

Autism and Non Sense Mediated Decay (NMD)

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AUTISM

Autism is a complex childhood developmental disability that causes problems with social interaction and communication [1,2]. “Infantile Autism” was first diagnosed and classified by Kanner [3] and for nearly 50 years was viewed as a relatively rare, low incidence condition. Autism has three unique features that includes (a) reduced motivation for social interaction, (b) restricted interests and repetitive behaviors, and (c) severe communication disorders. In the beginning a patient has to present with all three of the criteria to be diagnosed with autism. The percentage of diagnosis thus stayed very low until the 1980, where broadening of the diagnostic criteria and reclassifying autism as a spectrum was published. This diagnostic change lead to a dramatic increase in the number of cases diagnosed with autism reaching 10 to 20 per 10000 children with a decrease in intellectual disability diagnosis (US Centers for Disease Control, 2012).

Autism Spectrum Disorder includes an array of diseases in addition to autism such as Asperger’s syndrome, Pervasive Developmental Disorder-Not Otherwise Specified (**PDDNOS**), Rett’s syndrome, and childhood disintegrative disorder. In ASD, symptoms usually start at a very young age and can cause delays or problems in many different skills that develop from infancy to adulthood [2,4].

ASD is multi factorial, with many risk factors acting together to produce the phenotype. The dissimilarity between monozygotic and dizygotic twin rates suggests that some risk factors

interact and influence it at different levels, such as gene–gene or gene–environmental interactions [5]. In 10-15% of ASD cases is associated with known genetic causes. The most common causes include fragile X syndrome, where about 3 % of patients are also diagnosed with autism, tuberous sclerosis (2%) and various cytogenetic abnormal findings such as maternal duplication of 15q1-q13 (2%), and deletions and duplications of 16p11 (1%). None of these causes are specific to ASD, but rather are specific to a range of other autism spectrum phenotypes [5,2,6,7]

Only a few common variants have been identified as possible ASD candidate genes in linkage and association studies, pointing to the difficulty of finding common causes. The difficulty in finding robust common variants is not unique to autism spectrum disorder alone. Evidence for synaptic dysfunction as a unifying cause has come from findings of rare mutations in Neural Cell Adhesion And Synaptic Molecules Such As X-Linked Neuro Ligin 4 (**NLGN4X**), Neuro Ligin 3 (**NLGN3**) and SHANK3 genes [8-10]. The study of genetic factors associated with synaptic maturation is very important because the outcome from neuro imaging studies on autistic subjects demonstrates a defect in structural and functional brain connectivity [11].

At this point, autism could be caused by defects at a genetic level which includes all the susceptible genes, leading to dysfunction at protein synthesis level. This could lead to abnormal synaptic structure and function, which affects ASD core regions of the brain, causing atypical neural system in the whole brain.

Massive advances in sequencing techniques revealed large number of rare mutations associated with numerous conditions either neuro developmental or psychiatric. Many of these diseases could result from mutations that influence various aspects of mRNA metabolism, including processing, export, stability, and translational control. Example of these rare mutations causing the diseases include mutations in JPH3 which is associated with Huntington's disease, FMR1 which associated with fragile X syndrome, *UPF3b* which was associated with neuro developmental disorders, eIF4e in autism, DISC1 and HDAC9 with schizophrenia, and the list is ever growing.

UPF3B

UPF3b Mutations in Psychiatric Disorders Including Autism

A number of large-scale genetic screening studies were made on patients suffering from a number of neuro developmental disorders and their families to identify causes of the disorders they suffer from. These studies reported the presence of either missense or nonsense mutations in *UPF3b*. The nonsense mutations were caused by either a direct nucleotide change or a frame shift event leading to introduction of a stop codon. On the other hand, missense mutations are caused by a single nucleotide substitution only, leading to the production of a mutated form of *UPF3b*.

A number of mutations and deletions in the *UPF3b* gene have been identified in subjects with XLID. Tarpay and colleagues in 2007 reported a number of anomalies in 11 individuals from four families with one or more types of intellectual disability [12].

