presence of RNA, was conspicuously suggestive of a potential role for such aggregates in prion-like sequestration of soluble protein. The ability of these soluble, amyloid oligomers to nucleate *de novo* protein aggregation was tested by modified seeding protocols. These experiments are performed in two stages. First a standard equilibrium sedimentation assay is conducted at different protein to RNA ratios. Then, the time-resolved aggregation of soluble protein is followed in the presence of a small fraction (5-10% of the reaction volume) of RNA-free supernatant (SN), derived from the preceding stage (figure 15). The control seeding experiment consists of the SN-free standard kinetics of protein aggregation. An increase in the kinetic rate of a SN-containing reaction, with respect to the control experiment, indicates nucleated aggregation and therefore the presence of seed-like oligomeric species.

![Figure 15](image)

*Figure 15.* A schematic representation of a seeding experiment. The first stage in the process consists of equilibrium aggregation of soluble protein without or with RNA at a certain ratio. To ensure that the resulting soluble oligomers (if any) are free from bound RNA and separated from amorphous aggregates, the reaction is then RNase treated and spun at 14,000 rpm. Practically, stage II is a time-resolved LS experiment, which follows the aggregation kinetics of soluble protein in the presence of a small fraction of SN from stage I (5 to 10% of the total reaction volume).

Of the three model systems only HET-s was capable of seeding its own aggregation (figure 16A). Supernatant, derived from a RNA-free incubation of the protein, significantly accelerated aggregation. Still, SN form a 5:1, HET-s to RNA sample, stimulated aggregation even more. In contrast, SN from the respective RNA-free incubations of p53C and FL mrPrP, had no such effect (figure 16 B and C). This indicated that only the aggregation process of the fungal protein naturally leads to the formation of amyloid oligomeric seeds.
in the absence of an adjuvant molecule. Clearly, the other two proteins required RNA for their prionogenic behavior. Even more so, only specific protein to RNA ratios appeared to stimulate the oligomerization process. Lower p53C to RNA ratios (8:1) in the preceding aggregation reaction produced seeding inoculum, which shortened the lag phase for p53C aggregation in half (figure 16B). Higher protein to RNA ratios had almost no effect. Similarly, the kinetics of FL mrPrP aggregation remained virtually unaffected by the presence of SN derived from a 5:1 protein to RNA ratio (figure 16C). A 2.5-fold increase in RNA (2:1 ratio) doubled the aggregation rate for mrPrP (figure 16C and D). Finally, the prion protein aggregated 20 times faster in the presence of SN produced at equimolar amounts of FL mrPrP and RNA.

Figure 16. Kinetics of aggregation of the model systems in the presence of seeds, derived from equilibrium aggregation at different protein to RNA ratios. A, B and C – HET-s, p53C and FL mrPrP seeded aggregation, respectively. Inlet of C includes the extended, slower kinetics of mrPrP seeded aggregation. D – kinetic rates, derived from C.

Since amyloid oligomeric species play a key role in prion propagation, the above observations underline the importance of RNA as adjuvant in this process. The question remains, however, whether HET-s behaves the same when its aggregation is initiated from a folded state.
Interaction

All results, so far, are strongly suggestive of an interaction between RNA and the prionogenic model systems during aggregation. Even though this remains to be confirmed for p53C, the direct nature of the interaction between the other two proteins and RNA was demonstrated in papers II and III.

Several independent techniques were employed in pursuit of this goal in paper II. Most notably, a combination of NA and protein specific gel electrophoresis analyses of equilibrium sedimentation assays revealed that FL mrPrP co-aggregated with RNA in a concentration dependent manner (figure 17A). When RNA concentration was fixed, increase in the amount of prion protein, resulted in greater accumulation of both reaction components in the insoluble fraction of the assay. At a protein to RNA ratio of 20:1, almost all available protein and RNA were found in the pellet. The complementary CD spectra, of the aggregates produced at this ratio, provided further evidence for the interaction. Comparative CD analysis demonstrated conformational changes in both components of the mrPrP-RNA complex, with a notable decrease in $\alpha$-helicity for the protein (figure 17B). ITC, unfortunately, proved futile for quantification of binding, likely because interaction, aggregation and conformational rearrangements are events which happen simultaneously and cannot be completely isolated in time and space. Nevertheless, ITC reinforced the observation that small amounts of RNA are sufficient to stimulate mrPrP aggregation.
Figure 17. Sedimentation and CD analysis of FL mrPrP aggregated in the presence of DHFR mRNA. A – distribution of FL mrPrP and DHFR mRNA in the supernatant (SN) and pellet (P) at different protein to RNA ratios, assessed by SDS-PAGE (top) and agarose (bottom) gel electrophoreses. B – CD spectra of FL mrPrP-DHFR mRNA complexes produced at 20:1 protein to RNA ratio. Three of the spectra were collected experimentally – protein alone (red trace); RNA alone (green); mrPrP-RNA complex (blue trace). The other two are derivatives of the above spectra – sum of the protein alone and RNA alone spectra produced the cumulative spectra (yellow trace); the complex spectra minus RNA alone generated the difference spectra (dotted line).

