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Visions & Reflections

Disordered RNA chaperone proteins: from functions to disease[+]

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Abstract. RNA chaperones are ubiquitous proteins that play pivotal roles in cellular RNA metabolism and RNA virus replication. Here we propose that they act by organizing complex and highly dynamic networks of RNA-RNA, RNA-protein and protein-protein interactions. How this is achieved and how their malfunction may lead to disease will be discussed through the examples of human immunodeficiency virus type 1 nucleocapsid protein (NCp7), the fragile X mental retardation protein and the prion protein.

Key words. Intrinsically unstructured proteins; RNA-binding proteins; RNA chaperones; human immunodeficiency virus; NCp7; fragile X mental retardation; FMRP; prion diseases.

Intrinsic disorder and protein function

According to the classical structure-function paradigm of structural biology, the amino acid sequence of a protein defines a unique, fairly rigid and stable three-dimensional (3D) structure that in turn is a prerequisite for biological function [1]. This concept dominated thinking in molecular biology throughout the 20th century and has led to an extraordinary accumulation in structural data of biologically active proteins and enzymes, reaching more than 25,000 experimentally determined 3D structures to date [2]. Progress in structure determination has been instrumental in understanding protein function, as well as for the rational design of drugs aimed at inhibiting enzymatic activities. At the same time – and somewhat paradoxically – it has drawn attention to proteins and protein domains that stubbornly resisted structure determination attempts.

Cutting this Gordian knot, Wright and Dyson, in a landmark article, called for a reassessment of the structure-function paradigm, suggesting that a large number of proteins and protein domains may exist and exert their function(s) in the absence of a well-defined folded structure [3]. Indeed, according to the current view, proteins can exist in a number of thermodynamical states under physiological conditions, corresponding to globular, molten globule-like, pre-molten globule-like and unfolded (random coil) conformations [4, 5]. Importantly, protein function can arise from any of these conformational states or from transitions between them (fig. 1A). For example, the cyclin-dependent kinase (Cdk) inhibitor $p21^{Waf1/Cip1/Sdi1}$ was shown to lack stable secondary and tertiary structures in its free solution state, but its N-terminal kinase inhibitory domain adopted an ordered tertiary structure upon binding to Cdk2 [6].

Proteins or protein domains that do not adopt a unique 3D structure in their 'free' state are referred to as natively de-

