RAN translation of C9ORF72-related dipeptide repeat proteins recapitulates hallmarks of motor neurone disease and identifies hypothermia as a therapeutic strategy in zebrafish

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ABSTRACT

Objective: Hexanucleotide repeat expansions in the C9orf72 gene are the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). A large body of evidence implicates DPRs as one of the main drivers of neuronal injury in cell and animal models.

Methods: A pure RAN translation zebrafish model of C9orf72-ALS was generated. Embryonic and adult transgenic zebrafish lysates were investigated for the presence of RAN-translated DPR species and adult-onset motor deficits. Using C9orf72 cell models as well as embryonic C9orf72-ALS zebrafish, hypothermic-therapeutic temperature management (TTM) was explored as a potential therapeutic option for C9orf72-ALS.

Results: Here we describe a pure RAN translation zebrafish model of C9orf72-ALS that exhibits significant RAN-translated DPR pathology and progressive motor decline. We further demonstrate that hypothermic-TTM results in a profound reduction in DPR species in C9orf72-ALS cell models as well as embryonic C9orf72-ALS zebrafish.

Interpretation: The transgenic model detailed in this paper provides a medium throughput *in vivo* research tool to further investigate the role of RAN-translation in C9orf72-ALS and further understand the mechanisms that underpin neuroprotective strategies. Hypothermic-TTM presents a viable therapeutic avenue to explore in the context of C9orf72-ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterised by the progressive degeneration of both upper motor neurons in the motor cortex and lower motor neurons in the spinal cord. Disease onset begins with skeletal muscle degeneration and progresses to eventual respiratory failure and death within 2-4 years of symptom onset[1]. The majority of ALS cases are sporadic (sALS), which account for approximately 90-95% of cases. The remaining 5-10% of cases have familial history (fALS) and a strong inherited link[2]. The *C9orf72* gene is the most common identified gene associated with ALS pathogenesis. Following a landmark paper published in 2011, it was identified that hexanucleotide (GGGGCC)n repeat expansions, located in the first intron, between exons 1a and 1b of the *C9orf72* gene, is an important genetic cause of ALS that accounts for approximately 40% of fALS cases[3]. Although neurologically healthy people have ≤11 hexanucleotide repeats in the *C9orf72* gene, larger expansions of hundreds to thousands are observed in *C9orf72*-ALS patients[4]. The pathological pathways associated with *C9orf72*-ALS involve both loss-of-function and gain-of-function mechanisms. These have been broadly categorised into three non-exclusive pathogenic mechanisms and include (1) loss of function of the C9orf72 protein due to decreased transcription of the *C9orf72* coding region, (2) toxic gain of function from sense and antisense repeat RNA sequestering or altering RNA-binding protein function and (3) the production of toxic dipeptide repeat (DPR) proteins via repeat associated non-AUG (RAN) translation (for review please see[5]).

The development of novel animal models of *C9orf72*-ALS has given pre-clinical researchers the capacity to interrogate and gain further insight into the complex pathological mechanisms that contribute towards ALS pathogenesis and progression. In recent years, zebrafish (*Danio rerio*) have emerged as a useful pre-clinical tool to study gene function in the context of human disease, including *C9orf72*-ALS[6,7]. Zebrafish models offer a low-cost and higher throughput alternative when compared

to traditional rodent models and their rapid external development, translucent embryos and amenability to genetic manipulation facilitate the opportunity for high- to medium-throughput therapeutic screening. The zebrafish genome has been sequenced and zebrafish have 70% homology with human genes and over 80% of human disease-associated genes have a zebrafish gene counterpart[8], highlighting their utility as a pre-clinical model system for further understanding vertebrate gene function in health and disease.

Hypothermic-therapeutic temperature management (TTM), or therapeutic hypothermia, has been identified as an alternative, non-invasive therapeutic avenue for neurodegenerative disease. A protective role of hypothermic-TTM has been reported in a number of neurological and cardiovascular diseases, including stroke, traumatic brain injury (TBI), hypoxic-ischaemic encephalopathy and cardiac arrest[9]. Through these studies, clinical treatment protocols have been refined and the mechanisms that underpin this neuroprotection have been explored. Although these pathways have not yet been fully elucidated, profound effects on cellular metabolism, production of cold-shock proteins/hormones[10], free radical generation[11], synaptic plasticity[12] and BBB disruption[13] have all been identified as possible contributory mechanisms. The potential therapeutic effects of hypothermic-TTM has not yet been explored in the context of *C9orf72*-ALS.

This paper aims to build on this work by exploring hypothermic-TTM in the context of C9orf72-ALS by utilising cell and a novel zebrafish model of *C9orf72*-ALS. We first describe a novel pure RAN translation zebrafish model of *C9orf72*-ALS that exhibit significant RAN-translated DPR pathology and progressive motor decline, recapitulating clinical tissue pathology and clinical phenotypes. Further, we build on this previous work by demonstrating a profound reduction in DPR species following the implementation of hypothermic-TTM in C9orf72 cell models as well as embryonic *C9orf72*-ALS zebrafish.

MATERIALS AND METHODS

Transgene constructs

The pure repeats with no ATG start sites were cloned into an identical vector that was used to create our stable ATG-driven DPR transgenic zebrafish lines, as previously described[7]. Embryos were injected with a DNA construct containing either 45 repeats of the C9orf72 sense hexanucleotide GGGGCC (G4C2), 39 repeats of the antisense hexanucleotide CCCCGG (C4G2) or 2 repeats of the sense hexanucleotide repeat (2mer control), driven by a hsp70 promotor. As RAN translation uses codons that mimic the ATG start codon (CTG/GTG), we examined the potential start sites in the transgene that are upstream of the repeats in all three frames for both sense (G4C2, 45 repeats) and antisense (C4G2, 39 repeats) transgenes. Potential GTG start sites for the sense construct are located 12 nucleotides (nt), 17nt and none in +1, +2 and +3 frames. These produce poly(GA) (10.83kDa), poly(GP) (13.7 kDa) and potentially no poly(GR) peptides. Potential GTG start sites for the antisense construct are located 10nt, none and 5nt in +1, +2 and +3 frames. These produce poly(PA) (13.25kDa), poly(PR) and poly(GP) (10.94kDa) peptides. Lastly, a V5 epitope was included in all three frames following the repeat, so that RAN translation can be tracked in all three frames. The construct design is outlined in Figure 1. Creation and identification of transgenic zebrafish was performed as previously described[7] and maintained using established practices[14].

