RNA SPLICING, AGEING, TDP 43 AGGREGATION AND NEURODEGENERATION

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ABSTRACT

The role of splicing factors in pathological processes is increasingly documented. This is particularly evident in the pathologies affecting the nervous system where either the mis-folding or aggregation of RNA binding proteins is a key event in triggering the neurodegeneration. The aggregation of TDP-43 is the major distinguishing feature of most cases of Amyotrophic Lateral Sclerosis (ALS). There is still a significant uncertainty in regards to many aspects concerning the nature of the aggregates and their functional consequences. The biochemical and genetic aspects of TDP-43 aggregation need further definition, as new therapies could be directed towards them. A better understanding of TDP-43 structure and interactions is needed, from what we currently know it has been possible to construct cellular and animal models of TDP- 43 ALS like aggregation. Using these models drugs that can revert aggregation and recover function have been identified. Finally, ALS is a disease that occurs mostly during the fifth to the seventh decade of life. In this respect, it has been observed that the onset of the locomotion defect in an ALS fly model coincides with an age-related 4-fold drop in TBPH levels (the Drosophila TDP43 orthologous), similar TDP 43 reduction with age was observed in mice brain. Thus, understanding the relationship between aging and TDP-43 production and mis-folding in cell culture, animal models, and human tissues might provide further clues to explain the time of disease onset.

Keywords: Molecular Biology, gene expression, RNA splicing, Amyotrophic Lateral Sclerosis, Aging.

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Introduction

In this brief review I would like to describe how the research on basic molecular mechanisms of gene expression ends up making an impact on an apparently unrelated field such as neurobiology and ends up in translational work searching for effective therapeutic drugs to be used in neurodegenerative disease. To evidence these points as clear as possible I will spend a good part of the manuscript in describing the impact that the discovery of the RNA splicing process has had in molecular and developmental biology and enter in the role of splicing factors in neuro-degeneration.

RNA splicing, a fundamental and unexpected step in RNA metabolism, was observed for the first time in the late 70s. At that time, a peculiar RNA processing phenomena was described in the adenovirus 5'UTR; three pieces of noncoding sequences were joined together and the sequences between them eliminated to form the viral mRNA [1, 2]

This was soon followed by the observation that the recently cloned globin mRNA did not hybridize to a single band in a Southern blot but there clearly were other sequences, interrupting the coding sequences [3]. These genomic nucleotide fragments were even longer than the coding sequences. Finally, after the, at the time, laborious process of cloning higher eukaryotic genes, the sequencing of these clones showed that the presence of noncoding sequences that interrupted the coding sequences was the rule rather than the exception. The name of introns and exons was adopted for the intervening sequences and the coding sequences respectively and the prediction made that the protein coding content of genomes would comprise only a fraction of the total DNA [4]. The process of cutting the introns and joining the exons was called splicing. This discovery had immediate ramifications regarding biology, namely, how are the exons identified?

The first gene sequences showed, surprisingly, poor sequence homology at the junctions of introns and exons. The only universally conserved nucleotides were the GU at the 5' site of the intron and AG at its 3'site [5] obviously, these elements would be insufficient to direct the splicing process which is usually error free. In the following years other features were uncovered that increased the accuracy of exon/intron junction definition resulting in the so called consensus sequences around the exon/intron junctions (splice sites) and the intronic poly-pirimidine tract near the 3' splice site [6].

In the early 1980s it was discovered that there were variations in exon selection and that several mRNA isoforms could be produced from one pre-mRNA [7], this process was called alternative splicing and was followed by the discovery both in vitro and in vivo that auxiliary sequences overlapping with the coding sequences in the genomic context of an alternative spliced exon [8, 9]. Subsequently, specific cis-acting elements within these fragment were identified that according to their location and effect on splicing were called exon splicing enhancers or silencers (ESE and ESS respectively) and intron splicing enhancers and silencers (ISE and ISS respectively) whose effects are transduced through their interaction with RNA binding proteins (RBPs). In addition to the cis-acting element, other processes that influence splicing outcome exist. They include the effect on splicing of the RNA structure [10], the transcription rate [11], chromatin remodelling and epigenetic modifications [12].

