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Therapeutic strategies for *C9orf72* amyotrophic lateral sclerosis and frontotemporal dementia

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Abstract

Purpose of review: An intronic G_4C_2 expansion mutation in *C9orf72* is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9-ALS/FTD). While there are currently no treatments for this insidious, fatal disease, intense research has led to promising therapeutic strategies, which will be discussed here.

Recent findings: Therapeutic strategies for C9-ALS/FTD have primarily focused on reducing the toxic effects of mutant expansion RNAs or the dipeptide repeat proteins (DPRs). The pathogenic effects of G_4C_2 expansion transcripts have been targeted using approaches aimed at promoting their degradation, inhibiting nuclear export or silencing transcription. Other promising strategies include immunotherapy to reduce the DPRs themselves, reducing RAN translation, removing the repeats using DNA or RNA editing and manipulation of downstream disease-altered stress granule pathways. Finally, understanding the molecular triggers that lead to pheno-conversion may lead to opportunities that can delay symptomatic disease onset.

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Competing interests

GMH is an inventor on patents granted in the USA (US10801027B2) and Europe (EP3430143B1) for the use of inhibitors of SRSF1 to treat neurodegenerative disorders (WO2017207979A1). LPWR is an inventor on patents and pending patents related to repeat associated non-AUG (RAN) translation and the use of metformin and PKR inhibition to treat RAN protein disorders. The authors declare no other relationships, conditions or circumstances that present a potential conflict of interest.

Summary: A large body of evidence implicates RAN-translated DPRs as a main driver of C9-ALS/FTD. Promising therapeutic strategies for this devastating disease are being rapidly developed with several approaches already in or approaching clinical trials.

Keywords

C9orf72-ALS/FTD; therapeutic strategies; gene therapy; symptomatic management; small molecule inhibitors

Introduction

A GGGGCC hexanucleotide repeat expansion in the first intron of *C9ORF72* causes the most common forms of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (1, 2), genetically linking these two clinically distinct adult-onset neurodegenerative disorders. *C9ORF72* ALS, FTD or both ALS and FTD can occur in individual patients and within families (3). C9-ALS/FTD patients typically have hundreds to thousands of G_4C_2•G_2C_4 repeats while shorter tracts of 2–24 repeats are present in unaffected people (1, 2). ALS is characterized by motor neuron loss, muscle atrophy, progressive paralysis and usually death within 2–5 years of onset (4). In FTD, degeneration of neurons in the frontal and anterior temporal lobes can result in personality changes such as apathy, loss of empathy, disinhibition and executive function deficits (4). Therapeutic options are limited and there are no current treatment options that substantially change the course *C9orf72* ALS or FTD. The standard of care includes the anti-glutamatergic drug riluzole for ALS (5) and the anti-depressant fluoxetine or a related compound for FTD (6). In 2017, the free-radical scavenger drug edaravone was approved for use in ALS patients (7). Unfortunately, none of these treatments improve motor or cognitive deficits, however riluzole and edaravone have been shown to modestly slow disease progression in some ALS patients.

Similar to other microsatellite expansion disorders (8, 9), the C9orf72 G_4C_2•G_2C_4 mutation is bi-directionally transcribed and sense G_4C_2 and antisense G_2C_4 expansion transcripts form RNA foci and produce repeat associated non-AUG (RAN) proteins (10–15). Proposed disease mechanisms include: (i) toxic effects of the expansion RNAs (1, 16); (ii) toxic effects of sense (poly-GA, GR, GP) and antisense (poly-PR, PA, GP) dipeptide RAN proteins (10, 12–14) and (iii) haploinsufficiency of the C9ORF72 protein (17, 18). Overall, RAN proteins, particularly GA, GR and PR, have been shown to be toxic in a number of cell culture and animal models (for reviews (19, 20)).

The discovery of the *C9ORF72* expansion as the most common genetic cause of ALS and FTD (1, 2) has fueled an interdisciplinary worldwide research effort to understand the mechanisms and develop therapies for this disorder. *C9ORF72* expansions cause ~40% of familial and 6–8% of sporadic ALS cases and ~18% of familial and 6% of sporadic FTD cases (21, 22*) in European populations but are relatively rare in Asia (23). The relatively large numbers of C9-ALS/FTD patients worldwide, combined with multiple emerging therapeutic strategies have positioned C9-ALS/FTD well for breakthrough therapy development. Emerging therapeutic strategies include: targeting and removing the expanded

repeats; degrading, or preventing expression of expansion transcripts; reducing toxic RAN proteins: and modulating downstream affected pathways including nucleocytoplasmic transport and stress granules (Figure 1). These strategies and additional efforts to understand key molecular and physiological changes that trigger disease may provide insights that will lead to better disease management and help stratify the inclusion of the most informative patients for clinical trials.