Western blot

Whole zebrafish embryos were processed for western blotting following terminal anaesthesia using MS-222 (Sigma). Laemmli buffer was added to whole anaesthetised zebrafish embryos in the ratio of 8 µl per embryo, homogenised using the Precellys 24 (Bertin Instruments) tissue homogeniser (2500 rpm, 2x20 secs) and then centrifuged at 13,000 g for 10 mins. Supernatant was collected and boiled at 95°C for 10 min prior to SDS-PAGE and immunoblotting

Commercial mouse primary antibodies used were α-V5 (Sigma, R960), α-tubulin (Abcam, ab6046), as well as the rabbit primary antibodies α-DsRed (Clontech, 632-496), α-CIRBP (Proteintech, 10209-2-AP) and α-RBM3 (Proteintech, 14363-1-AP) . All other DPR-specific rabbit antibodies targeting poly(GP), poly(GR) and poly(PR) were custom-generated (Eurogentec) and purified in-house using affinity membrane chromatography. Species-specific HRP-conjugated secondary antibodies were used and immunoblots imaged by chemiluminescence using LiCor Odyssey® Fc imaging system.

Swim-tunnel analysis

Zebrafish swimming ability was tested using a swim tunnel with an initial flow-rate of 2 L/min, increasing in 2 L/min increments every 5 min until the maximum flow rate of 11.6 L/min was achieved. Data were analysed as previously described[7]. 5 min post-testing, the spontaneous swimming behaviour of the fish was measured for 30 minutes using a camera linked to ZebraLab software (ViewPoint Behaviour Technologies). Speed thresholds used were slow ($x<60$ mm/sec), intermediate ($60<x<120$ mm/sec) and fast ($x>120$ mm/sec).

Cell culture and transfection

Cells were maintained in a 37°C incubator with 5% CO_2. HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (Sigma), supplemented with 10% foetal bovine serum (FBS) (Gibco) and 5 U ml^{-1} Penicillin-streptomycin (Lonza). Neuro-2a (N2A) (ATCC) cells were cultured in Dulbecco's Modified Eagle Medium (Sigma), supplemented with 10% FBS (Gibco), 5 U ml^{-1} Penicillin-streptomycin (Lonza) and 5 mM sodium pyruvate. HEK293T, HeLa and N2A cells were transfected with 700 ng of plasmid using 3.5 µg PEI/ml media and one tenth media volume of OptiMEM in a 24 well format. Approximately, 50,000 HEK293T cells, 50,000 HeLa cells and 75,000 N2A cells were seeded per well of the 24 well plate.

Hypothermic treatment

In vitro

24 h post-transfection, cells were washed and complete media replaced. Plates were incubated for 48 h at 37°C (normothermia) or 32°C (hypothermia) before proteins were extracted 48 h post-treatment. Cells were washed in ice cold phosphate buffered saline (PBS) and subsequently lysed in ice cold lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail (Sigma)) for 10 min on ice. Extracts were then centrifuged at 17,000 g for 5 min at 4°C. Extracts were quantified using Bradford Reagent (BioRAD), resolved by SDS-PAGE, electroblotted onto nitrocellulose membrane and probed with the relevant primary antibodies.

Zebrafish

Embryos were manually dechorionated and transgenic positive fish identified by DsRed expression at 48 h. Only normally developed and healthy appearing embryos, with expected fluorescence patterns, were selected for screening. Embryos were incubated at 28°C (normothermia) or 22°C (hypothermia) at 2 days post-fertilisation until 5 dpf. The zebrafish larvae were then terminally anaesthetised using MS-222 (Sigma) and homogenised in the Precellys 24 Tissue Homogeniser in 1X RIPA solution (Merck-Millipore) before being centrifuged at 17 000 g for 15 min. Supernatant was collected for downstream analysis.

MSD poly(GP) immunoassay

A poly(GP) Meso Scale Discovery (MSD®) enzyme-linked immunosorbent assay (ELISA) was established using a custom made rabbit α-GP antibody (Eurogentec) and based on previously described methods[16]. Briefly, 30 µl of poly(GP) antibody (1 µg ml^{-1}) diluted in 1X tris-buffer saline (TBS) was added per well of a 96-well SECTOR plate (MSD) and incubated overnight at 4°C. Plates

were washed three times in 1X TBS-Tween®-20 (0.1%) and blocked for 1 h shaking in 50 µl 3% milk-TBS-T solution (RT, 700 rpm). Plates were washed again in TBS-T and 50 µl $GP_{(7)}$ standard or zebrafish lysate was added per well and incubated for 2 h shaking (RT, 700 rpm). Plates were washed again in TBS-T and 50 µl MSD® SULFO-TAG labelled streptavidin (1 µg ml^{-1}) and biotinylated poly(GP) antibody (1 µg ml^{-1}, Eurogentec) added per well diluted in blocking solution. Wells were washed again, 150 µl MSD® read buffer-T added and plates imaged using the MESO SECTOR 2400 imager.

Quantification and statistical analysis

Data were analysed by one-way ANOVA with Tukey's post hoc test or two-way ANOVA with Sidak's post hoc test for multiple comparisons, t-test or Kaplan Meier analysis as indicated in the appropriate figure legend. Significance is denoted as * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$.