For the first family, which had two brothers who were diagnosed with FG syndrome (OMIM 305450) and a carrier mother were reported with four nucleotide deletion of 674-677delGAAA leading to a frame shift translational change producing a stop codon, that will lead to production of *UPF3b*R225fs*22 protein form. The second family which was diagnosed with Lujan-Fryns syndrome (LFS-OMIM 309520), a type of syndromic XLID with phenotypical features, in three brothers and a cousin, with a number of female carriers in the family. This family had two nucleotide deletions in 867-868delAG, leading to a frame shift change and production of *UPF3b*G290fs*2. The third family, which was also diagnosed with LFS, in 5 male members over three generation and three of them, had carrier mothers. Again this family had a single nucleotide mutation 1288C>T, forming a PTC and a subsequent *UPF3b*R430*. The fourth family screened, which is the most interesting one, were diagnosed with XLID in two brothers, and also a carrier mother. This family had a missense mutation, where T is substituted with G at location 478, leading to a mutated full length *UPF3b*Y160D mutation. Interestingly the 160 location where this mutation occurred is highly conserved in species from mammals to plants, indicating the importance of tyrosine (Y) at the 160 location. Furthermore, more analysis of the family found that the grandmother has no mutations, indicating it's a *de novo* mutation in the mother. Another interesting result is that in both the 3rd and 4th family, analysis of the carrier females showed a high percentage of X-chromosome inactivation. Thus in total, mutations leading to open reading frame truncation were identified in three of the four families, in addition to the missense mutation reported in the members of the fourth family [13,12]. Interestingly, five patients out of the 11 studied exhibited autistic features. Until recently the genetic basis for both FG, and LFS was not known, but these findings provide evidence of an overlap [7].

In 2010 another large scale screening study of 372 families with XLID and 25 individuals with Lujan-Fryns syndrome, three families were found with *UPF3b* mutations. In this study, the choice of the families with neuro developmental disorders was stricter; the presence of at least two brothers or two boys from different generations was essential. The first family is a rather large family of 47 in total, 10 men from different generations were diagnosed with non-syndromic XLID. Further analysis showed that they all had a mutation in *UPF3b*, 1081C>T leading to the formation of a PTC and production of *UPF3b*436*. Additionally, in the same family, 15 carrier females were also found with high percentage of chromosome X inactivation 85:15%. The second family analyzed was Dutch and presented with two brothers diagnosed with ID. A single nucleotide substitution was found in both brothers, 1136G>A which leads to production of a mutated *UPF3b* protein, Arg379His (R379H). The third family, is from Belgium and one brother was presented with ID and autism; it was found that he has a single nucleotide substitution in *UPF3b* 1103G>A forming Arg368Gln (R368Q). The mother of the boy was found to be a carrier too. Interestingly, the missense mutations in the 2nd and 3rd family (368 and 379) are at highly conserved locations throughout a large number of species indicating its importance [14].

In another recent study, a family with two brothers diagnosed with childhood onset schizophrenia “a severe and very rare form of schizophrenia with undefined genetic cause”, autism, and attention deficit hyperactivity syndrome. A scan of several hundred of probable genes revealed a novel four nucleotide deletion in *UPF3b* 683-del686AAGA leading to a frame shift translational effect and production of Q228fs*18 [15].

Another missense mutation in *UPF3b* was reported in 2011 [16] in a patient with schizophrenia. The mutation is a single nucleotide substitution of G to A at 764 resulting in an amino acid substitution of arginine to lysine (*UPF3b*R255K).

Recently a study was done on a four-generation Chinese family with three members diagnosed with XLID. A nonsense mutation in *UPF3b* was found in the affected members where C is substituted to T leading to a PTC causing translational frame shift producing *UPF3b*R430*. Additionally, three female carriers from the same family were identified with high percentage of chromosome X silencing [17].

For this research, the missense mutations affecting *UPF3b* were chosen for further analysis. These mutations lead to the amino acid changes *UPF3b*Y160D [12].

UPF3 Variants

Humans have two genes encoding *UPF3* protein; the first gene, *UPF3a*, is located on chromosome 13 and the second gene, *UPF3b*, is located on chromosome X. There is a high similarity between *UPF3A* and *UPF3b* proteins reaching up to 60%. The N-terminal amino acids from 38 to 236 are the most conserved with up to 86% similarity. On the other hand, C-terminal amino acids 202 to 453 are considerably more divergent and they contain one of several Nuclear Localization Signals (**NLS**) [18].

Both *UPF3* proteins are nucleo cytoplasmic shuttling proteins containing three Nuclear Localization Signals (**NLS**) and a single Nuclear Export Signal (**NES**) and are primarily localized in the nucleus [18,19].