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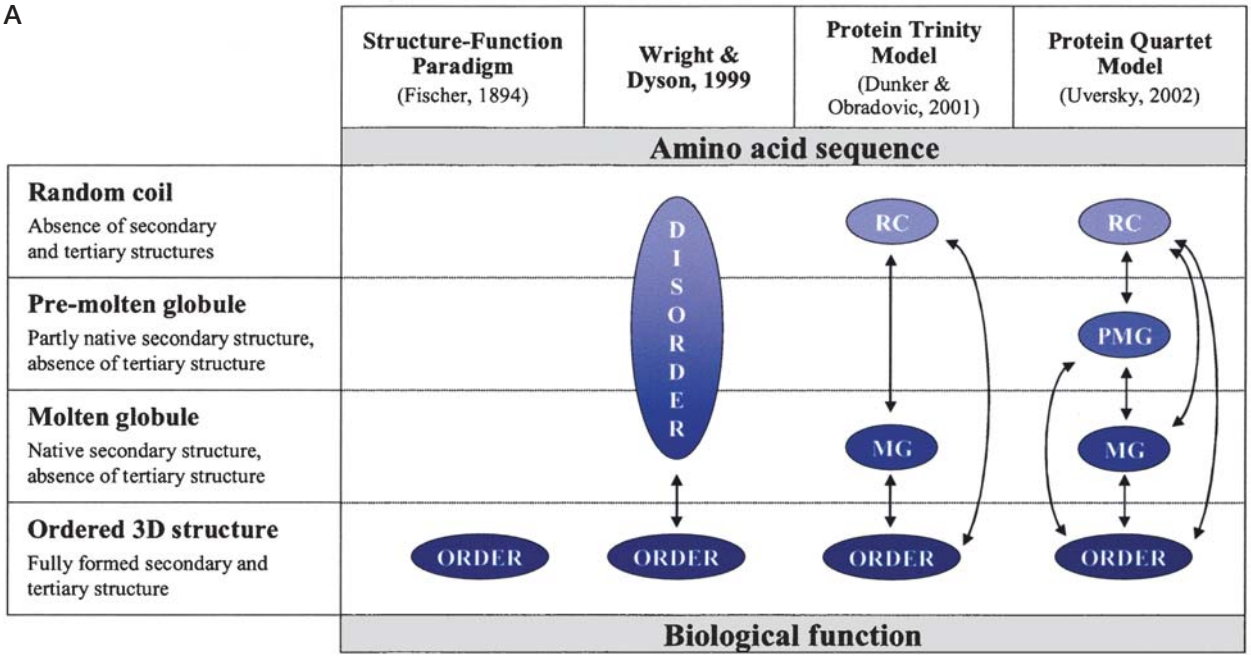
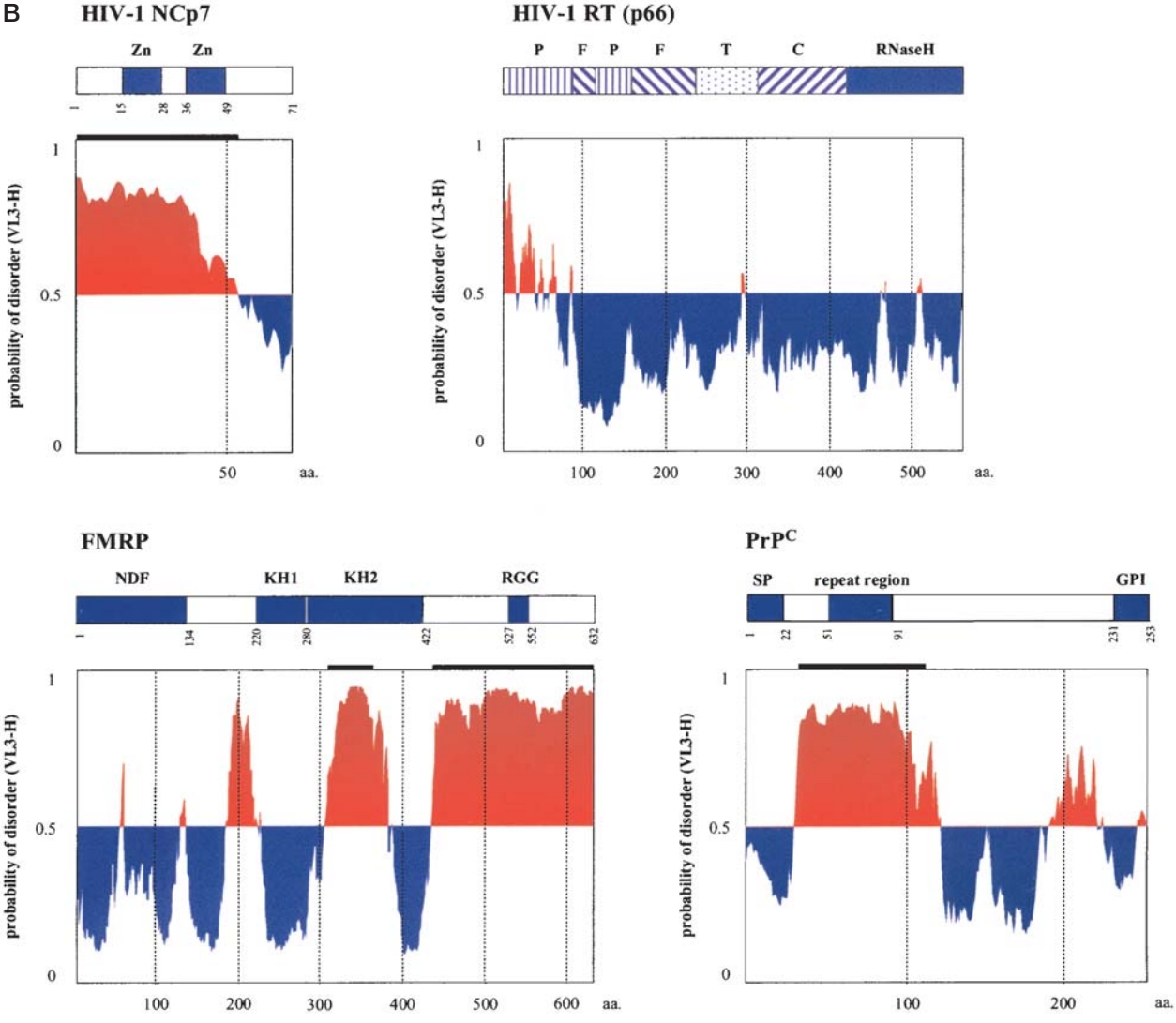
A