Targeting expansion transcripts for degradation

Sense C9ORF72-repeat transcripts.—Almost immediately following the discovery of the C9orf72 expansion mutation efforts to develop antisense oligonucleotide (ASO) drugs to knock-down the repeat expansion RNAs began. These nucleic acid-based drugs are chemically modified and in some applications take advantage of the nuclear RNase H1 pathway to degrade double-stranded sequences that form when ASOs bind to targeted gene transcripts (24**). For *C9ORF72*, specific ASOs were shown to selectively reduce sense G_4C_2 RNA foci in patient cells without reducing the levels of *C9ORF72* mRNA (11, 16). BAC transgenic mice treated with single-dose ASOs that selectively target sense expansion RNAs but not mRNAs encoding C9orf72 protein, decreased sense RNA foci and sense DPRs and improved behavioral abnormalities (25). Together these results paved the way for a phase I clinical trial to test the safety, tolerability, and pharmacokinetics of the Ionis/Biogen BIIB078 ASO in adults with C9ORF72 ALS (NCT03626012).

More recently, stereopure ASOs were shown to increase RNAse H activity *in vitro* and *in vivo* compared to stereorandom ASOs (26). A lead candidate stereopure ASO showed selective degradation of sense G_4C_2 expansion containing transcripts without reducing the variant 2 isoform which lacks the repeat. Treatment of BAC transgenic mice with these ASOs reduced RNA foci and RAN GP proteins but not C9ORF72 protein levels. Additionally, these oligonucleotides selectively protected motor neurons harboring *C9ORF72*-expansion mutations from glutamate-induced toxicity (27**). In an alternative approach, miRNAs targeting sense *C9ORF72*-repeat transcripts using adeno-associated virus serotype 5 (AAV5) delivery, which reduced levels of sense expansion transcripts and RNA foci in FTD iPSC-derived frontal brain-like neurons and BAC transgenic mice (28**). Interestingly, AAV-mediated miRNA-depletion of SOD1 was recently reported in 2 patients with SOD1-ALS (29**), providing proof of concept data that intrathecally delivered microRNAs can be used as a potential treatment strategy for ALS.

Inhibiting transcription of C9-expansion transcripts.—Several different strategies are being actively pursued to decrease the transcription of C9-repeat expansion transcripts. For example, SUPT4H, SUPT5H and RNA polymerase II associated factor 1 complex (PAF1C) are transcription factors that play important roles in the elongation of RNAs containing expanded repeats (30, 31). Decreased expression of SPT4 encoded by *SUPT4H* was shown to decrease the levels of sense and antisense *C9orf72* expansion transcripts and GP-RAN proteins in *C. elegans, Drosophila* and iPSC-derived models of C9ORF72-ALS/FTD and to ameliorate neurodegenerative phenotypes in *Drosophila* (32). Similarly, depletion of PAF1C reduced expression of G_4C_2 expansion RNAs and poly(GR) in a

Drosophila model (31). Although interesting, the therapeutic potential of these strategies may be limited by off-target effects (33*).

In other approaches, CRISPR-Cas9 deletion of the promoter region driving expression of the *C9ORF72* repeat-containing transcript isoforms 1 and 3 led to the efficient reduction in expression levels of all three sense DPRs in C9-ALS patient-derived motor neurons (34*). Pinto et al., showed targeting *C9ORF72* expansions at the DNA level using deactivated Cas9 (dCas9) efficiently inhibits transcription and reduced the levels of GP RAN protein in reporter cells (35). Similarly, use of RNA-targeting deactivated-Cas9 (RCas9) allows degradation of C9ORF72 expansion transcripts in cell models (36), although it is possible that this strategy also blocks transcription by binding of the dCas9-PIN-gRNA to the DNA repeats as in dCas9 strategy.

While there is great hope that decreasing the levels of G_4C_2 expansion transcripts will be sufficient to improve disease, several studies suggest that it will be important to increase efforts to also target antisense transcripts. For example, antisense RNA foci preferentially accumulate in regions of pathology in a *C9ORF72* BAC transgenic mouse model suggesting antisense expansion RNAs or RAN proteins may be more toxic than their corresponding sense products (37). ASOs that target sense C9ORF72 expansion transcripts did not correct widespread transcriptomic defects found in patient-derived cells (11), suggesting strategies targeting both transcripts may offer the best outcomes.