Human *UPF3b* has two isoforms variants due to an alternate splicing event, variant 1 and 2. Variant 1 is 1451 nucleotide long, coding for a 283 a.a. long *UPF3b* protein. On the other hand, variant 2 is 39 nucleotides shorter (amino acids 270 to 283 present in variant1 are missing here) and codes for a 470 a.a. long *UPF3b* protein. Both isoforms of the *UPF3b* gene are highly expressed in a large number of tissues in the human body, which includes the adrenal gland, bone marrow, cerebellum, whole brain, kidney, lung, placenta, prostate, salivary gland, skeletal muscles, testis, thyroid gland, uterus, small intestine and the lymphoblasts [18].

UPF3b protein contains an RNA Recognition Motif (**RRM**) in the N-terminal region between a.a 42 and 143 [20,21] and within this sequence, the region between a.a 52 to 57 is required for binding to UPF2. Furthermore, the Exon Junction Complex (**EJC**) core proteins recognition and binding domain is located between a.a 421 and 434, close to the C-terminus [22,23,24]. The

binding of *UPF3b* to both the EJC and UPF2 is essential for initiation of nonsense-mediated decay. The structure of *UPF3b* with all the domains is seen in Figure 1.

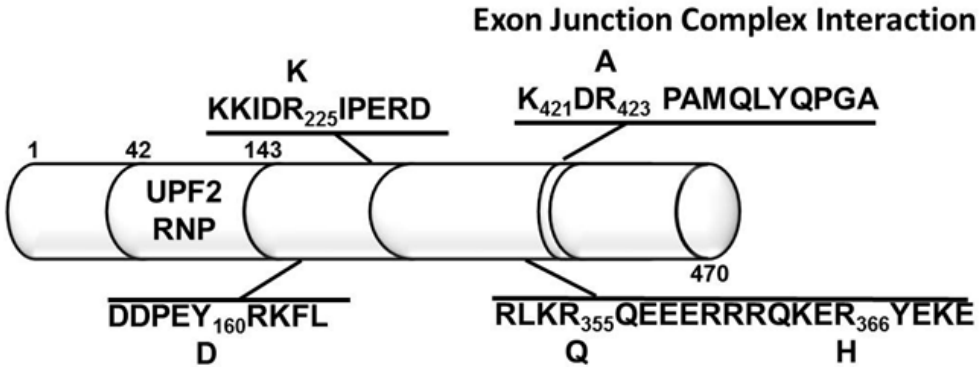


Figure 1: *UPF3b* protein structure with the missense mutations sites used in this study. A schematic representation of *UPF3b* protein structure. *UPF3b* has two isoforms as a result of alternative splicing, isoform I is 483 aa and isoform II is 470 aa long. Amino acids 270 to 283 are missing in isoform II. From aa 42 to 143 is the RNA Recognition Motif Domain (**RNP**), which has the UPF2 binding domain within it from aa. 52 to 57. From aa 421 to 434 is the exon junction recognition and binding domain, it specifically binds to Y14 of the EJC. Within the EJC binding domain is the artificial single point mutation R423A that was demonstrated to inhibit NMD. For this study I used the *UPF3b* isoform II with the missense mutations reported. The first two reported missense mutations, Y160D and R255K, are located close to the RNP motif. Additionally, the first mutation Y160D is also close to the UPF2 binding site. Furthermore, the third and fourth reported missense mutations, R355Q and R366H, are located close to the Exon Junction Complex (**EJC**) binding site.

It has been shown that there is some but not complete redundancy between *UPF3a* and *UPF3b* proteins in NMD. Knockdown of *UPF3b*, which is thought to be the primary active form, is partially compensated for by *UPF3a*, on the other hand; knocking down *UPF3a* has no effect on *UPF3b* level or activity [25,26]. Thus *UPF3b* protein is an important, but not an essential, component in NMD [27]. This is possibly also due to the presence of the UPF1 dependant alternative NMD pathway. In 2007, Tarpey [12] analysed the cells of the patients who were diagnosed with neuro developmental disorders and had *UPF3b* mutations, and found an elevation in one of the targets of the classical NMD pathway (GADD45B), with no change in targets of an alternative UPF1 dependant NMD pathway (PANK2 and SMG5).1

UPF Proteins And NMD

In recent years, a cellular surveillance process known as NMD was characterized. NMD identifies and degrades mRNAs with a premature termination codon (PTC), upstream of a normal termination codon produced from transcription of genes with mutations leading to

the generation of a PTC, or by splicing that creates shorter open reading frames. In addition to targeting transcripts with premature termination codons (aberrant), NMD also regulates a number of normal transcripts. These targets have NMD-inducing features that render them a target for degradation. These features include Upstream Open Reading Frames (**uORF**), a long 3' Untranslated Region (**UTR**) (>1.5kb), or PTC-containing isoforms generated by alternative splicing [28-32].