Structure-Function Paradigm (Fischer, 1894)
Wright & Dyson, 1999
Protein Trinity Model (Dunker & Obradovic, 2001)
Protein Quartet Model (Uversky, 2002)
Amino acid sequence
Random coil
Absence of secondary and tertiary structures
Pre-molten globule
Partly native secondary structure, absence of tertiary structure
Molten globule
Native secondary structure, absence of tertiary structure
Ordered 3D structure
Fully formed secondary and tertiary structure
DISORDER
RC
PMG
MG
ORDER
Biological function

B


HIV-1 NCp7
Zn Zn
1 15 28 36 49 71
probability of disorder (VL3-H)
1
0.5
0
50
aa.
HIV-1 RT (p66)
P F P F T C RNaseH
100 200 300 400 500
FMRP
NDF KH1 KH2 RGG
1 134 220 280 422 527 552 632
100 200 300 400 500 600
PrP^C
SP repeat region GPI
1 22 51 91 231 253
100 200

natured/unfolded or intrinsically unstructured/disordered [3–4, 7–8]. Disordered regions frequently – but not necessarily – undergo disorder-to-order transition upon binding to a physiological partner [9, 10].

Intrinsic disorder can confer numerous functional advantages over a rigid, 'frozen' structure [3, 7, 11]. Flexible regions may easily adopt different conformations to allow stable interactions with multiple, structurally heterogeneous substrates (one-to-many signalling) [6]. They are also essential for a fine-tuned regulation of protein function by mediating highly specific, but low-affinity (*i.e.* reversible) binding to partners [12] and by providing target sites for post-translational modifications [13] and for degradation by proteases [14]. Conformational flexibility is required also for macromolecular assembly, including oligomer formation [9, 15]. Finally, native disorder might help preserve protein function under extreme temperature and pH conditions.

Given the multiple advantages of disorder, it is not surprising that a high proportion (30–60%) of eukaryotic proteins are predicted to contain long (≥40 consecutive residues) disordered regions [16, 17]. Large unstructured segments are frequent in regulatory proteins such as signalling and cancer-associated proteins, transcriptional regulators and RNA-binding proteins, but they are relatively rare in proteins with a catalytic function [17, 18] (fig. 1B).

Intrinsic disorder in RNA chaperone proteins

RNA chaperone proteins are ubiquitous in living organisms where they perform essential functions in gene expression and regulation. These proteic factors are also abundant and contain large flexible domains [19, 20]. RNA chaperones bind RNA with broad sequence specificity and help RNA molecules reach their most stable conformation by preventing misfolding or by resolving misfolded RNA species [19, 21–22]. Since RNAs are able to adopt a multitude of rather stable, but non-functional conformations (*i.e.* they may easily become trapped in local energy minima within an energy landscape), chaperone-assistance is indispensable for proper RNA function. Indeed, RNA chaperones guide RNA molecules in every step of cellular RNA metabolism, playing pivotal roles in transcription, RNA processing, transport and translation. Well-characterized cellular RNA chaperones include the fragile X mental retardation protein FMRP, hnRNP A1, RNP YB-1/p50, p53, La protein and the cellular prion protein PrP^C, to name just a few [19].