Immunotherapy strategies targeting RAN proteins

There is strong evidence that RAN DPRs, particularly GA, GR and PR, are one of the main drivers of disease (for reviews (19, 20)) and hence decreasing their levels is an attractive therapeutic approach. Several immunotherapy approaches have focused on the GA RAN proteins. In patient fibroblasts and primary neurons, α-GA antibodies reduced GA aggregate formation and blocked aggregate seeding activity of cerebellar extracts from *C9ORF72* autopsy tissue (38). Vaccination of GA-overexpression mice with ovalbumin-$(GA)_{10}$ peptides elicited the production of α-GA antibodies, lowered GA protein levels and prevented microglial activation and motor deficits (39**). Using BAC transgenic mice that express sense and antisense transcripts and multiple types of RAN proteins, Nguyen et al., (40**) showed passive immunotherapy with α-GA antibodies improved behavioral deficits, increased survival and decreased neuroinflammation and motor neuron loss. These peripherally injected antibodies crossed the blood brain barrier and co-localized with GA protein aggregates. Glycosylation of the Fc antibody region was important for cell entry and GA proteins were reduced in a TRIM-21-, and proteosome-, and autophagy-dependent manner (40**). In addition to reducing GA, the α-GA1 treatment surprisingly also reduced GP and GR proteins, likely through increased proteosome function. No changes in sense or antisense RNA levels or foci were observed in α-GA1 treated mice providing strong support that RAN proteins drive C9-ALS/FTD (40**).

Decreasing RAN protein levels

Several groups have shown that activation of the integrated stress response (ISR) and increased p-eIF2α levels increase RAN translation (41-44, 45**). Zu et al. showed

G_4C_2 and other repeat expansion RNAs activate ISR protein kinase R (PKR) and that PKR inhibition dramatically reduces RAN protein levels (45**). Zu et al., went on to show Inhibition of PKR using AAV-delivered dominant negative PKR-K296R or the FDA-approved drug metformin decreased p-PKR and RAN protein levels and improved behavior and neuropathology in C9 BAC transgenic mice (45**) without changing sense or antisense transcript levels. There is an active clinical trial to test safety of metformin in C9ORF72 ALS patients and its effects on RAN protein levels (NCT04220021). Inhibition of the SRSF1-dependent nuclear export of both sense and antisense *C9ORF72*-repeat transcripts and subsequent RAN translation was also reported as a promising gene therapy approach in preclinical models including patient-derived motor neurons and *Drosophila* models of disease (46).

Other strategies to reduce RAN protein levels include stimulating their clearance. For example, heat shock protein family B member 8 (HSPB8) has been shown to promote autophagy-mediated removal of several misfolded C9 RAN proteins from motoneurons (47). Although the therapeutic potential of this approach is uncertain, clearance of protein aggregates could have applications for a wide variety of neurodegenerative diseases. Taken together, these data support the therapeutic potential of targeting RAN translation and RAN proteins for C9-ALS/FTD as well as other RAN protein associated disorders.

Targeting the genomic *C9ORF72* hexanucleotide-repeats

Correcting the GGGGCC•GGCCCC repeat expansion mutation should theoretically address all deleterious mutation effects, including effects from sense and antisense RNAs and DPRs. Current gene editing techniques have focused primarily on clustered regular interspaced short palindromic repeats (CRISPR)-associated (Cas) systems, although application to C9-ALS/FTD has so far been limited (48, 49*). In iPSC-cells CRISPR/Cas9 editing and homology-directed repair (HDR) replacement of the expansion with a wildtype repeat resulted in restoration of *C9ORF72* gene expression and methylation and reduced intron retention and downstream pathogenic phenotypes (49*). In iPSC-derived motor neurons, CRISPR/Cas9 correction abolished GluA1 AMPA receptor (AMPAR) mediated excitotoxicity (48). Targeting regions outside the repeat or the entire *C9ORF72* gene have also been tested with varying degrees of success. For example, deletion of a portion of the upstream *C9ORF72* promoter prevents the production of exon 1a expansion containing transcripts and the activation of neurodegenerative pathways (34*). Unfortunately, this approach does not prevent expression of antisense RNA and associated antisense DPRs, which likely contribute to disease. While correcting the expansion mutation seems to be a straight-forward idea, adequate delivery to affected tissues/cell types and the accuracy of emerging CRISPR based approaches will be critical for effective therapy development.