In paper III, the specific sites, through which domain V rRNA binds to unfolded HET-s, were mapped by primer extension assay. Five reverse transcription “roadblocks” in the sequence of domain V rRNA were identified with this method after UV cross-linking of the complex between rRNA and HET-s. Those nucleotide elements matched the reverse transcription “roadblocks” derived from control, protein-rRNA complexes and closely resembled the previously described rRNA sequences involved in PFAR – figure 18 and [50]. This experiment illustrates the direct association between unfolded HET-s protein and rRNA. Furthermore, in the context of PFAR, it provides a possible explanation for the suppressive action of domain V rRNA on the aggregation of the fungal prion. Likely, under the experimental conditions of paper III, the rRNA directly interacts with the unfolded protein, effectively decreasing aggregation and fibrillation by means of PFAR assisted refolding.
Another extremely important question, partially met in this work, addresses the specificity of the interaction between RNA and the model prionogenic systems, during aggregation.

Specificity of p53C and FL mrPrP toward a particular nucleotide sequence remains unlikely, since a wide range of RNAs, of different size and composition, had analogous effects on the aggregation process of both prionogenic systems (figure 12). This assumption is supported by the complete inertness of free ribonucleotides in the aggregation process. Moreover, the apparent inverse relationship between RNA size and effective concentration in the equilibrium assays, is somewhat suggestive of the importance of a defined polynucleotide surface, rather than sequence in RNA induced aggregation. Nevertheless, the involvement of particular nucleotide elements, as the ones demonstrated for HET-s, should not be completely dismissed for the interaction between p53C or FL mrPrP with RNA.

The crucial observation of paper II that RNA co-aggregates with FL mrPrP in a concentration dependent manner (figure 17A) is a direct demonstration of the interaction between the two polymers. When coupled to limited protease
and nuclease digestion, this assay also revealed that the nucleic acid is an integrated component of the aggregated species. The association between RNA and mrPrP was strong enough for a population of low molecular weight polynucleotide (a little less than 100 bases) to remain unaffected by RNase A even after extensive treatment (figure 19). This was observed for in vitro transcribed, as well as N2a extracted, RNAs. For both, the protected region was of comparable size, indicating specificity towards a common motif in the secondary structure of the RNAs.

![Figure 19. RNase A resistance of aggregate samples of FL mrPrP at 20:1 protein to RNA ratio, analyzed by agarose gel electrophoresis.](image)

In contrast, a clear indication that the fungal prion interacts with rRNA in a sequence specific manner was provided in paper III. Mutating one of the highly conserved nucleotide elements involved in interaction between domain V rRNA and HET-s, resulted in corresponding loss of signal on the “road-block” map of the primer extension assay. In addition, LS and ThT assays revealed a partial decrease in efficiency of suppression of HET-s aggregation by the rRNA, indicating the relevance of this site for the RNA effect on the process. However, that same mutation was previously shown to almost completely abolish domain V rRNA PFAR in protein refolding assays [50]. Therefore, the definitive validation of the causative relationship between rRNA-HET-s interaction and PFAR mediated inhibition of aggregation requires the consecutive mutation of all interaction sites.