In the virus world, viral RNA chaperones such as retroviral nucleocapsid proteins mediate the obligatory structural rearrangements of the viral genome that take place during the early and late steps of virus replication [23, 24]. Other nucleocapsid proteins in distant virus families, like that of hepatitis C virus [25] and hantaviruses [26], may also have similar functions.

In a recent review, Tompa and Csermely pointed out that among all the functional protein classes examined so far, RNA chaperones possess the highest frequency of intrinsically disordered regions, with more than half of all amino acid residues situated in putative unstructured segments [20]. Based on this observation, the authors propose an elegant model (the entropy exchange model) for RNA chaperone function. According to this model, binding of an RNA chaperone to the misfolded RNA is accompanied by local folding of the protein, with a concomitant melting (unfolding) of an RNA segment. The RNA molecule is then free to explore the conformational space again and releases the chaperone. Successive disorder-to-order and order-to-disorder transition cycles – driven by a reciprocal entropy transfer between the RNA molecule and the chaperone molecules – will eventually lead to the formation of the most stable RNA conformation required for function [20].

The human immunodeficiency virus nucleocapsid protein network

Retroelements, from Ty retrotransposons in yeast to the pathogenic lentivirus HIV in humans, are widespread in

Figure 1. (*A*) Evolution of protein structure-function relationship models**.** Emil Fischer's lock-and-key concept emphasized the importance of a well-defined 3D structure for protein function that dominated molecular biology for more than a century. However, the last decade has led to the realization that a large number of proteins do not require a rigid 3D structure for their function, and exist in a 'disordered' state – with variable amounts of pre-formed secondary and tertiary structures, corresponding to any of four thermodynamical states (random coil, pre-molten globule-like, molten globule-like, and ordered) – under physiological conditions. This diversity in the native conformational states of proteins and the interconversions between them may be instrumental in highly regulated cellular processes. (*B*) Prediction of intrinsically unstructured regions in RNA chaperones. Disordered regions in HIV-1 nucleocapsid protein, the fragile X mental retardation protein and the cellular prion protein were predicted using the DisProt VL3-H predictor developed by Dunker and Obradovic (http://divac.ist.temple.edu/disprot/predictor.php [86]). An amino acid with a disorder score above or equal to 0.5 is considered to be in a disordered environment, while below 0.5 to be ordered. Note that all these proteins are predicted to contain long, putatively disordered segments and that the same regions were identified as critical for RNA chaperone activity. Disorder prediction for HIV-1 reverse transcriptase is included to illustrate that proteins with enzymatic activities usually require a well-defined 3D structure for their function. Disordered regions confirmed by several independent prediction methods are indicated with a black bar on top of the charts. The overall accuracy of prediction is corroborated by the available structural data for NCp7 [27, 28], RT [87], PrP^C [76] and FMRP [43]. The domain structure of proteins is indicated above the charts (where Zn stands for zinc finger, P for palm domain, F for fingers domain, T for thumb domain, C for connection domain, NDF for the N-terminal domain of FMRP, KH for hnRNP K-homology domain, RGG for arginine-glycine rich region and SP for signal peptide).