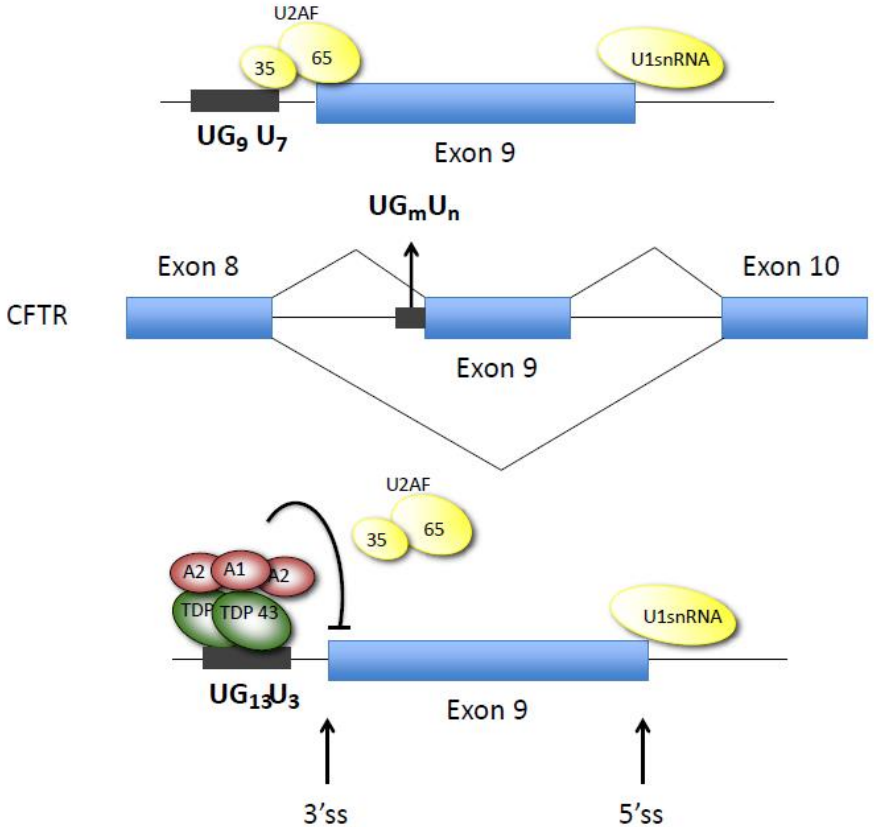
Alternative splicing in development and ageing

All organisms during development and reaching a mature reproductive stage undergo slight changes and adjustments in the cellular and tissue molecular and physiological processes that taken to the extreme can be called an aging process. One metabolic area where these alterations can be clearly seen is in the RNA splicing process and in particular in the alternative splicing variations. This is because RNA splicing is a ubiquitous regulatory mechanism that the cell utilize to produce more than one mRNA transcript from a single gene. Alternatively spliced transcripts can be translated into different protein isoforms with diverse functions and/or

localizations, they can also occur in untranslated regions affecting mRNA stability, localization, or translation. Indeed, alternative splicing is not a rare event: in humans more than 90% of the genes are estimated to undergo alternative splicing. From the ~20,000 human protein-coding genes, high-resolution mass spectrometry analyses have shown that ~37% of them generate multiple protein isoform [13] evidencing alternative splicing contribution to protein diversification. These alternative splicing events are in part directed by a series of trans acting factors that bind a variety of exonic and intronic sequences and in this way can heavily influence splice site choice and exon inclusion. Indeed, the physiological importance of alternative splicing is highlighted by the enormous number of human diseases caused by mutations in cis-acting RNA-sequence elements, trans-acting splicing factors or spliceosome components. Indeed alternative splicing is a main player in cell linage and tissue-identity acquisition and maintenance, cell differentiation, and tissue/organ development. Molecular understanding of developmental transitions has also revealed important bases of pathological mechanisms in diseases where normal networks are mis-regulated. Multiple mechanisms regulate splicing in nature, in particular during development [14].