Finally, a truncated variant of mrPrP provided some insight into the site-specific interaction of RNA with the prion protein. This construct was devoid of the NTD, which is characterized by a set of octapeptide repeats. Under the exact same conditions as the FL protein, ΔNTD mrPrP aggregated to a much lesser extent on its own. More importantly, presence of RNA did not contribute to farther aggregation (figure 20). This result is in direct agreement of the previously proposed relevance of the NTD for RNA induced aggregation of mrPrP [110] and highlights a possible interaction site for the nucleic acid on the OR region.
Figure 20. Equilibrium aggregation of FL mrPrP (A) and ΔNTD mrPrP (B) in the presence of RNA, followed by LS.
Conclusions

A number of neurodegenerative disorders and many of the cancers, which plague humanity, share a causative relationship with protein misfolding and aggregation. The molecular events which precede the development of such conditions are strikingly akin to those in the heart of the prion phenomenon. Although notorious for their pathogenic implications, many species, of a wide phylogenetic distribution, employ prions as key regulators of physiology. The prion process, therefore, bridges the void between functional and disease related aggregation, and poses the question of the principles of organized and function-tailored, protein polymerization in the context of a crowded cellular environment.

One important feature of prion-like aggregates is the lasting or transient incorporation of nucleic acids [114], [115]. As a consequence of this observation, RNAs have been proposed as the most likely adjuvants in the prion process [32]–[34]. By elucidating the effects of RNA on the aggregation of three, unrelated prionogenic proteins, the present work provides further evidence in support of this hypothesis. Papers II and III present proof for the direct interaction of RNA with the mammalian and fungal prion, respectively. Together with the consequent analysis on the effect of the interaction on aggregation, this illustrates a generic role for RNA in the prion process.

The most obvious conclusion, drawn by this study, relates the pronounced influence of RNA on the aggregation of each of the model systems. The observed effects depended on RNA concentration and significantly diverged from the native levels of aggregation of all three proteins. All RNAs tested either augmented or suppressed the aggregation of p53C and only stimulated the process for the prion protein. HET-s aggregated much less, but only in the presence of ribosomal RNA.

The established bimodal impact (conductive or suppressive) of RNA on the extent of p53C aggregation was clearly an attribute of the molar ratio between protein and RNA. Although only stimulating, this effect on the mammalian prion protein aggregation was maximal at higher molar ratios and abated with increase in RNA concentration. This dependence of aggregation on the stoichiometry of the two-component reaction suggested that RNA effectively modulates the mechanism of the process. Evidence for this was provided by time-
resolved and qualitative experiments, which demonstrated that RNA concentration played a decisive role on the kinetics and, consequently, products of aggregation. This observation is conspicuously reminiscent of the executive RNA function in the assembly of ribonucleoprotein granules [57]. In RNPs, both RNA conformation and concentration, seem critical for the structural and physicochemical properties of the intracellular assemblies. It is therefore likely that, in an *in vivo* setting, either of the two proteins, are involved in RNP associated, liquid-liquid phase transitions. The physiological participation of cytosolic PrP in RNP structures has already been described [116].

Another essential conclusion of this study correlates RNA presence with the formation of seeds during the aggregation of the model systems. Once again, for p53C and the mammalian prion protein, this process depended on RNA concentration. Seeded-nucleation is a crucial molecular mechanism, which drives prion propagation and amyloidogenic aggregation [43], [117]. In that sense, this work provides another direct evidence for the adjuvant role of RNA in the prion process. Furthermore, the indication that RNA stimulates the seeded aggregation of p53C could prove insightful for the mechanistic understanding of the dominant-negative and gain-of-function properties of tumor suppressor mutants, associated with highly invasive and metastatic cancers [82], [118], [119].

Altogether, the produced results on the aggregation of p53C and FL mrPrP in the presence of RNA can be summarized in figure 21. The two aggregation profiles are slightly different, however. Unlike p53C, the mammalian prion protein aggregates at a minimal rate, on its own, leading to the formation of much smaller, but seemingly amorphous, aggregates.

For now, HET-s aggregation in the presence of varying RNA concentrations cannot be included in this scheme. All experiments in paper III were conducted with unfolded protein and reflect on refolding coupled aggregation, making it impossible to draw parallels with the results for the other two model systems. In that sense, the results of this paper can only be discussed in the context of ribosome assisted refolding.
Figure 21. A schematic representation of the aggregation process of p53C or FL mrPrP in the presence of RNA. When the soluble monomers of both proteins reach critical concentration, aggregation ensues. In the absence of RNA (at the tip of the RNA concentration gradient) p53C forms large, amorphous aggregates, while the prion protein forms much smaller species. The presence of a small amount of RNA greatly stimulates the amorphous aggregation of both proteins. At higher RNA concentrations the aggregated species significantly decrease in size and the formation of oligomeric species prevails. Such aggregates can function as nucleating seeds, effectively decreasing the kinetic barrier for aggregation.
The unanswered questions

The prion phenomenon seems invariably enigmatic and growing increasingly complex with every report of a novel prion-like protein, prion function or hypothesis on prion propagation. Nonetheless, no other field in biology holds as much potential for expanding our knowledge beyond the boundaries of conventional science, and as Richard Feynman once exclaimed “I would rather have questions that cannot be answered than answers that cannot be questioned.” Feynman also once ventured into experimental molecular biology, but surrendered to the tediousness of the method, opting for the more elegant (according to him) field of theoretical physics. That must have been during the early days of prion research.