The NMD machinery has the capability to discriminate NC-containing mRNAs from normal mRNAs, and to inhibit translation and activate decay of the NMD targeted mRNAs. It is assumed that one of the functions of this system is to avoid the synthesis of truncated proteins that may have cellular dominant negative effects or new unwanted effects [33-35]. It is clear that the NMD pathway has important dual roles in the prevention of synthesis of truncated proteins, and in the regulation of gene expression.

The detection of NCs is helped by a protein complex called the Exon Junction Complex (**EJC**). During mRNA splicing, introns are spliced out and exons are joined. Close to the site of the exon-exon joint, a number of proteins are recruited that form the EJC [34,36,33,38]. Proteins identified as part of the EJC core include MAGOH, Y14, and eIF4AIII, MLN51 (also known as BTZ or CASC3) [38,36].

EJC proteins play important roles in post-splicing events including mRNA export, cytoplasmic localization, and nonsense-mediated decay [39]. Recent evidence suggests that mRNA translation is also influenced by the splicing history of the transcript [40]. The next paragraphs, the role of EJC in NMD will be explained.

Normal translation occurs in two stages or rounds, the pioneer and the bulk round of the translation [41]. In the pioneer round of translation, the heterodimer cap binding complex composed of cap-binding proteins CBP20 and CBP80 is bound to 5' end of the mRNA, facilitating the loading of the first ribosomes on the mRNA [41, 42]. This first ribosome then moves along the mRNA, producing protein and every time this ribosome reaches an EJC it dissociates it and releases it from the mRNA. At the end of the pioneer round, normal RNA transcripts are no longer associated with EJC proteins. Subsequently CBPs are replaced by eIF4E that binds to eIF4G, which binds to PABP (poly A binding protein) closing the mRNA ring. The replacing of the CBPs with eIF4E initiates the bulk round of translation, which directs steady state rounds of mRNA translation and production of an abundance of correct proteins (Figure 2), [43,36,44].