The hnRNP TDP 43

There is a myriad of splicing factors that can act positively or negatively on exon inclusion. Many of them belong to the RNA binding proteins family, the positive factors in splicing are often the Serine-Arginine (SR) proteins and the negative factors are Heterogeneous Nuclear Ribonucleoproteins (hnRNPs). These splicing factors are involved in several pathological processes among these neurodegeneration.



The case of the hnRNP TDP 43 (TAR DNA Binding Protein) is a paradigm of these phe nomena. Our laboratory characterized TDP 43 as an hnRNP with a role in splicing events leading to Cystic Fibrosis [15]. In fact it was known that a polymorphism in the 3'splice site of CFTR exon 9 composed by a variable number of UG repeat and U runs was associated with milder clinical presentation of Cystic Fibrosis (Fig 1, middle panel).

Figure 1: Schematic representation of the region of exon 8, 9 and 10 of the CFTR pre mRNA (Middle panel). Exon 9 is included in variable proportions in different individuals. If there is no interference of the TDP 43/A2 complex with the 3'ss U2AF complex is predominantly included (Upper panel). On the other hand if the U2AF-pre mRNA complex formation is hampered by the TDP43/A2 complex, exon 9 is predominantly skipped (Lower panel).

The UGm and Un may exist in different combinations in normal individuals, the approximate range of UGm is from 5 to 13 while that of Un is from 3 to 9. They can exist in different combinations but not all of them are compatible with normal CFTR RNA processing and consequently function. For example UG9 U7 yields normal exon 9 splicing (Fig 1, upper panel), UG13 U5 results in higher proportion of exon 9 skipping but the residual exon 9 inclusion prevents the development of classical CF and is associated with single defects such as vas deferens obstruction or bronchiectasis. Finally the polymorphism configuration UG13 U3 produces total skipping of exon 9 (Fig 1, lower panel). Hence a non-functional CFTR protein and full blown CF [16]. Investigating the molecular basis of this phenomena we found that hnRNP TDP 43 bound to the UG sequence and interacted with other hnRNPS such as A2 that in turn inhibited the recognition of the 3'ss by the U2AF complex (Fig 1).
Subsequent studies showed that TDP 43 is involved in multiple processes in RNA metabolism ranging from splicing, micro RNA processing mRNA transport and stability Fig 2. In addition it shuttles between nucleus and cytoplasm and regulates its cellular levels by a novel mechanism involving an unusual splicing event in the 3'UTR of the pre mRNA. A decade ago TDP 43 has jumped to fame when it was identified as the main component of the protein inclusions seen in the brain of people affected by Amyotrophic Lateral Sclerosis (ALS) and Fronto Temporal Lobar Degeneration (FTLD) [17].