Stress granules and nucleocytoplasmic transport

TDP43 plays important roles in transcriptional regulation, alternative splicing of pre-mRNAs, axonal transport of mRNAs, translational regulation and miRNA processing. TDP-43 also associates with stress granules (50), which constitute dynamic membrane-less organelles that promote cell survival by halting translation of non-essential mRNAs in response to cellular stress (51). Stress granules are composed of RNA and RNA-

binding proteins with low complexity domains (LCDs) that mediate liquid-liquid phase separation (LLPS). Mutations in the LCDs domains of TDP43, Ataxin-2 and other RNA-binding proteins involved in ALS/FTLD stimulate their self-assemblies leading to the formation of persistent cytoplasmic stress granules leaving aggregated proteins which contribute to disease pathogenesis (52). Interestingly, arginine-rich C9ORF72 DPRs impair stress granules assembly dynamics by undergoing LLPS and further inducing the phase separation of stress granule proteins (53) and promote nucleocytoplasmic transport disruption by stimulating the recruitment of nucleocytoplasmic transport proteins to stress granules (54). TDP-43 proteinopathy, aggregation of stress granule proteins (G3BP1, ataxin-2), nucleocytoplasmic defects, neuronal loss and motor/cognitive deficits were observed in an AAV-driven overexpression mouse model of expanded G_4C_2 repeats (55). DPRs also promote nucleocytoplasmic transport disruption by stimulating the recruitment of nucleocytoplasmic transport proteins to stress granules (56). In fact, many nucleocytoplasmic transport factors are localized to stress granules when exposed to stressors or mutant proteins implicated in ALS pathogenesis, leading to impaired nucleocytoplasmic transport (56).

Targeting nucleocytoplasmic transport deficits

In 2015, Zhang et al. demonstrated increased nuclear export in *C9orf72* ALS iPSN model shown as abnormal cytoplasmic RanGTPase accumulation (57). RanGTPase is important in nucleocytoplasmic protein transport (reviewed in (58)). Abnormal expression and localization of nuclear pore proteins found in C9ORF72 autopsy tissue and patient-derived iPSNs (57, 59). Modulating the expression of nuclear pore proteins or transport associated proteins affects G_4C_2 expansion transcripts and arginine containing RAN protein toxicity (57, 60, 61). Overexpression of importin or inhibition of nuclear export with RNA inhibition of (Exportin 1) XPO1 or pharmacologically ablating XPO1 function using KPT-276 rescued *C9orf72* toxicity in the *C9orf72* fly model (57). Another XPO1 inhibitor, KPT-350, designed by Karyopharm Therapeutics and acquired by Biogen (BIIB100) has been used in pre-clinical studies of many neurological diseases and demonstrated neuroprotective and anti-inflammatory roles (62-64*, 65).

Trophic support supplementation

Neurotrophic factors (NTFs), a family of biomolecules that support neuronal growth, survival and differentiation, have been explored for decades as therapeutic strategy for neurodegenerative diseases (31428042), including ALS. Various neurotrophic factors have been tested in preclinical rodent models of SOD1-ALS, including Brain-derived Neurotrophic Factor (BDNF), Insulin-like Growth Factor 1 (IGF-1) and Vascular Endothelial Growth Factor (VEGF). Small molecule agonist of the BDNF receptor (66), VEGF injections (67, 68), lentiviral and AAV-mediated delivery of NTFs (69–72) and stem cell therapy of NTF secreting cells (73–81) have shown promise in SOD ALS models. Despite these promising results, including several ongoing clinical trials (82*), there has been little to no direct studies looking at NTF therapeutics in C9-ALS/FTD. While some NTF clinical trials in ALS likely include C9-ALS/FTD participants it is important, given the unique disease mechanisms of C9-ALS/FTD, to directly examine NTF specifically in the C9 context.