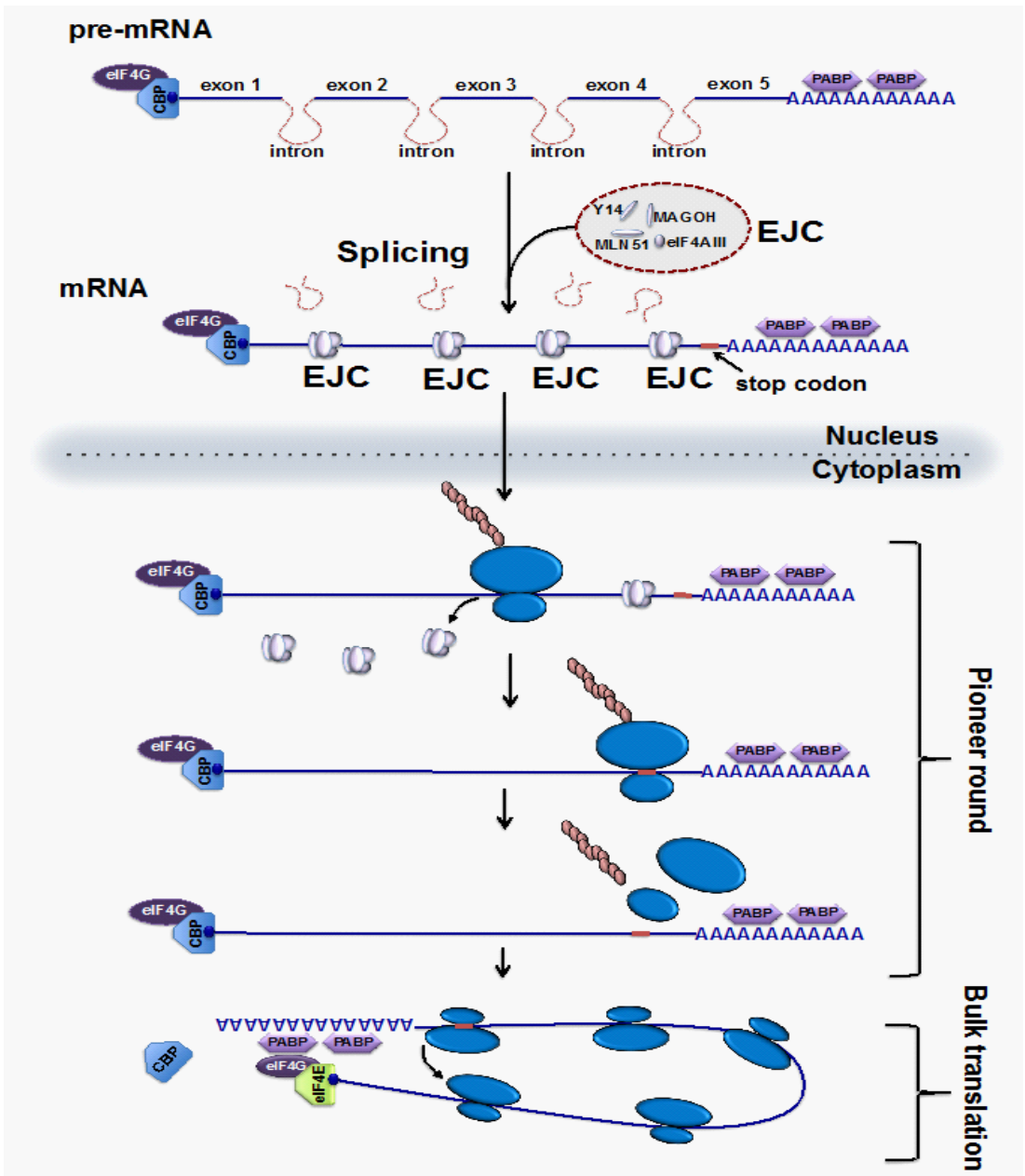


Figure 2: Normal Translation. The Exons are joined during mRNA splicing in the nucleus. Close to the site where the exons are joined, a complex of proteins is deposited composed of Y14, MAGOH, MLN51 and eIF4AIII proteins. This complex is known as the Exon Junction Complex Proteins (EJC). The processed mRNA with Cap Binding Proteins (CBP) complex at the 5' end moves to the cytoplasm to start the pioneer round of translation. The two ribosomal subunits attach to the mRNA to form a ribosome, which then starts reading through and building

the peptide chain. Every time the ribosome reaches an EJC, this complex is dissociated. When the ribosome reaches the stop codon, it stalls and then is released as is the newly synthesized peptide chain. For bulk translation, the CBPs are replaced by eIF4E that is bound to eIF4G, a further component of the translation initiation complex. The poly (A) binding proteins then bind to eIF4G, forming a closed mRNA circle. This facilitates translation initiation, allowing the efficient translation of mRNA. Abbreviations: CBP, cap binding proteins; EJC, exon junction complex; PABP, poly(A)binding protein.

On the other hand in the case of the presence of a PTC, during the pioneer round of translation the ribosomes stall at the PTC, triggering the early molecular events of the NMD process. It is thought to start with the recognition of the NC by the eukaryotic release factors 1 and 3 (eRF1 and eRF3) due to the stalling of the ribosome, which in turn recruits UPF1, another NMD factor. UPF1 binds to the protein kinase SMG-1 and together with eRF1 and eRF3 forms a protein complex known as SURF [45]. The formation of this complex leads to the phosphorylation of UPF1. This phosphorylation is required for binding with to EJC core proteins located downstream of the NC. At this point, UPF1 binds to UPF2, and then UPF2 by its third and last MIF4G domain binds to *UPF3b*. A bridge-like structure is then formed when the bound *UPF3b* attaches to the Y14 part of the EJC, linking UPF1 to the EJC via UPF2 and *UPF3b* [30,36,35,46]. The phosphorylation of UPF1 recruits SMG-5/SMG-7 which in turn recruits PP2A, resulting in UPF1 dephosphorylating and dissociation. All of these reactions trigger subsequent later steps and degradation of the mRNA either by exonucleases or endonuclease activity. The exonucleases degradation starts by the loss of the mRNA 5' cap by the decapping enzyme component DCP2 and dead enylation of the poly (A) tail by deadenylase poly (A) ribonuclease (PARN). Followed by a rapid decay of the doomed mRNA by the 5'-to-3' exonuclease Xrn1 and/or the 3'-to-5' exonuclease complex exosome, which is followed by recycling of the release factors, UPF proteins, and the 40S and 60S ribosomal subunits follows. Endonucleolytic degradation of mRNA involves the binding of the phosphorylated UPF1 to SMG-6, leading to its de-phosphorylation by PP2A recruitment. SMG-6, which is an endonuclease, cleaves the mRNA close to the PTC, resulting in the formation of two fragments. One of those fragments is degraded by Xrn1 and the other by the exosome (Figure 3), [34,47,48].