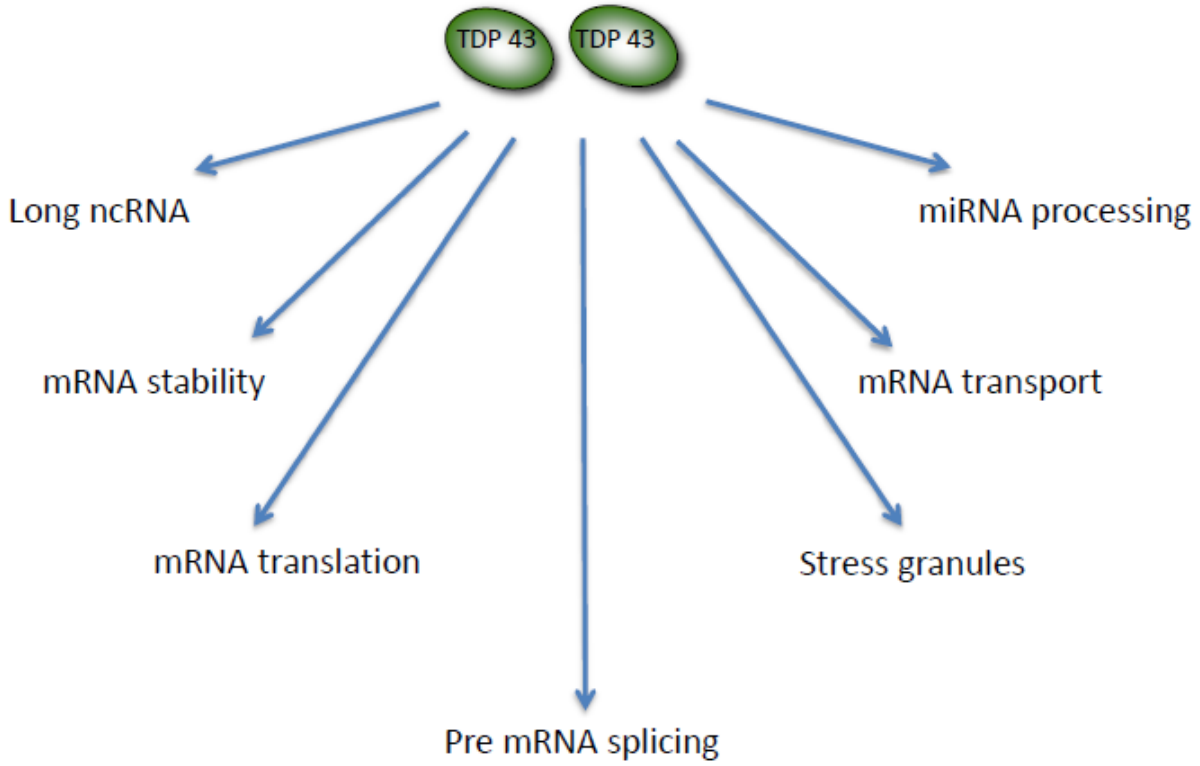


Figure 2: Functions of TDP 43 in the RNA metabolism, the best documented one is its role in pre mRNA splicing. The involvement in stress granules may be the path that leads to further aggregation and the formation of the large inclusions seen in the brain of ALS and FTLD patients.

TDP 43 role in ALS

Histological analysis of patient's brain showed TDP 43 inclusions in many neurons some in the nucleus, some in the cytoplasm and even in their axons. TDP 43 is a mostly nuclear protein

seen diffusely in the nucleoplasm. However most of the cells containing inclusions have nucleus that were negative for TDP 43 staining. Two hypothesis were proposed for the pathogenesis of ALS: One that this was due to the toxicity of the inclusions and the other one that the lack of TDP 43 in the nucleus, due to the aggregation of the protein in the cytoplasm, provokes a loss of function in the multiple processes that this protein is involved in, such as RNA splicing, mRNA transport and stability, miRNA processing [18] (Fig 2) We have gathered a substantial body of evidence that shows that the latter hypothesis may be the correct one. In fact depletion of TDP 43 in tissue culture cells, obtained by siRNA treatment, produces a series of changes that affect the viability of the cells and splicing variations are clearly visible. Furthermore removing the gene in mice produces disorganization of the embryo and is an early lethal mutation. The fruit fly Drosophila has a homolog of TDP 43 called TBPH, deletion of the gene allows the development of the larva up to the pupa but the adult fly has serious locomotion defects that do not allow its exit from the pupa [19]. If the fly is removed manually its inability to move properly can be seen and they die within 1-2 days. This data pointed to a lack of function model of the disease. This is what should be expected if the aggregates sequester TDP 43 and prevent its functionality. We decided to model the aggregation process in tissue culture cells and in flies.

Modelling ALS like TDP 43 aggregation

Aside the animal models, several attempts were undertaken to mimic the TDP-43 aggregation in cells in culture. Such models are valuable to investigate the impact of aggregation on the cellular metabolism, as well as to evaluate new therapeutic strategies to overcome aggregation. It was observed early on that the TDP-43 C-terminal tail contains a Q/N rich region that is involved in the protein-protein interactions [20] Moreover, it was demonstrated that expression of C-terminal fragments of TDP-43 is sufficient to generate cytoplasmic aggregates [21]. The importance of the Q/N rich region within the C-terminal tail of TDP-43 in the self-aggregation process was also confirmed in separate experiments where this sequence was shown to be essential for polyQ induced aggregation [22]. In fact the importance of the C-terminal is also supported by the fact that the majority of the mutations that have been found in ALS patients are localized in the C-terminal tail and the aggregation tendency is enhanced by these ALS-linked TDP-43 mutations [23]. In addition, the protein is cleaved, generating C-terminal fragments that are associated with cellular toxicity and/or increased TDP-43 mis-localization [24]. Based on these findings, and with the aim of looking for methodologies that could model the disease, our laboratory developed a cellular model of aggregation using a 30 amino acid TDP-43 C-terminal peptide to promote TDP-43 aggregation [23,25].