Prevention and functional management

For C9-ALS/FTD there is a relatively long-period of apparent good health prior to disease onset, often in the fourth or fifth decade of life and extending this period of good health has been gaining considerable attention. Both preclinical animal studies and human studies demonstrate that moderate exercise regimens improve functionality and ameliorate disease symptoms for ALS in general (reviewed in (83)). However, the role of exercise is complex. In a retrospective study, patient-reported exercise history was inversely correlated with age-of-onset in C9orf72 but not other forms of ALS (84), although additional studies that examine the impact of specific types of exercise will be important. Targeted training may be key. In a randomized, sham-controlled clinical trial, Plowman et al. showed, expiratory muscle-strength training is well tolerated in ALS patients and improved bulbar function in a longitudinal C9orf72 case-study and also in larger cohorts of genetically undefined ALS patients (85, 86**). Longitudinal studies suggest that lifestyle modifications (e.g. smoking cessation, maintaining a healthy body-mass index) at a younger age may lower the risk of developing ALS (87**). Recent studies have identified C9ORF72 expansions in1.6% (n = 8/487) of cases with possible idiopathic normal pressure hydrocephalus (iNPH) but no controls (n = 0/432) >65 years. Clinically significant shunt response was detected in 6 out of 7 shunted C9ORF72 expansion carriers. Additional studies are needed to understand the frequency of NPH in C9ORF72 expansion carriers and the potential utility of shunts to drain excess cerebrospinal fluid in these patients. Dietary studies specific to C9-ALS/FTD are rare, although a larger study focused on ALS in general has demonstrated that increasing fruit and vegetable associated fiber, antioxidants and carotenes was associated with improved function (88). The results of many of these broader ALS studies are complicated by the complex genetics and phenotypic presentation of ALS, increasing the call for preventative and lifestyle studies that focus on C9orf72 or other single ALS mutations.

Stratification and efficiency in clinical trials

While there are multiple therapeutic approaches for C9-ALS/FTD in the pre-clinical pipeline, there are only two C9orf72-ALS/FTD specific clinical trials registered in ClinicalTrials.gov: (1) an antisense oligonucleotide (ASO)-based clinical trial of BIIB078 (Biogen), which targets C9orf72 expansion containing transcripts for ASO-induced RNase H-mediated degradation (NCT03626012) and; (2) a clinical trial (NCT04220021), to test the safety and tolerability of metformin in C9orf72 ALS patients and the drug's ability to decrease RAN protein levels. Several additional trials are open to but not specific to C9-ALS/FTD patients. These include a phase 1 clinical trial of BIIB100 to reduce excessive nuclear export (NCT03945279) in C9 and other ALS patients, to assess safety, tolerability, pharmacokinetics, and pharmacodynamics. A phase 2 clinical trial examining the safety, tolerability, PK and PD of AL001, a recombinant human anti-human sortilin (SORT1) monoclonal IgG1 in FTD patients with either granulin or C9orf72 mutations (NCT03987295). Sortilin is a type I membrane glycoprotein involved in proganulin trafficking that is expressed in the central nervous system (89). Frontotemporal degeneration can be caused by mutation in the progranulin (GRN) gene or the C9orf72 hexanucleotide expansion repeat and there are rare patients with mutations in both (90). More recently, Wave Life Sciences has been reported to seek regulatory approval for WVE-004, an

investigational stereopure ASO targeting the expansion transcript of C9-ALS/FTD (27**). It is interesting to note the current batch of clinical trials, which focus on different pathogenic pathways, could potentially be used together.

Conclusions

Despite the mechanistic and clinical complexity of *C9ORF72* ALS/FTD, intense research efforts over the 10 years since the expansion mutation was identified have led to a remarkable number of novel therapeutic approaches in pre-clinical and clinical trial stages. The breadth and diversity of these approaches provide hope for C9-ALS/FTD patients, who currently have limited therapeutic options focused on supportive care. The pace of research focused on the root causes of this disease has been remarkable and is likely to accelerate and uncover additional new therapeutic targets and treatment strategies that will significantly impact C9-ALS/FTD and the larger family of repeat expansion disorders.

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Summary

- Hexanucleotide repeat expansions in *C9ORF72* gene are the most common known genetic cause of ALS and FTD, a spectrum of debilitating and incurable neurodegenerative diseases.
- Repeat associated non-AUG (RAN) translation which leads to the production of dipeptide repeat proteins is a major driver of neuronal injury and disease.
- A number of therapeutic strategies aimed at reducing the impact of expansion transcripts, RAN proteins, nuclear transport deficits or stress granule biology have shown efficacy in preclinical models of disease.
- Approaches aimed at modifying environmental factors and lifestyle are promising complementary avenues for improving the primary care of patients.
- Intense research efforts have resulted in several clinical trials and others are expected to start soon.