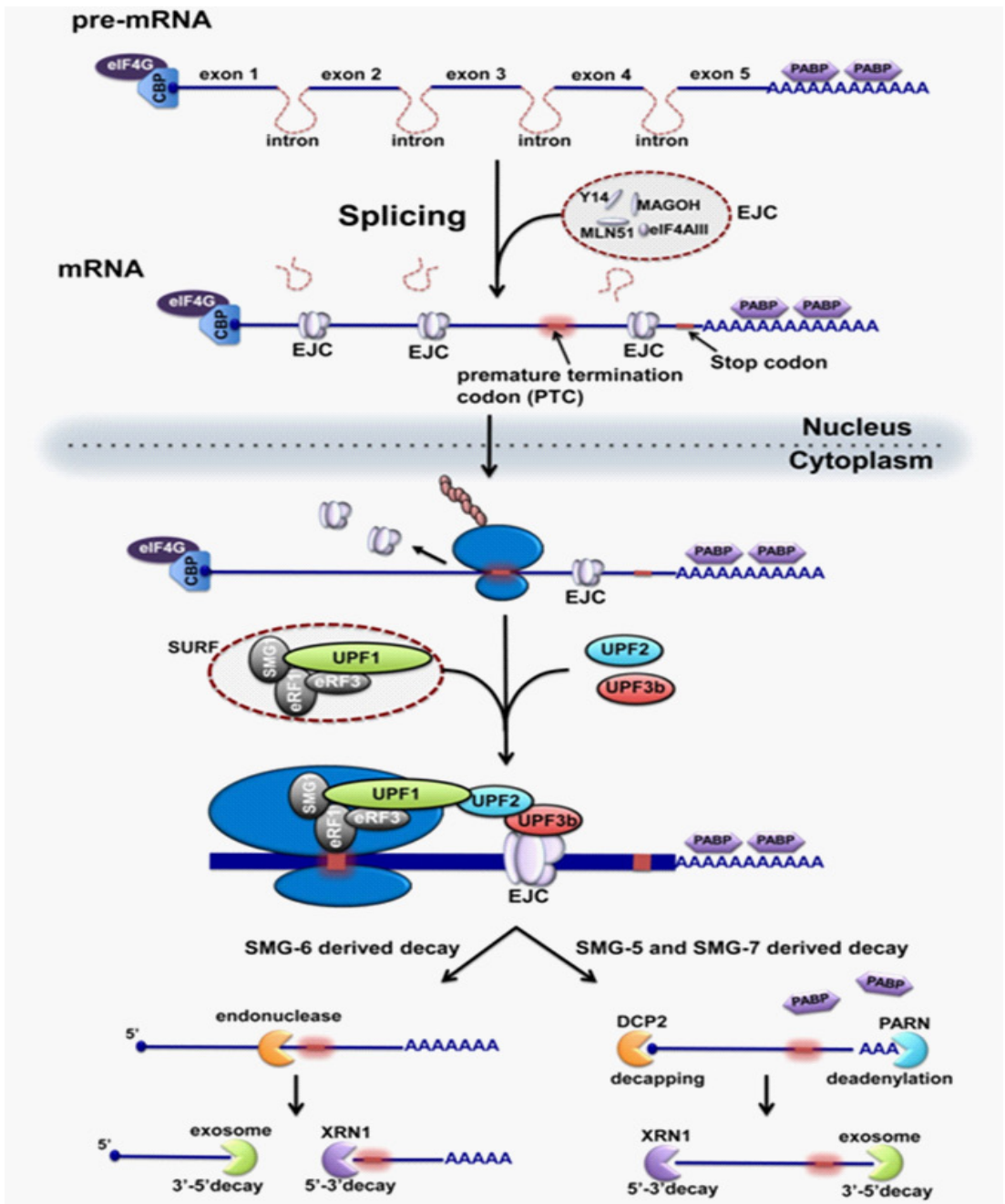


Figure 3: Nonsense Mediated Decay. Exons are joined by removal of introns during mRNA processing in the nucleus. A PTC can be present in the mRNA for any number of reasons. In the cytoplasm during the first round of translation, the ribosome moving along the CBP bound mRNA dissociates every EJC it reaches. When the ribosome reaches the PTC, it stalls and this gives rise to a cascade of events leading to the decay of the aberrant mRNA known as Nonsense

Mediated Decay (**NMD**). The surf complex composed of eRF1, eRF3, UPF1 and SMG-1, a kinase that phosphorylates UPF1, binds close to the PTC on the ribosome by eRF1. UPF1 binds to UPF2, which in turn binds to *UPF3b*. *UPF3b* in its turn binds to the Y14 part of the EJC, forming a bridge like structure linking the ribosome to the EJC. The formation of this structure starts the degradation process of the mRNA, either via the endonuclease SMG-6, or exonucleases in SMG-5 and SMG-7 dependant decay. SMG-6 dephosphorylates UPF1 and binds close to the PTC and cleaves the mRNA. The two resulting fragments are then further degraded by XRN1 in a 5'-3' decay and exosome in a 3'-5' decay. The other decay pathway, exonucleolytic decay, involves SMG-5 and SMG-7 promoting the dephosphorylation of UPF1 by the recruitment of PP2A. UPF1 dephosphorylation leads to hydrolysis and decapping of the mRNA by DCP2 and deadenylation of the poly (A) tail by Poly(A)Ribonuclease (**PARN**). The resulting mRNA is then degraded 5'-3' by the exonuclease XRN1 and from 3'-5' by exosome. Either pathway results in degradation of the doomed mRNA and recycling of the ribosome subunits, the UPF proteins, and the cap proteins. **CBP**: Cap Binding Proteins; **EJC**: Exon Junction Complex; **PABP**: Poly(A)Binding Protein; **ERF1 AND ERF2**: Eukaryotic Release Factor 1 And 3; **PTC**: Premature Termination Codon.