These models are based on tandem repeats of TDP-43 Q/N rich amino acid sequence 331-369 (12xQ/N) linked to EGFP reporter is able to trigger the formation of predominantly cytoplasmic aggregates, capable of sequestering either exogenous or endogenous full-length TDP-43, recapitulating some of the features of the inclusions present in patients, such as ubiquitination and phosphorylation .

However, there was no detectable splicing function deterioration in the presence of these TDP-43 aggregates induced by EGFP-12xQ/N, suggesting that they were not efficient enough in trapping endogenous TDP-43 to cause a loss of function in the short interval measured in a cell system. In fact, it can be seen in that there is still TDP-43 present in the nuclei of EGFP-12xQ/N expressing cells.

In order to generate a model that could accomplish the nuclear loss of function of TDP-43 that is characteristic of ALS, a new variant of the previous model was generated. This new model is based on the TDP-43 molecule itself linked to the tandem repeats 12xQ/N (TDP-12xQ/N).

The TDP-12xQ/N model was shown to induce TDP-43 aggregation that was accompanied by TDP-43 nuclear depletion and consequent alteration of its splicing function [26].

Our cell-based models of ALS are useful tools for the identification of active agents capable of reducing TDP-43 inclusions. In fact as a proof of principle we have tested a series of tricyclic anti depressive drugs that showed a reasonable activity in eliminating aggregates by stimulation of the cell clearing systems and restoring TDP 43 functionality. This model is now used in a wide new molecule screening for drugs and is showing optimal results.
We have now also generated an animal model based on this 12xQ/N construct. We have used, in the first instance Drosophila melanogaster a powerful model to study human neurodegenerative diseases. Several characteristics make Drosophila the organism of choice. Among them, the short generation time (approximately 10 days) and short life span (around 60 to 80 days). In particular these features make Drosophila amenable to study age-related disorders. In addition, approximately 75% of human genes known to be associated with disease have a Drosophila orthologue. In most neurodegenerative disease, specific neuronal regions begin to degenerate late in life. In order to study this, several methods are available to express genes in a spatially and temporally restricted manner. Moreover, synaptic activity can be measured using electrophysiological and imaging techniques from the neuromuscular junction and adult central nervous system, making Drosophila particularly amenable to study motor neuron diseases, such as ALS.
The Drosophila 12xQ/N transgene showed that its locomotion was compromised with aging and its lifespan was shorter. The animals with the severe locomotion phenotype present a sharp atrophy and retraction of the Neuro-Muscular Junction. The onset of the locomotion phenotype around day 15 happens in mature flies, a similarity with the rather early onset of ALS in humans. The onset of the locomotion phenotype in Drosophila coincides with a four-fold reduction in the levels of brain TDP43 (TBPH in flies) relative to the one day old fly [27].

The decrease in TDP 43 tissue levels is physiological and evolutionary conserved
The investigation of tissue levels of TDP 43 and other RNA binding proteins in function of age was extended from Drosophila to zebrafish and mice. A similar decay was observed in these species. In particular the mice showed also a programmed fourfold reduction of TDP 43 in the brain between 10 and 90 day old animals, a compatible age range to the Drosophila equivalent. The mice studies revealed in addition that the decay was tissue specific and have different rate of progress in different tissues (Pacetti, De Conti et al, 2018, submitted). In fact while in liver the TDP 43 levels were maintained through age, in brain there is an age related decay similar to the one observed in Drosophila and in muscle there is an early sharp decrease of TDP 43 levels. This reduction is seen in both TDP 43 protein and mRNA.