Today the questions which stand before molecular biologists in the prion field are of the caliber Feynman might have not overlooked. Some of those are:

- How does prion conversion/replication occur and what is the role of cofactors in this process?
- What is the epigenetic factor behind prion strain variation and, essentially, how do prions convey neural degeneration?
- Can the prion principle be applied to other misfolding diseases and, if so, are they truly non-infectious?
- What is the natural prevalence and evolutionary significance of the prion phenomenon?

At least one of those outstanding questions is addressed in papers II and III, where the clear demonstration of prion-RNA interaction was correlated with aggregation and propagation. Altogether, the observations of this work indicate that RNA is, indeed, a likely adjuvant in the prion process.

How exactly does RNA influence amyloidogenic conversion, remains to be shown. In any case, the importance of a defined polynucleotide surface, seems to prevail over sequence, in at least two of the described model systems. Electrostatic interaction with this polyanionic scaffold could facilitate hydrophobic contacts between adjacent monomers. Alternatively, RNA binding could
also stimulate intramolecular protein rearrangements, through induced-fit effect [114]. Either way, RNA appears to manifest a template-like function in the process of prion conversion, or in protein aggregation as a whole. To better understand this mechanism, a critical future direction in this work would be the complete, spatial and thermodynamic, characterization of the interaction between RNA and the model systems. A similar approach on mapping the protein binding sites on RNA would either confirm or dismiss the proposed, in papers I and II, sequence independent interaction for p53C and mrPrP.

Finally, quantification of the thermodynamic and kinetic stability parameters of the model systems during unfolding/refolding in the presence of RNA could further contribute to understanding the role of the nucleic acid in the misfolding events, which lead to prion-like aggregation.

Investigations on the modulatory activity of RNA on the aggregation of other prionogenic model systems are essential for the demonstration of a general role of RNA in conveying structural information in biological systems. It is becoming increasingly clear that RNA is an instigator of intracellular, liquid-liquid phase separation of proteins into dynamic RNP granules [55], [57]. The proteins which undergo such transitions have been speculated to be particularly prone to amyloidogenic aggregation [56], [120]. Moreover, the specific sequestration of ribosomes in potentially cytotoxic, stress-related aggregation has recently emerged [121]. Therefore, it can be anticipated that an integrated study on the modulatory role of RNA in protein aggregation will reveal a common mechanism for the assembly and disassembly of both physiological and pathogenic protein deposits.
Samtidigt som människosläktet har utvecklats till att uppnå en exceptionellt hög levnadsnivå har degenerativa sjukdomar som Alzheimers sjukdom, cancer och diabetes stegvis ersatt infektioner som de främsta begränsande faktorerna för vår livslängd. Det både stör och fascinerar att sådana sjukdomar orsakas av den allra vanligast förekommande biomolekylen – protein. Faktiskt är inte proteinerna bara de allra vanligaste, utan även de mest funktionella och samtidigt sämst förstådda av de biopolymerer som våra celler innehåller.

Av den anledningen är det kanske inte så konstigt att allt fler av de mest allvarligaste sjukdomar som drabbar människor på något vis har att göra med felaktigt beteende hos proteiner. En mekanism som leder till en sådan sjukdom drivs av hur protein interagerar med varandra på molekylär nivå. Den naturligt förekommande och ibland funktionella process som kallas proteinaggregation är en hjälpväg i den livsnödvändiga veckningen av protein. Som ett resultat av att cellens insida är helt fylld med molekyler, är proteinveckning och aggregering några av de minst förstådda biomolekylära processerna. Om man lyckas förklara den ena gäller detsamma den andra, men ännu viktigare är att den ökade kunskapen skulle ge mekanistisk insikt i hur degenerativa proteinavvikelser uppkommer.