A recent report indicates that NMD is not restricted to the pioneer round of translation, but it also happens in the bulk round of translation. NMD in the bulk round of translation could happen when the pioneer round ends and the bulk round starts while there is still a ribosomal unit stalling at a PTC, and the NMD degradation was not initiated yet. Rufener in 2013 reported that eIF4E bound mRNAs are targeted by nonsense-mediated decay in human cells, due to lagging of the initiation of NMD, and the change between the CBP and eIF4E happens early [43].

There are two different NMD pathways the cause degradation of mRNAs with PTCs, either EJC dependent or EJC independent. The EJC dependent NMD, is what was earlier explained, the presence of a PTC upstream (>30nt) of EJC, triggers NMD due to staling of the ribosomes and the cascade of events that follows. The theory of the presence of another pathway independent of EJC was proposed when it was observed that some mRNAs with PTC after the last EJC still gets degraded. Depletion of a number of NMD factors showed that UPF1 and SMG-1 are essential in both pathways but not SMG-5 or SMG-7. It was concluded from that study that two partially redundant PTC triggered decay pathways in HeLa cells exist, one being SMG-6 dependent and the other is SMG-7 dependent [49].

Additionally, another study in 2013 reported that PNRC2 has the ability to promote the decapping of PTC containing transcripts in a SMG-5 dependent and SMG-7 independent manner, these findings suggests the existence of even a third distinctive RNA degradation route in NMD [50].

It could be concluded that NMD targets aberrant mRNAs and degrades them, regardless at which point in translation the transcript is, either the pioneer or bulk round of translation. *UPF3b* plays an important role in the actual process of recognition and initiation of degradation.

NMD And Neurons

NMD has an important function in the development of the brain as evident by a number of reports. Most important of all the patients who were reported with *UPF3b* mutations, suffered from a type or another of neuro developmental disorder [14,16,15,12,13].