RESULTS

RAN translation and stress response in 5 dpf control, sense and antisense transgenic zebrafish

Five DPR species are generated in C9orf72-mediated disease and have been shown to be toxic in a number of CNS *in vitro* and *in vivo* models[17]. Therefore, our first aim was to characterise the DPR species produced in each of the generated transgenic zebrafish lines. Western blot analysis of 5 dpf zebrafish embryo lysates was performed to determine the presence of RAN-translated DPR species in founder transgenic control (2mer, A1-3), sense (G4C2, B1-5) and antisense (C4G2, C1-4) zebrafish lines. Western blot analysis with antibodies against the V5 epitope, which tags all translated DPR species, showed varying levels of RAN-mediated V5 expression between the generated lines (Figure 2A). These ranged from high, moderate to low expression levels. No V5 expression was detected in any of the three transgenic founder 2mer control lines A1-3 generated.

V5 expression does not allow us to identify the composition of the specific DPR species produced in each line. Therefore, generated sense and antisense lines that showed high V5-DPR expression were chosen for further characterisation of specific DPR species. Poly(GP) expression was common in all sense and antisense transgenic lines tested (Figure 2B-C). Interestingly, poly(GR) appears to be expressed in sense lines B1-2 as expected, but also in antisense line C1 tested (Figure 2B), indicating potential bidirectional transcription in this antisense line. Furthermore, multiple, higher molecular weight bands are predominant in the antisense lines, indicating that ribosomal slippage is more frequent in the antisense transgenic lines. Poly(PR) was highly expressed in antisense lines C2 tested and to a lesser extent antisense line C1 (Figure 2C).

Embryonic and adult C9orf72 zebrafish express multiple RAN-translated DPR species

Following this initial screening of generated lines, chosen lines were renamed and will be referred to as G4C2-1 (sense), C4G2-2 (antisense) and 2mer-3 (control). These were chosen for further analysis due to high expression of DPR species that correlated with the DsRed stress response.

V5 immunostaining demonstrated the production of multiple V5-tagged DPR species at different molecular weights in both G4C2-1 sense and C4G2-2 antisense lines, with no detectable V5-DPR species detected in the 2mer-3 control line (Figure 3A-B). The sense line expresses poly(GP) DPRs as well as poly(GR) DPRs, albeit to a lesser extent (Figure 3A). Compared to the sense line, the C4G2-2 antisense line exhibited very strong poly(GP) and poly(PR) expression (Figure 3B).

The presence of DPR species in embryonic 5 dpf embryos is a valuable tool for studying the mechanisms of DPR pathology and for screening tool compounds that may modulate this deleterious pathway. This said, C9orf72-mediated toxicity mainly occurs within the CNS and muscle tissue of clinical ALS patients. Therefore, the next aim was to determine and characterise the presence of DPR species in adult zebrafish muscle and brain tissue. V5-tagged DPR species were detected in both adult sense and antisense zebrafish lines in both the brain and muscle tissue (Figure 3C-D). DPRs were observed at a higher molecular weight in the antisense line when compared to the sense line in both brain and muscle tissue.

In summary, sense line G4C2-1 and antisense line C4G2-2 produce multiple DPR species at 5 dpf and 12 months. Western blot analysis evidenced ribosomal slippage and frameshifting may be producing multiple DPR species above the predicted molecular weight. The locality of DPR expression replicates that seen in human C9orf72-ALS patients and therefore further evidences the utility of this model as a screening tool for modulating DPR production in embryonic and adult zebrafish.

Swimming endurance

Swimming endurance was tested to evaluate the neuromuscular integrity of adult transgenic zebrafish. Swim tunnel performance analysis showed that at both 6- and 12-month timepoints, swim tunnel performance was decreased in both sense and antisense zebrafish, when compared to the transgenic 2mer control line (Figure 4A-B). Quantification of endurance deterioration showed that swim tunnel performance significantly worsened between 6 and 12 months in both sense and antisense fish. This worsening was not observed in the 2mer control (Figure 4C-E). In summary, deficits in swim tunnel performance are seen as early as 6-months-old and this loss of swimming endurance worsens up to the 12-month-old time point tested. The presence of adult-onset swimming endurance deficits provides an important phenotypic parameter to measure disease progression.

Hypothermic cooling reduces DPR expression in C9orf72 in vitro models

To build on this work, hypothermic conditioning and subsequent induction of the cold shock response was investigated as a mechanism to specifically reduce RAN-translated DPR production. To test this, HEK293T, N2A cells and HeLa cells were transfected with the same C9orf72 expansion construct used for generating the C9orf72-ALS transgenic zebrafish lines. In all cell models, western blot analysis shows that transfected cells show significant RAN-translated V5-DPR production in both sense and antisense cell lysates. No detectable V5-DPRs were observed in each of the controls. Hypothermic cooling to 32°C significantly reduced V5-DPR expression in all cell models in both sense and antisense cell models when compared to normothermic (37°C) conditions (Figure 5A-D). Furthermore, immunostaining for CIRBP (cold-inducible RNA-binding protein) and cold shock protein RBM3 (RNA-binding motif 3) were both significantly increased in hypothermia-treated cells, indicating activation of the cold-shock response in all cell models (Figure 5E-G). Taken together, hypothermic

cooling reduces RAN-translated V5-DPR expression in three separate *in vitro* models which is accompanied by a significant increase in the cold shock response.

Hypothermic-TTM reduces DPR expression in embryonic C9orf72 zebrafish

To build on this, we further applied this hypothermic cooling protocol to embryonic C9orf72 zebrafish to assess *in vivo* efficacy. Embryos were placed in normothermic (28°C) and hypothermic (22°C) conditions from 2 to 5 dpf. Embryonic lysates analysed by western blot showed a significant decrease in V5-DPR expression in hypothermic sense (G4C2-1) (normothermia 100% ± 29.83 v hypothermia 18.6% ± 13.93, ***P = 0.0006, Figure 6A-B). Poly(GP) expression in hypothermic antisense (C4G2-2) embryos was also significantly decreased when compared to normothermic control embryos (normothermia 100% ± 33.54 v hypothermia 58.58% ± 23.16, *P = 0.0320, Figure 6E-F)).