På senare år har forskningen arbetat mot detta mål, och i synnerhet har den riktat in sig på en särskild proteinklass, som, vid första ögonkastet, verkar kunna fortfarande sin egen, onormala aggregering. Dessa proteiner, vanligtvis kallade prioner, existerar i åtminstone två olika former. Vad gäller den männsliga prionen så är den ena varianten av proteinet funktionell och godartad, medan den andra är en infektios och aggregerande prion som kan fortfarande sig och är sammankopplad med en grupp undantagslöst dödliga, neurogenerativa sjukdomar. Händelserna prionsjukdomar många likheter med andra proteinassosierade nervsjukdomar. Detta faktum, samt den intresseväckande idén att prioner på något vis vistas i gråzonen mellan proteinveckning och aggregering, har gjort att många molekylärbiologer är fast beslutna att ta reda på mekanismen bakom deras uppkomst och fortfarande. Den här avhandlingen gripur sig anspekt av prionfenomenet – den möjliga påverkan av en molekylär kofaktor.
RNA har presenterats som en av de många faktorer som möjligtvis deltar i processen där prionproteiner förvandlas till sin aggregeringsbenägna form. Deras framträdande sekundärstruktur och negativt laddade ytor tillåter RNA att fritt delta i flera intermolekylära samspel. Till exempel kan ibland strukturell information överföras genom dessa interaktioner. Denna avhandling sammanfattar hur olika RNA, med varierande storlek, sektions och ursprung, påverkar aggregeringsprofilerna hos tre, obesläktade prionmodellsystem.


HET-s å andra sidan är det första proteinen som är funktionellt i sin prionform. Även om det hittills är oklart om HET-s är aktivt i sin icke-aggregerade form, bidrar HET-s i urskiljandet av ’själv’ och ’främmande’ bland svampceller då prionerna bildar större komplex. Det här är ett tydligt exempel på att prionfenomenet är evolutionärt konserverat, och ger även insikt i andra mer kompllicerade processer, som exempelvis minnesbildning, där proteiner prionliknande beteende har tillskrivits en roll.

Med dessa tre, distinkta prionframkallande proteiner som modellsystem ger denna avhandling en inblick i RNA:s roll i prionaggregeringsprocessen. Sammanfattningsvis pekade resultaten på en allmän funktion av RNA i den molekylära process som styr uppkomst och fortpföljning av fiberlikna proteinaggregat. Varje aspekt av aggregeringsprocessen i alla de tre modellerna verkade undantagslätt påverkas av nukleinsyror.

Å ena sidan verkade koncentrationen av RNA reglera omfattningen och hur snabbt aggregeringen sker. Å andra sidan, om mängdsförhållandet mellan RNA och proteiner varierades i en aggregeringsanalys resulterade det i aggregat med olika struktur. Aggregaten hade en distinkt benägenhet att agera ‘frö’ för
ny aggregering av lösliga protein, vilket antagligen är relevant för den allmänna mekanism som ligger bakom prionfortplantning. Dessutom interagerade HET-s och PrP direkt med RNA, men bara i det första fallet påverkades interaktionen av nukleinsyrasekvensen.

Även det tumördämpande proteinet p53 verkar binda till RNA, på samma vis som PrP. I denna process verkar nukelinsyramolekylens yta, och möjligtvis struktur, vara avgörande. Därtill gav en rad RNA av olika ursprung, storlek och sekvens liknande effekter på aggregeringsprocesserna av både PrP och det tumördämpande proteinet p53, vilket föreslår att den enkelsträngade och negativt laddade RNA-molekylen har en generell funktion i processen.

Sammanfattningsvis påminner resultaten i denna avhandling om RNA:s reglerande roll i de så kallade ‘ribonukleotidproteinkorn’, som påvisats spela en roll i lagring, bearbetning, nedbrytning och transport av mRNA. Här belyses även en tidigare förbisedd roll för nukelinsyror i den molekylära process som styr proteinaggregering och möjligtvis fortplockning av prioner.
Disclosure and acknowledgements

To those of you who worked their way to this page, your effort is greatly appreciated, and I admire your resolve! To those who stumbled upon it, I hope you at least browsed through the cool figures. In any case, welcome to the full disclosure! The preceding pages are the required component of a PhD thesis and, for the most part, I really enjoyed putting the right words together. This thesis, however, is a means to an end – the end of my PhD. What follows is the part dedicated to the PhD itself and what it meant to me.