Furthermore, studies on animal embryos have demonstrated that inhibition of one or more of the NMD factors results in embryonic death and sever brain malformations. For example the double knockout of the NMD factors UPF1, UPF2, and Magoh (EJC) in mice resulted in early embryonic death [51,52,53].

Huang in 2011 reported that all factors involved in NMD are NMD targets themselves due to the presence of long 3' UTR in their transcripts. *UPF3b* mutant mice were generated and their organs analyzed for the expression levels of NMD factors, interestingly it showed that they were significantly elevated in the brain and spleen tissues only, indicating that *UPF3b* activity is tissue specific [54].

Depletion of UPF1 in zebra fish leads to aberrant eye and brain patterns and up to 80-85% increase in embryonic death [55]. In *Drosophila*, UPF1 and UPF2 loss of function inhibits cell growth and causes death in a *UPF3*-independent manner [56]. On the other hand, mutations in UPF2, SMG-1 and SMG-6 disrupted the formation of the neuromuscular junctions and blocks neurotransmission [57]. This demonstrates the high importance and the essentiality of NMD pathway for embryonic development and most importantly brain development.

On cellular level, it's been demonstrated that brain development might depend on NMD activity. During in vitro neurogenesis the up-regulation of miR-128 (micro RNA) is required for reduction in UPF1 levels and NMD, which otherwise inhibits the neuro genesis process [58]. Interestingly, one of NMD factors, *UPF3b*, was significantly reduced in RNA extracted from the brain of human embryos from different stages of human embryonic development (Carnegie stage 15, 20 and 25) which points towards a repressed NMD during human brain development [59].

The role of compromised NMD in neurons was also reported in a number of papers. Laumonier in 2010 showed that *UPF3b* is distributed throughout the neurons from the nucleus and cell body reaching the dendritic spines, where all the postsynaptic structures are located [14]. This distribution throughout the neuronal structure gives an indication of the importance of *UPF3b* in NMD and probably other functions in neurons. In another study, it was demonstrated that some of the NMD components are enriched in the axonal growth cones and that NMD acts locally to influence axonal path finding, by regulating the translation of local mRNAs. Mice embryos with knockout of UPF1 or UPF2 genes exhibit axons with aberrant trajectories, meaning axons lose their guidance after crossing out the spinal cord border [60].

Additionally, it was reported that the presence of mutated *UPF3b* in neuronal differentiating stem cells, leads to decreased neuronal branching and growth. This observation is similar

to what was reported in neurons obtained from patients diagnosed with autism and other neurodegenerative disorders [61].

A number of transcripts, whose functions are closely related to neuronal morphology and development, have been identified as natural targets for NMD. RoBo3.2 is one of these transcripts and has a PTC upstream of an EJC due to alternative splicing. RoBo3.2 is important in axonal guidance, but if not degraded by NMD post commissioner crossing will render neurons unable to elongate in the correct direction. It has been reported that NMD target and degrade RoBo3.2 pre-commissioner crossing only, indicating that NMD sometimes work at different locations and different mRNA pools [60]. The arc transcript has two introns in the 3' UTR region, which makes it a natural target for NMD. It has been reported that knockdown of either UPF1 or eIF4AIII, an EJC component, increase the mRNA levels of arc. The increase in arc leads to an increase in neuronal excitatory transmission and GLUR1 receptors at synapses [62]. ARHGAP24 isoform 1 is a further natural NMD target. ARHGAP24 mRNA was found elevated in lymphoblasts of an *UPF3b* deficient patient. Further investigation of ARHGAP24 showed that it's a natural target for NMD because it has both a long 3'UTR and a 5' Upstream Open Reading Frame (**uORF**). Additionally, it showed an inverse mRNA expression to *UPF3b* at different human embryonic stages, suggesting it is regulated by NMD [59]. Most importantly, ectopic over expression of ARHGAP24 isoform 1 in primary hippo campal neurons resulted in a decrease in axonal out growth and the number of dendritic and axonal termini. Also its over expression in PC12 differentiating cells leads to inhibition of neurite outgrowth [59]. Other examples of NMD targets with neuronal functions include GADD45B, which is essential for adult neurogenesis [63,12], and SIX3, which encodes a master regulator of the visual cortex and forebrain development [64,59].

Thus it could be concluded that NMD may act to provide an important regulatory tool for neuronal and synaptic homeostasis in the brain. Altering this may pre-dispose to neuro developmental disorders [65].

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