Embryonic lysates were further analysed for poly(GP) expression using the MesoScale Discovery (MSD) ELISA platform, which uses electrochemiluminescence (ECL) as a detection technique. Similar to the western blot analysis, MSD ELISA analysis showed a significant reduction in poly(GP) DPR species following hypothermic cooling when compared to their normothermic counterparts. This effect was seen in both sense (G4C2-1) embryos (hypothermia 17.7% ± 1.32, ****P < 0.0001, Figure 6C-D)) and antisense (C4G2-2) embryos (hypothermia 53.1% ± 7.00, ***P = 0.0003, Figure 6G-H)). Taken together, this further shows the strong effect of hypothermic cooling on RAN-translation and subsequent DPR production.

DISCUSSION

We describe a novel transgenic zebrafish RAN-translation model of *C9orf72*-ALS that expresses multiple RAN-translated DPR species in both embryonic and adult transgenic zebrafish. This recapitulates a major pathological cellular hallmark as well as the marked motor decline that is observed in C9orf72-ALS patients. Taken together, this model offers a novel pre-clinical research tool to further illuminate the pathological mechanisms of RAN-translation in the context of *C9orf72*-ALS, facilitating the screening and development of novel therapeutic approaches. We highlight this utility by demonstrating that reducing environmental temperature through hypothermic-TTM reduces RAN-translated DPR species in both RAN-translation C9orf72-ALS cell models as well as the RAN-translation C9orf72-ALS zebrafish models. This is the first study to demonstrate hypothermic-TTM as a viable therapeutic avenue to explore in *C9orf72*-ALS.

Multiple RAN-translated DPR species can be detected in sense and antisense zebrafish embryos at 5 dpf. Incorporation of a V5 epitope tag in all three reading frames following the repeat expansion further facilitates higher throughput for screening changes in global RAN-translation in a more complex organism than cellular models. This pure repeat uninterrupted model closely recapitulates the hexanucleotide repeat expansion RAN-translation mechanisms that are observed in *C9orf72*-ALS patients[18]. This zebrafish model will further our understanding of how RAN-translation and the production of toxic DPR species may precipitate and/or exacerbate ALS progression and therefore how these mechanisms may be targeted for therapeutic intervention.

Zebrafish models of ALS

Zebrafish have emerged as a major pre-clinical research tool in recent years, particularly in neuroscience research studying complex brain disorders[19]. Zebrafish share 70% gene homology with humans and the structure of the zebrafish CNS has all the major mammalian brain domains and many

of the same neurotransmitters[20]. Furthermore, the zebrafish motor system is largely similar to that of humans and many of the genes that are associated with ALS risk and development are found in zebrafish, including C9orf72[21].

A number of transient and stable ALS zebrafish models have been developed to recapitulate aspects of the cellular and behavioural phenotypes that are observed in clinical patients. A transient transgenic zebrafish model expressing 32 and 72 sense (GGGGCC) repeats exhibits increased apoptotic markers and DPR expression at 24 hpf when compared to transgenic embryos expressing 8 repeats, where these markers were absent. This highlights the requirement of a minimum repeat number threshold to produce DPR species and induce cell death[22]. Other studies investigating motor performance and repeat expansion length found expansions with 10 or less repeats exhibit no motor-axon defects or loss of motor performance, whereas transgenic lines containing 37, 70 or 90 repeats exhibited significant motor-axon abnormalities[23]. This data is supported by our previous paper detailing significant motor decline and eventual early mortality in transgenic ALS zebrafish stably expressing 89 hexanucleotide repeats[7]. In this study, we observe no evident expression of RAN-translated DPR species or decline in motor performance in the transgenic control line expressing 2 sense repeats (2mer-3). Expression of either 45 sense G4C2 (line G4C2-1) or 39 antisense C4G2 (C4G2-2) repeats results in the expression of multiple DPR species as well as progressive motor performance decline, supporting this previously published research. Taken together, our data as well as previously published literature suggest that the loss of motor performance described is not an artefact of gene manipulation and is due to the production of toxic RAN-translated DPR species.

Hypothermic-TTM – cellular mechanisms

Targeted temperature management (TTM), previously termed therapeutic hypothermia, is the intervention of core body temperature to prevent fever, induce hypothermia or maintain

normothermia. The neuroprotective effects of hypothermia have been known since the ancient world and these have been further explored in a number of pre-clinical and clinical studies targeting neurodegenerative disease. Through these studies, treatment protocols have been optimised and the mechanisms that underpin this neuroprotection have been interrogated. Although these mechanisms have not been fully elucidated, a number of neuroprotective pathways have been reported.

One of the first cold-shock proteins identified was RNA-binding motif protein 3 (RBM3)[24]. Following Hypothermic-TTM, RBM3 expression is increased through BDNF-TrkB signalling. This inhibits the action of phosphorylated ERK branch activation and is an important pathway in neuronal structural plasticity[25]. Furthermore, RBM3 expression localises to dendrites and increases local dendritic protein synthesis[26] as well as global protein synthesis[27] through binding of ribosomal subunits, specifically RTN3. RTN3 is involved in synaptic formation and neuronal plasticity[28]. *In vitro* studies have shown that RBM3 is substantially responsible for the neuroprotective effects of hypothermic-TTM. Using neuron-like PC12 cells, Chip et al. showed that blocking RBM3 through specific siRNAs abolished the neuroprotective effects of cooling to 32°C. Furthermore, RBM3 overexpression reduced apoptotic markers in the absence of hypothermia[29]. The role of RBM3 has been further explored in animal models of neurodegeneration. Hypothermic-TTM significantly improves neurological outcomes in mouse models of Alzheimer's disease and prion disease, however this neuroprotective capacity is reduced following lentiviral-mediated knockdown of RBM3. Furthermore, lentiviral-mediated overexpression promotes neuroprotection, with RBM3 over-expressing mice showing a marked increase in lifespan, later onset of neurodegeneration and improved behavioural outcomes when compared to shRNA knockdown mice[30]. Lastly, a recent paper identified that RBM3 pre-mRNA undergoes alternative splicing that is sensitive to changes in temperature. A previously uncharacterised exon (exon3a) is not included in the mRNA at 34°C, however exon3a-containing

mRNA isoforms are present at 38°C. Furthermore, antisense oligonucleotide administration targeting exon3a results in a sustained increase in RBM3 expression as well as neuroprotection in a mouse model of prion disease[31]. These mechanisms could be further explored in the context of C9orf72-ALS.