If I had to explain what PhD is in one sentence, I would have to say that doing a PhD is like living a life in 5 or less years. You start it all by yourself, confused, clumsy and uncoordinated. For a while you look up to those around you and the group takes you in as foster home would. You spend some time (a lot of time) here and there, one day by one person’s bench/dinner table, the next on another. You pay attention, mimic, repeat and make mistakes, then repeat some more trying to understand and fit in. Little by little you take your first steps and then you are off to school – teaching. As you learn how to teach, and you get your own bench/dinner table you get to be more and more confident. You become boisterous and you want to prove yourself. Your foster home sends you away travelling with the hope you tell everyone you meet about the big family name you got. You do meet lots and lots of people and you do tell them you come from a great, great house (which is, basically, run by you). You make many friends. You have great, beautiful friends – the best friends; you have lots of fun, amazing fun. If you are lucky, you fall in love. If you are even luckier, you have some children and start a family. Things get faster and faster. Suddenly, but late enough, you start working. You work as hard as you can. You work until your peripheral muscles atrophy and your clothes don’t fit, and all you can do is pipette and type. In the blink of an eye it is all over.

Whether there’s life after PhD, is the topic of another chapter. What remains, however, are a few indecipherable lab books and a lot of defining memories.
**A defining memory**

A couple of years back, on the day of my half time seminar – a sort of a progress check, midway/ish through a PhD student’s short but prolific career, I had found myself in an average-sized, bright-lit room, packed with people I knew. I had always had many friends, but our relationship was usually one devoted to informal dealings, which involved lots of entertainment. On top of that I had never considered myself nearly as dedicated a scientist as 90% of the people I knew in academia. For that reason, I rarely allowed myself to discuss my projects in private circles, unlike my fellow, researcher friends who often went on to share experimental ideas half way through a round of drinks. So, there I was, naturally perplexed by the surge of interest toward my academic life, as I watched 30 or so of my close friends walk in and take up every single seat in the room, 5 minutes before my first slide. It finally dawned on me – I was in for a performance, the intimacy of which I had not anticipated. A usually composed presenter I suddenly felt somewhat claustrophobic and out of breath. I clearly remember having the idea to try and pull myself together by awkwardly commanding Alex to sit right in front of me - for courage. Unfortunately, as he conceded, he automatically proceeded to stare at me with his huge, soft, dark eyes, filled with urgent questions, such as “What do you know about science Prase?”. The five longest minutes of my life ended the moment I laid eyes on fabulous Fabio, who had barely walked in and was already staring straight in my soul with a friendly and bewildered grin. Eyes wide open and eyebrows stretched over his tall forehead he was equal amounts surprised and amused by the sizable turnout and my awkward demeanor. I was momentarily reminded who I was and what I was supposed to do. I introduced my topic, I cracked a joke and I gave my talk.

Remarkably, at my last slide my friends were all wide awake and eager to ask questions. In fact, if I recall correctly, my academic opponent had somehow felt their eagerness and hurried to let the audience have a swing at me. By the end of my seminar, I had enjoyed 55 of the best and, as it naturally happens with good time, shortest minutes of my life. Questions were flying left and right. Every time I tried to answer one person I could catch, with the corner of my eye, Mirthe or Rasa or Alex or David or someone else getting ready to go. People were so enthusiastic to discuss, ask and give suggestions that I suddenly realized what being a scientist meant for me.
Acknowledgements

I will always look back on this moment as the first time in my life, where I consciously felt complete, genuine and uninhibited. In that brief period of clarity, I had defined myself through my friends and, years before my graduation, I knew I had accomplished the goal of my PhD.

In these final lines, I would like to thank you my friends! Throughout the years, you have been my support, my inspiration, frustration and consolation, confusion and bewilderment, my passion and my life. We have laughed, cried, danced, shared, dreamed and conspired together. With you by my side I have lived through countless moments worthy of acknowledgement and that is what my PhD was about!

This opportunity would have never been possible without my supervisor – Prof. Sanyal. I have learned tremendously, from our interaction, not only on the subject of science, but also about life!

At the very end, I would like to express the great sense of pride I feel for my “baby” brother. Through the darkest moments of this journey you have always been my backbone, my moral compass and a source of hope! Although convention prescribes the opposite approach towards younger siblings, I have always looked up to you and sought after your advice. You have grown to become an outstanding man I can only aspire to share your resolve, generosity and kindness!
References


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