living organisms, and all of them encode a unique nucleic acid-binding protein (NABP) which is an RNA chaperone with many functions in virus replication and spread. HIV type 1 (HIV-1) nucleocapsid protein, named NCp7, is probably the best-known example of these small retroviral NABPs with potent chaperoning activities. HIV-1 NCp7 is fairly disordered on its own but becomes essentially structured upon binding to small nucleic acid targets [27, 28]. How does NC work and function? Clearly it needs to bind the target RNA molecule, not as a single protein molecule, but coating the RNA in the form of oligomers. This in turn is thought to define the degree of RNA occupancy and, by way of consequences, several distinct NC functions from virus assembly to genome replication: (i) a small number of NCs probably operate to select HIV genomic RNA at the start of virus assembly; (ii) NC oligomers coat and dimerize the HIV genome, ensuring virus core formation and structure, and upon virus infection oligomers chaperone the conversion of the genome into proviral DNA by the viral reverse transcriptase (an enzyme with a well-defined structure; fig. 1B); and (iii) NC oligomers completely cover the newly made viral DNA, providing protection against cellular nucleases and possibly facilitating integration into the host genome by integrase.

A major conclusion is that HIV NC operates, most probably in the form of oligomers, within a complex network of interactions. In addition, NC appears to be a factor integrating many signals from viral and cellular protein, enzyme and nucleic acid partners in order to orchestrate virus morphogenesis, replication and dissemination. This leads us to speculate that NC is the key organizer of a network that pilots major steps of virus replication and the concomitant generation of virus diversity. As a consequence, disrupting HIV-1 NC by genetic or chemical means would severely impair virus replication and structure, stressing the importance of screening for drugs aimed at inhibiting NC [29]. Indeed, a recent therapeutic vaccine strategy used with some success inactivated HIV-1 virions in which NC was disrupted by aldrithiol-2 [30]. In addition, as conformational flexibility seems to be important for all of NC's varied functions – enabling RNA chaperoning, promiscuous binding necessary for network organization and self-assembly in virion formation – drugs confining NC flexibility may be efficient in combating HIV by acting at several stages of virus replication.

The fragile X mental retardation protein, a potent RNA chaperone involved in neuronal development

Among the most sophisticated RNA chaperones that are present in the cell, the fragile X mental retardation protein (FMRP) is one of great interest since its absence in humans is related to the fragile X syndrome. This syndrome, an X-linked disease, is typically due to the transcriptional silencing of the fragile X mental retardation 1 (*FMR1*) gene and the subsequent lack of its gene product [31–35]. FMRP possesses all the features of a complex RNA-binding protein: it contains a tandem heterogeneous ribonucleoprotein (hnRNP) K-homology (KH) domain and an RGG amino acid motif. Due to the presence of nuclear localization and nuclear export signals, FMRP is able to shuttle between the nucleus and the cytoplasm, where it is mainly associated with poly(A)+ messenger RNPs (mRNPs) present in actively translating polyribosomes [36–41].

Similarly to HIV NC, FMRP possesses RNA-binding and chaperoning activities *in vitro* under physiological conditions [42]. However, FMRP is much larger than NC and is thought to contain both structured and disordered domains [43] (fig. 1B). The chaperone activity of FMRP has to be considered in a cellular context which is infinitely much more complex compared with what is observed *in vitro* in a test tube. In order to understand the role of FMRP in translation control, a combination of *in vitro* and *in vivo* studies have been conducted, aimed at the identification of FMRP-interacting RNAs and proteins. *In vitro* approaches that were used to select messenger RNAs (mRNAs) with the highest affinity for FMRP identified two classes of transcripts containing either a G-quartet structure or U-rich sequences [44–47]. Search for *in vivo* FMRP targets yielded a large number of mRNAs coding for proteins with a variety of functions, including many RNA-binding proteins and factors involved in signal transduction and gene expression regulation [48, 49]. Furthermore, recent reports described the interaction of FMRP with the microRNA/siRNA (small interfering RNA) pathway [50–53] and with the small non-coding BC1 and BC200 RNAs [54]. These findings suggest that FMRP may regulate mRNA levels by several independent (or interconnected) mechanisms, either directly binding to target RNAs or mediating RNA-RNA interactions.