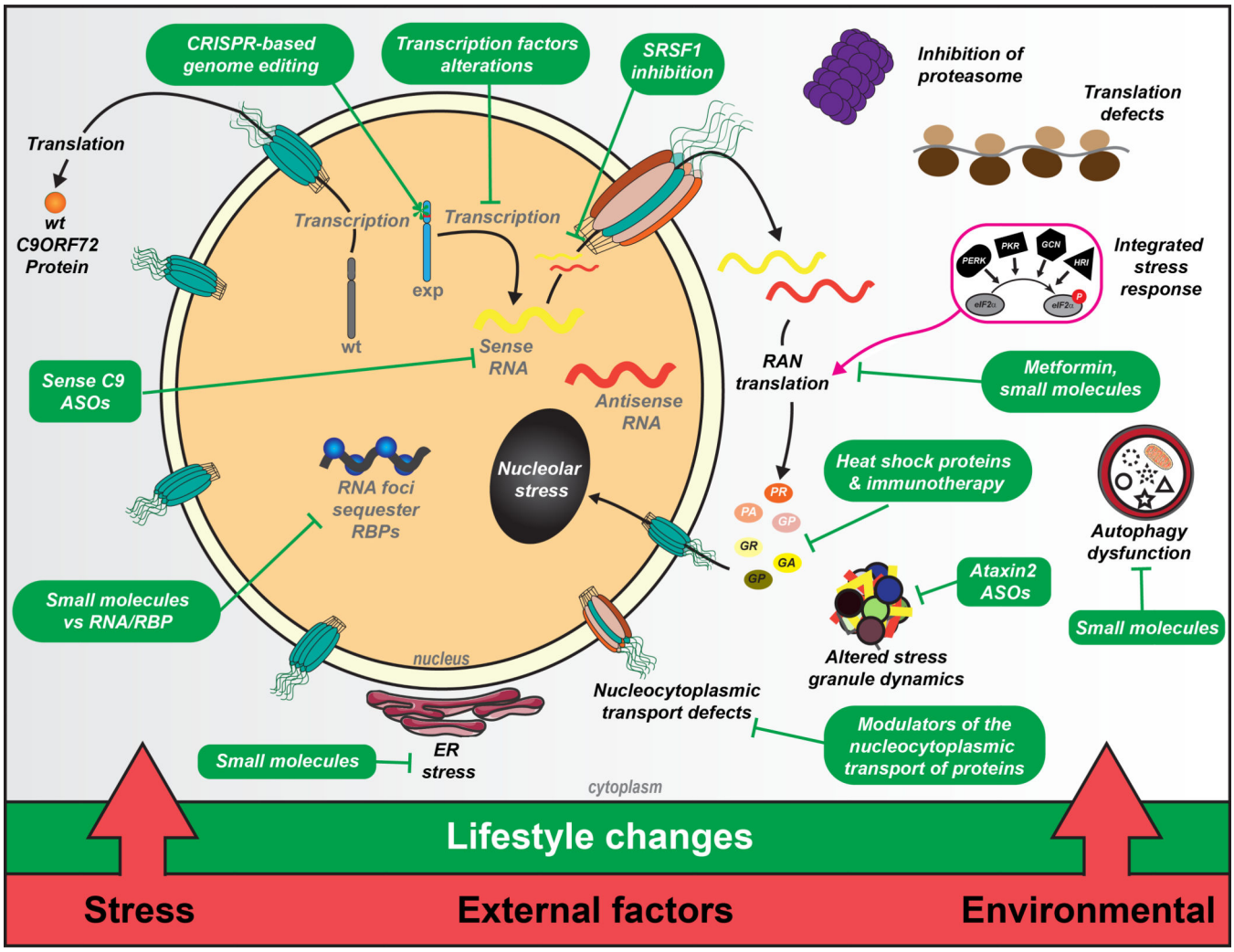

Figure. Cellular consequences and therapeutic approaches for C9orf72-ALS/FTD

Expression of sense and antisense expanded C9 transcript RNA and dipeptide repeat (DPR) proteins affect a wide array of downstream cellular pathways including ER stress, nucleocytoplasmic defects, altered stress granule dynamics, autophagy dysfunction, translational defects, proteasome inhibition, RAN translation, nucleolar stress, RNA foci formation. Different therapeutic approaches (green boxes) target the C9 RNAs and RAN DPRs directly as well as the downstream pathways. Additionally, external factors, such as patient lifestyle, stress, comorbid diseases and environmental factors (bottom red bar) can influence cellular events with exercise and lifestyle changes offering mixed therapeutic potential. Some content modified from Servier Medical Art (smart.servier.com) under creative common license.