The cold-inducible RNA binding protein (CIRBP) also plays a key role in the neuroprotective effects of hypothermic-TTM. CIRBP is an RNA-binding factor and expression is increased following hypothermia[32]. Mild hypothermia increases CIRBP expression in the cortex, hypothalamus and hippocampus[33]. *In vitro* studies using rat primary cortical neurons showed that CIRBP expression is protective against H_2O_2-induced apoptosis[34]. Experimental models of cerebral ischemia evidence a significant reduction in ROS and jugular NO release following mild hypothermia when compared to normothermic conditions[35]. Furthermore, using a rat model of TBI, the anti-apoptotic effects of mild hypothermia were abolished following CIRBP siRNA silencing[33], indicating a significant neuroprotective role of CIRBP in hypothermic-TTM.

Using cell models of C9orf72-ALS, cooling to 32°C significantly increased protein expression of both RBM3 and CIRBP and this is associated with a significant decrease in DPR expression. This potent effect of hypothermic-TTM on DPR expression was further observed in our C9orf72-ALS zebrafish model. Future research using these models aims to further investigate the relationship between hypothermic-TTM protocols and other C9orf72-ALS disease outcomes.

Hypothermic TTM - clinical perspectives

Pre-clinical research efforts have demonstrated key pathways involved in ameliorating tissue pathology, as well as demonstrated efficacy in pre-clinical models of disease. Progression of this research into randomised clinical trials has shed light on the utility of hypothermic-TTM in human

disease. Hypothermic TTM has found most of its application in severe acute injury and is applied over a short period. The most widely cited and implemented use of hypothermic-TTM is in cardiac arrest (CA) patients. Following a large-scale multi-centre randomised control trial (RCT) the Hypothermia after Cardiac Arrest (HACA) Study Group found that mild hypothermia improves neurological outcome and reduces mortality[36]. Hypothermic-TTM to a core body temperature of 32-34°C is now a recommended treatment protocol for CA patients by the American Heart Association (AHA)[37].

Hypothermic-TTM has also been translated into the clinic for the treatment of hypoxic-ischaemic encephalopathy (HIE)[38,39]. A recent meta-analysis of 28 RCTs found that hypothermic-TTM reduced the risk of mortality in neonates with moderate to severe HIE, concluding that healthcare professionals should consider this as a treatment for neonates with HIE[39].

Hypothermic-TTM has also been explored in the context of ischaemic stroke. A meta-analysis conducted by van der Worp et al. concluded that therapeutic hypothermia improves outcomes by approximately one third in animal models of cerebral ischaemia, in conditions that are achievable in a clinical setting[40]. This clear evidence warranted further investigation in human patients, however to date, this pre-clinical research has not been successfully translated into an effective therapy for ischaemic stroke patients. A meta-analysis comprising twelve studies found no additional benefit in hypothermic-TTM patients. Furthermore, treated patients experienced higher adverse events, which may be due to the presence of patient co-morbidities increasing risk for adverse event development. The influence of comorbidities on treatment outcome needs to be addressed in future studies[41]. Research efforts to standardise treatment protocols and outcome measurements as well as further understand the influence of age, gender and comorbidities on these outcomes are needed to translate this pre-clinical work into a safe and effective treatment option[42,43].

Efforts have been made to improve therapeutic hypothermic-TTM protocols, including the use of hydrogel-coated water-circulating transfer pads[44] or cold fluid intravenous infusions[45] to improve upon more conventional cooling systems. Supplementary treatment during periods of hypothermic cooling may also reduce the risk of adverse event development. These alternative and refined cooling approaches, alongside supplementary treatment approaches, allow more controlled hypothermia induction, maintenance and re-warming, which may provide more safe and practical options for prolonged use in future clinical studies[46]. To what extent these different treatment modalities influence experimental and clinical outcomes needs to be defined in disease-specific contexts, thus careful study-design is paramount to ensure patient safety and determine clinically efficacy.

Conclusions

The transgenic model detailed in this paper provides a medium throughput *in vivo* research tool to further investigate the toxic role of RAN-translation in C9orf72-ALS, and further understand the mechanisms that underpin any neuroprotective strategies. Future work aims to further explore the mechanisms of hypothermic-TTM in the context of C9orf72-ALS by utilising the C9orf72-ALS cell models cell and the RAN-translation zebrafish models described. This will allow us to further understand how hypothermia influences ALS tissue pathology and motor performance. Additionally, combining hypothermic-TTM protocols with other neuroprotective strategies may provide synergistic effects, as highlighted by previous studies of neurodegenerative disease[47]. The ultimate aim of this research is to eventually translate this pre-clinical data into an effective C9orf72-ALS therapy. The vast array of safety and efficacy data that has been published to date clearly demonstrates the potential benefit of hypothermic-TTM in neurological disease.

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FIGURES

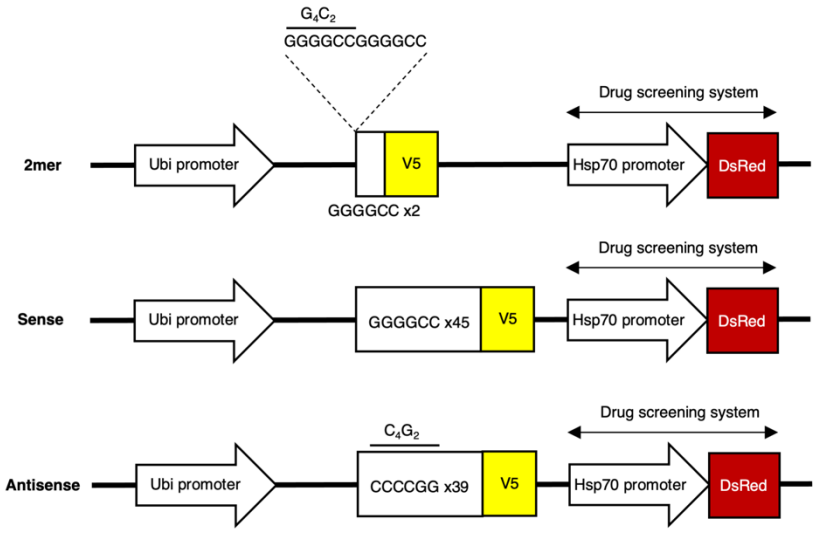

Figure 1 Schematic of transgene used to create pure repeat RAN transgenic zebrafish lines.