FMRP has the ability to interact with a series of proteins either directly or indirectly through protein-protein interactions. FMRP direct interactors such as FXR1P, FXR2P, NUFIP, 82-FIP, CYFIP1 and CYFIP2 have been described (reviewed in [33]). In addition, other proteins such as nucleolin, YB-1/p50, Pur-α, myosin Va, kinesin and Staufen have been detected in complexes containing FMRP, but it is not known whether they interact directly or indirectly with FMRP [55–58]. These interacting proteins might modulate the affinity of FMRP to different classes of mRNAs by inducing structural changes in conformation, thus exposing differentially the RNA binding domains as proposed in [59]. Although the FMRP version lacking the protein-protein interaction domain (ΔPPId) located in the N-terminus still binds to synthetic RNA, it fails to associate with polyribosomes *in vivo* [59]. Deletion of this

protein-protein interaction domain also strongly impairs the RNA chaperoning properties [42], indicating that self-association (oligomerization) mediated by this domain is important for FMRP function(s).

According to the 'window of activity scheme' (see fig. 2), FMRP is proposed to pilot a network that could mediate RNA transport, modulate translation or recruit mRNAs into cytoplasmic granules thus inhibiting translation, as a function of an increasing degree of RNA occupancy by FMRP molecules. The *in vitro* data also indicate a narrow window of activity, suggesting that small differences in FMRP levels could modulate the fate of the RNA from transport to expression or repression. In agreement with this model, high levels of FMRP induce translation repression of reporter constructs *in vitro* using the rabbit reticulocyte lysate system [60, 61]. Also, high levels of FMRP expressed in STEK cells lacking FMRP induce trapping of mRNA into silent granules and translation repression of co-transfected reporter genes [62]. Interestingly, the highest levels of FMRP are observed in neurons [63] and particularly in granules containing repressed mRNA that have to be translocated from the cell body to distant locations such as neurites [62, 64–67]. The pleiotropic phenotypes caused by the lack or low levels of FMRP support the hypothesis that FMRP is probably required to help build up mRNP networks to achieve dynamic and efficient RNA transport, translation and maintenance in neurons.

Prion protein, an enigmatic RNA chaperone

Prion proteins are associated with transmissible spongiform encephalopathies, a group of invariably fatal neu-