The decrease of TDP 43 levels is due at least in part to a reduction of the transcription rate
We are also investigating the mechanism(s) behind the reduction of TDP 43 levels that we found to be tissue specific. In liver TDP 43 levels are maintained through the lifetime of the animal, but there is a mild constant reduction in brain and a sharp reduction in muscle. The modulation of TDP 43 expression seems to occur through an increase of methylation in the promoter of the gene with age, observed specifically in those tissues where there is a decrease of protein levels. As expected there is an inverse correlation with the time and size of the reduction: With age TDP 43 levels are lower in muscle<brain<liver while the degree of methylation goes the opposite way muscle>brain>liver.
In conclusion a possible pathogenic mechanism for the onset of ALS is schematically shown in Fig 3. TDP 43 participates in several RNA metabolism steps in the cell (Fig 2), thus keeping its cellular levels within a restricted range is extremely important and a self-regulation mechanism is in place [28], as both excess and lack of TDP 43 are harmful to the cell [28]. During the lifetime in long lived cells such as the neurons a stress may occur that results in

protein mis-folding and aggregation and consequent alterations in its proteostasis. The cell has mechanisms to clear these aggregates such autophagy and ubiquitin protease pathways, however it is possible that chronic stress and/or damage to the protein degradation mechanism results in an incomplete clearance of the aggregates which in turn start to sequester newly synthesized TDP 43.