The control line contains 2 repeats of GGGGCC (G4C2), the sense line contains 45 repeats of G4C2 and the antisense line contains 39 repeats of CCCGG (C4G2). A V5 epitope tag is in all three frames following the repeats and transgenes also carry the hsp70-DsRed transgene in tandem as a screening tool. No ATG codon exists that could allow the translation of the repeats in all three frames. Potential translation through GTG sites may produce poly-GA, poly-GP and poly-PA DPRs, whereas no such codon exists for poly-GR and poly-PR DPRs.

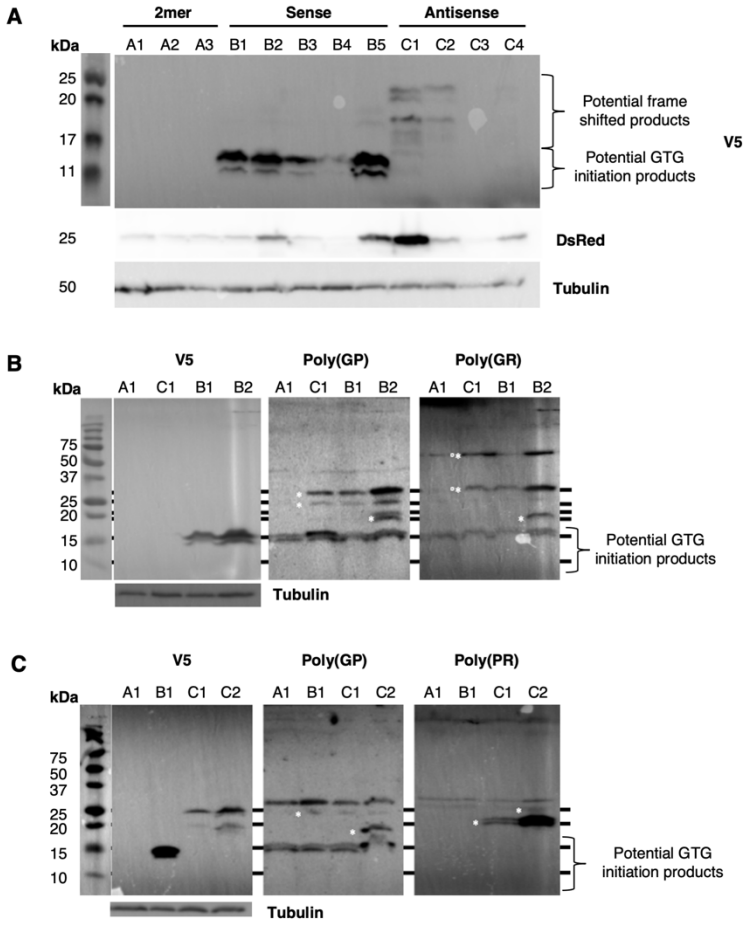

Figure 2 Western blot of RAN-translated products in 5 dpf control (2mer), sense (G4C2(45)) and antisense (C4G2(39)) embryonic zebrafish

(a) Three control (2mer, A1-3), five sense (G4C2, B1-5) and four antisense (C4G2, C1-4) generated transgenic lines were probed with anti-V5, anti-DsRed and anti-tubulin (loading control) antibodies to detect RAN translation in +1, +2 and +3 frames. (b) Sense RAN-translated DPR species poly(GP) and poly (GR) and (c) antisense RAN-translated DPR species poly(GP) and poly (PR). Asterix (*) indicate protein bands which are proposed to be driven by RAN-translation.

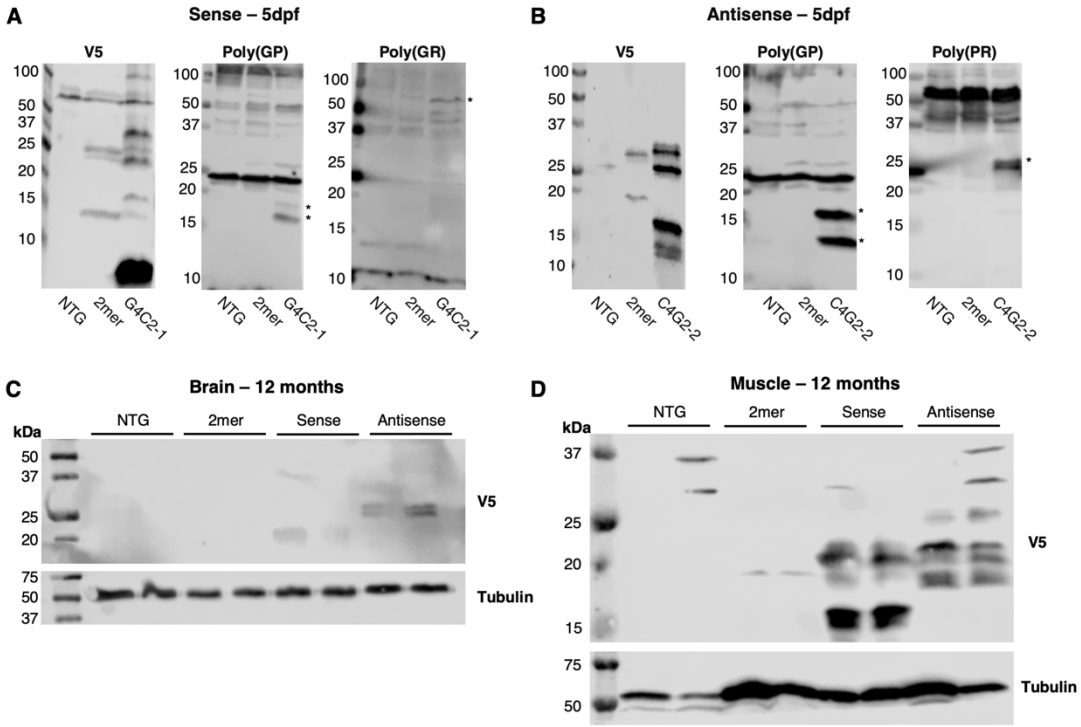

Figure 3 Embryonic and adult C9orf72 zebrafish lines produce multiple RAN-translated DPR species.