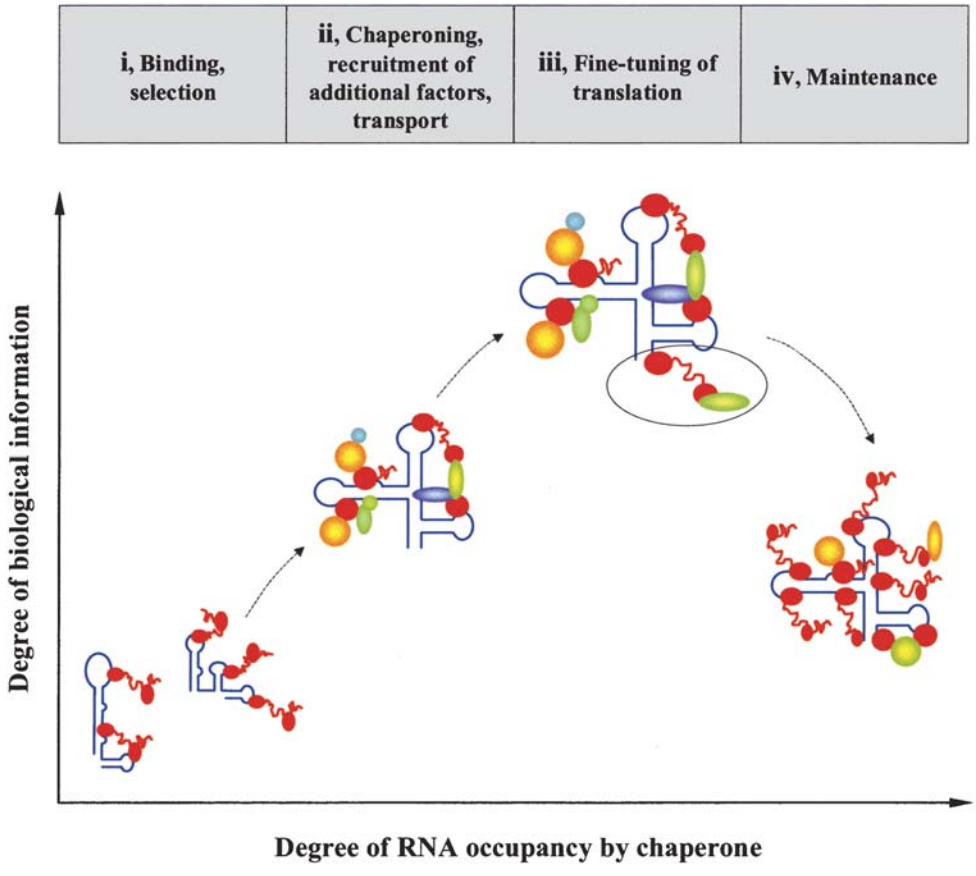


Figure 2. Speculations on the relationships between the degree of RNA occupancy by an RNA chaperone and expected functions**.** Through the example of FMRP, this simple scheme illustrates different degrees of RNA occupancy by RNA chaperone molecules and the expected functions associated with four different modes depicted here: (i) Binding of a limited amount of FMRP to RNA molecules is believed to specifically target U- and G-rich mRNA involved in neuronal development and maintenance. (ii) When more FMRP chaperone molecules are available, they bind RNA, which in turn causes transconformational processes to take place by virtue of chaperoning and the concomitant recruitment of protein and RNA partners. It is speculated that such processes are necessary for mRNP transport and translation. (iii) At this stage the level of translation is believed to be fine tuned by supplementary FMRP molecules in minimal quantities or of other ubiquitous RNA chaperones such as YB-1/p50. (iv) If available, even more FMRP molecules will bind mRNPs, 'freezing' RNA structures, suppressing translation and redirecting RNA to cytoplasmic granules. For retroviral NC proteins, the binding-to-chaperoning transition is accompanied by viral RNA folding and dimerization [19, 23, 88].

rodegenerative disorders, which include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goat, and Creutzfeldt-Jakob disease (CJD) in humans [68]. A hallmark of prion diseases is the accumulation of PrP^{Sc}, an abnormally folded conformational variant of the cellular prion protein (PrP^{C}), in the central nervous system. While PrP^{C} is rich in α-helices and easily degraded by proteinase K (PK), PrP^{Sc} is characterized by high β-sheet content and partial PK resistance. Although the exact mechanism of prion disease transmission and progression is still hotly debated, PrP^{Sc} is considered to be a major component of the infectious agent, known under the acronym prion [68].

Despite considerable efforts, the physiological function of PrP^{c} remains elusive. A number of *in vitro* and *in vivo* studies implicated PrP^{C} in diverse cellular processes such as copper metabolism, protection against oxidative stress, signal transduction and regulation of apoptosis (reviewed in [69]). In addition, recombinant prion protein was shown to bind and chaperone nucleic acids *in vitro* [70–75]. Remarkably, prion protein behaved as a molecular mimic of HIV-1 nucleocapsid protein in a number of functional tests, such as tRNALys annealing to the primer binding site of HIV and retroviral RNA dimerization [73–75]. The nucleic acid binding and chaperoning activities mapped to the unstructured N-terminal region of PrP (fig. 1B). Notably, the random coil-like nature of this region is a conserved feature of PrP's from amphibians to mammals despite the poor sequence identity (~30%) between non-mammalian and mammalian prion proteins [76, 77], suggesting that intrinsic disorder plays an important role in PrP function(s).

Binding of PrP to either DNA or RNA *in vitro* not only changes nucleic acid structure, but at the same time induces formation of stable RNP complexes and profound conformational rearrangements in the prion protein itself, resulting in a partially proteinase-resistant isoform (PrP^{Res}) reminiscent of the pathogenic PrP^{SC} [74, 78–79]. Furthermore, transconformation of PrP^{C} to PrP^{Res} *in vitro* by protein-misfolding cyclic amplification (PMCA) was shown to require the presence of host-encoded RNA but not DNA. RNA derived from invertebrate species was not able to stimulate the conversion, indicating that specific RNA(s) are involved in this process [80].