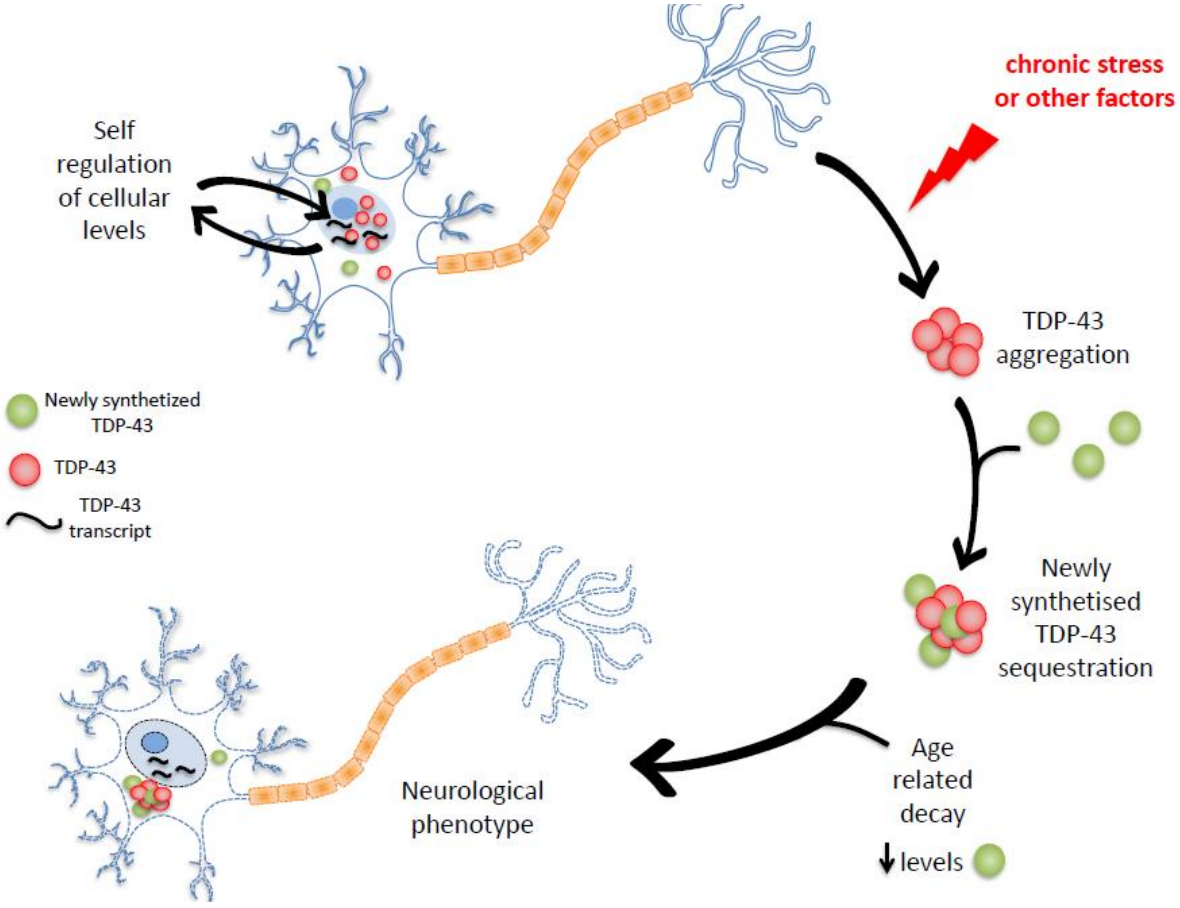


Figure 3: Schematic representation of a possible pathogenic mechanism leading to ALS/FTLD-U. It is based on observations made in cell in culture, Drosophila and Mice systems and should be considered as a model to be confirmed for the human pathology.

The growth of the aggregates and their increased capacity of capturing the newly synthesized TDP 43 combined with the decrease of TDP 43 synthesis observed during aging may lead to a situation of total depletion of functional TDP 43 in the nucleus that results in damage to the cell in particular, in the case of a motor neuron, of the Neuro Muscular Junction (NMJ). It is possible that preventing or reverting the aggregation process we could recover TDP 43 functionality.

We have identified two potential therapeutic approaches for TDP 43 proteinopathies: The increase of TDP 43 protein levels or the clearance of the aggregates. The latter option is the more promising with the identification of compounds that in our cellular model are very efficient in aggregate clearance and restoration of TDP 43 functionality. It remains to be explored the potential of increasing the production of TDP 43 by interfering with the promoter methylation in the hope that the higher levels will overcome the loss of functionality.

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Prof. Francisco E. Baralle es Dr. en Química (UBA) y Dr. en Medicina (UBA/Nápoles). Hizo su trabajo post-doctoral en el MRC Laboratory of Molecular Biology Cambridge UK (1974-1980) y de 1980 a 1990 fue Profesor de Patología en la Universidad de Oxford (UK) y miembro del Magdalen College. De 1990 al 2014 fue Director del International Centre for Genetic Engineering and Biotechnology (ICGEB). Del 2014 al presente es PI del grupo de RNA Biology en la misma institución.

Sus principales contribuciones a la Biología Molecular incluyen la primer secuencia completa de un mRNA eucariote (beta- globina) y el aislamiento y caracterización del gen humano de la globina embriónica epsilon. Al inicio de la decada del 80 describió por la primera vez el procesamiento alternativo de un pre mRNA celular (fibronectina) y reprodujo este fenómeno en sistemas celulares con el uso de mini genes. Desde entonces su principal interés son los mecanismos moleculares del procesamiento del pre-mRNA en el metabolismo normal y patológico. Ha estudiado extensivamente los defectos moleculares a la base de enfermedades hereditarias causadas por mutaciones en un solo gen, como talasemia, fibrosis quística, neurofibromatosis y en enfermedades poli factoriales como dyslipoproteinemias, hipertensión y neurodegenerativas. En este último campo se focaliza su investigación corriente en particular sobre la proteína TDP 43 caracterizada en su laboratorio y luego identificada como el componente de las inclusiones neuronales típicas de los pacientes con Sclerosis Lateral Amiotrofica y algunas formas de demencia incluso una fracción de pacientes con Alzheimer. Recientemente ha coordinado el proyecto *mis splicing and disease* dentro del European RNA Alternative Splicing Network of Excellence (EURASNET).

En su laboratorio del ICGEB además de llevar a cabo investigación básica en los temas citados anteriormente, se llevan adelante proyectos aplicados a la producción de proteínas recombinantes para uso terapéutico que se transfieren a instituciones y/o empresas de los países miembros del ICGEB. Productos como Erythropoietina, Interferones, insulina producidos con la tecnología ICGEB están ya en el mercado en varios países particularmente en países de Latinoamérica y Asia.

En 1980 dicto en Argentina el primer curso de manipulación genética, que fue replicado bianualmente hasta 1988. Como director del ICGEB desde 1990 ha tenido numerosas colaboraciones extremadamente fructíferas con grupos científicos e industriales Latinoamericanos.