Western blot analysis of V5-DPRs and poly-repeats species GP, GR and PR in (a) sense (G4C2-1) and (b) antisense (C4G2-2) 5dpf embryonic lysates compared to non-transgenic (NTG) and 2mer-3 control. 12-month (c) brain and (d) muscle lysates express multiple V5-DPR species.

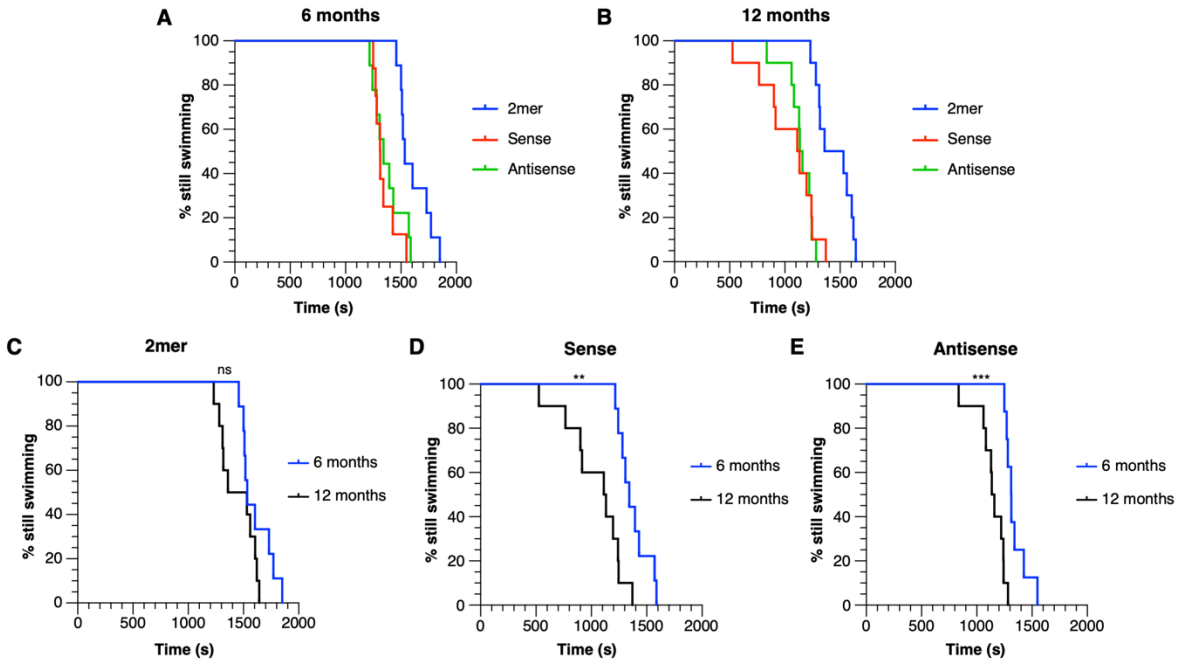

Figure 4 Adult C9orf72 zebrafish show reduced swimming endurance

Kaplan-Meier analysis of swim tunnel performance in (a) 6-month (b) 12-month-old zebrafish. (c) Control (2mer-3) zebrafish show no significant decline in swimming endurance between 6- and 12-month timepoints. Both (d) sense (G4C2-1) and (e) antisense (C4G2-2) zebrafish lines significantly declined in swimming endurance between 6- and 12-months old. N=9 2mer control, N=10 sense (G4C2-1) and N=10 antisense (C4G2-2). Log-rank (Mantel-Cox) test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