What could be the *in vivo* relevance of the finding that the Prion protein is a potent RNA chaperone? Our recent results show that PrP^{C} is associated with ribonucleoprotein complexes and translating polyribosomes in human cell lines. Moreover, PrP^{C} expression influences the translation of co-transfected reporter genes in a manner similar to FMRP [our unpublished data]. Thus, one of the physiological functions of the prion protein might be to regulate RNA transport and translation, especially in neural cells where PrP^{C} accumulates to high levels. Thus, it is tempting to speculate that the loss of RNA binding and chaperoning properties or changes in these activities upon PrP^{C}-to-PrP^{Sc} conversion might impair mRNA transport and translation in neuronal cells, resulting in neuron malfunction and spongiform encephalopathy and ultimately in death.

Conclusions and future prospects

A simplistic and reductionist view proposes that mRNAs might be under two diametrically opposed conditions in the cell; either active or inactive. Naked mRNA does not exist in the cell, it has to be stabilized, packed and protected by a series of RNA-binding proteins in the form of mRNP. The transcriptome made up of mRNAs and other untranslated RNAs assembled in RNPs [81] is in constant remodelling through the interaction of a changing repertoire of proteins [82, 83]. Transition of mRNA from active to silent state or *vice versa* is under the control of *trans*-acting elements, *i.e.* repressing or activating factors, including RNA-binding proteins.

RNA chaperones are ideally suited for organizing such a complex and dynamic process since (i) they are able to interact with a plethora of RNAs, as it has been demonstrated for FMRP, and (ii) their intrinsically unstructured regions provide a platform for interactions with a variety of proteins, making possible the precise regulation of RNA metabolism both in time and space. Understanding cellular RNA metabolism – and its consequences in health and disease – would require a detailed knowledge of chaperone interacting partners and target RNAs, as well as elucidation of the structural basis of RNA chaperone function. For example, phosphorylation and the C-terminal chaperoning domain appear to regulate FMRP-mediated association of mRNPs to ribosomes and consequently the level of translation. In this context, how post-translational modifications, protein binding, RNA transconformation, and degree of RNA occupancy by FMRP and other abundant RNA chaperones are interconnected is not yet understood.

Even for well-known retroviral chaperones such as HIV-1 NCp7, we are far from understanding how they function at the molecular level. RNA chaperones most probably act in the form of oligomeric structures, homo-oligomers as for HIV NC and other retroviral NCs and probably both homo- and hetero-oligomers for FMRP and PrP. These chaperone oligomeric structures are found in a test tube, but their existence remains to be shown in a cellular context. Furthermore, the conformation and 3D structures of HIV-1 NC and FMRP oligomers are totally unknown except for some electron microscopic pictures showing dense globular structures along a nucleic acid molecule [72–75].

RNA chaperones are candidate targets for fighting viruses and possibly treating neurodegenerative disorders. How-

ever, the current drug design strategies are most efficient at inhibiting enzymes with a well-defined structure and a small number of specific partners. Redirecting or impairing RNA chaperone functions for therapeutic purposes requires a more global thinking about the role of disorder in biology and how RNA chaperones integrate and deliver a series of signals critical for mRNA synthesis, expression and decay. New types of drugs, either modulating or freezing the intrinsic flexibility of these proteins [18], or simultaneously targeting several key connections in their interaction network [84, 85], might be a good choice for treating viral or genetic diseases. To this aim, precise mapping of the protein and RNA connecting motifs within this interaction networks is indispensable, as it is in due course for FMRP.

"Like water molecules coaxing clouds into snow crystals but cloud to snow crystals but far more sophisticated are RNA chaperones conducting RNA molecules from disorder and misfolding to order and function."

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