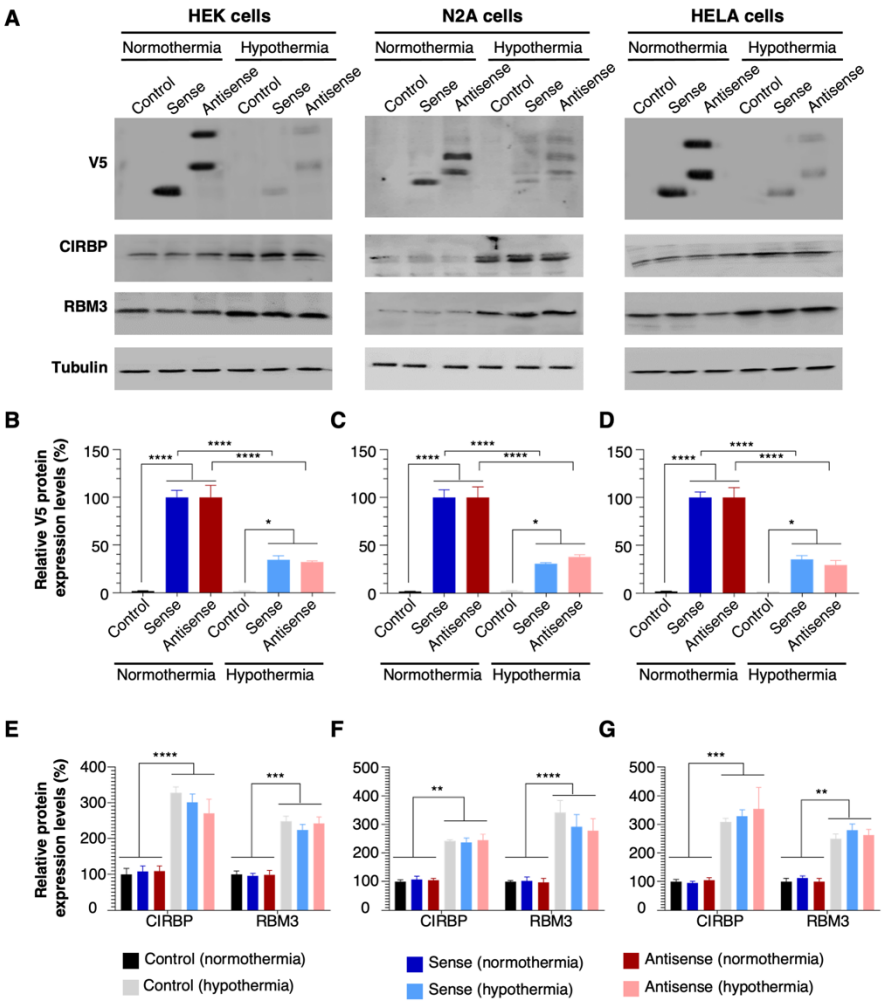

Figure 5 Hypothermic cooling reduces DPR expression in C9orf72 cell models

(a) HEK293T, N2A and HeLa cells were transfected with control, sense and antisense constructs and protein lysates were collected following 48 h hypothermic cooling (32°C). Western blot analysis and quantification of (b-d) V5-DPRs and (e-g) cold shock proteins CIRBP and RBM3. Data presented as mean ± SD. One-way ANOVA; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

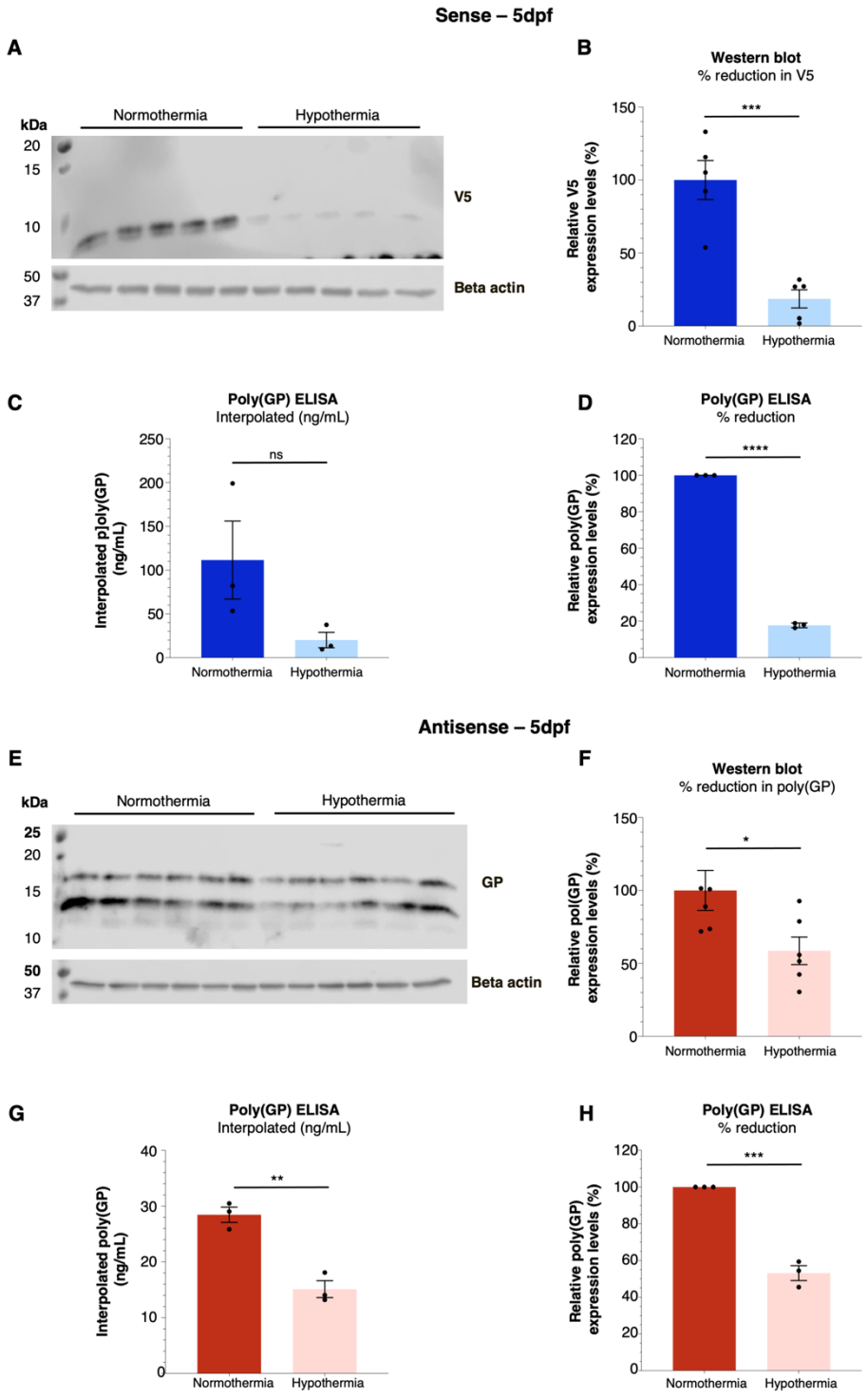

Figure 6 Hypothermic-TTM reduces DPR production in embryonic C9orf72 zebrafish

Western blot and MSD poly(GP) ELISA analysis of 5 dpf embryonic lysates following hypothermic cooling. (a-b) anti-V5 immunostaining and (c-d) MSD poly(GP) ELISA analysis of sense (G4C2-1) embryonic lysates. (e-f) anti-poly(GP) immunostaining and (g-h) MSD poly(GP) ELISA analysis of antisense (C4G2-2) embryonic lysates. Western blot – N=5-6 clutches per treatment group, poly (GP) MSD ELISA – N=3 clutches per treatment group. Data presented as mean ± SD. Unpaired t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.