Developing an antisense oligonucleotide treatment for Spinocerebellar Ataxia Type 3

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Discussion

In this thesis AONs were investigated as a potential therapy for the neurodegenerative disorder SCA3. Currently, only symptomatic treatment is available, and there is thus an immediate need for therapies preventing disease onset and disease progression. The monogenetic nature of SCA3 makes it a good candidate for generation of genetic therapies such as AONs. Additionally, AONs have proven versatile molecules that show particular promise for use in the CNS (chapter 2). In this thesis, we have investigated two different AON based exon skipping therapies for SCA3. In the first approach, the aim was to prevent the formation of toxic polyQ protein fragments by skipping exons encoding the proteolytic cleavage sites (chapter 3). In the second approach we directly targeted the CAG repeat containing exon of *ATXN3* for exclusion, and tested this approach in a SCA3 mouse model (chapter 5). Finally, we assessed potential side effects of phosphorothioate modified AON treatment in the mouse brain with RNA expression profiling (chapter 6). The results obtained in this thesis will be discussed both in the broader context of SCA3 molecular pathogenicity, and with regards to the use of AONs as a potential therapy for SCA3.

RECENT DISCOVERIES ON SCA3 PATHOLOGY

Gain of toxic protein function

Aggregation and formation of neuronal inclusion bodies containing the expanded polyQ protein region was one of the first pathologic hallmarks discovered in SCA3 brain material [1]. In part due to this observation, it was hypothesized that the expanded polyQ region of the mutant protein leads to gain of toxic function, perhaps due to sequestration of other proteins into the aggregates or through direct toxicity of the aggregates themselves. The opposing viewpoint has also been argued, namely that the polyQ expansion induces a loss of ataxin-3 protein function, in turn contributing to pathogenicity. A loss of the deubiquitinating activity of mutant ataxin-3 seems unlikely however, as we and others have shown that mutant ataxin-3 can both bind (chapter 3) and cleave (chapter 5) ubiquitin chains similar to wild-type ataxin-3. Similarly, mutant ataxin-3 can still interact with VCP [2], as well as the DNA damage response proteins RAD23 [3] and PNKP [4, 5]. Since interaction for these proteins with mutant ataxin-3 is retained, this argues against a "loss of wild-type function" pathologic model in SCA3.

In favor of the gain of toxic protein hypothesis, it was for instance recently discovered that mutant ataxin-3 inactivates PNKP, resulting in inefficient DNA repair and culminating in apoptosis [5]. Furthermore, an RNA expression profiling experiment compared a SCA3 mouse containing the mutant human ataxin-3 protein to an *ATXN3* knockout mouse, with the knockout mice showing fewer differentially expressed genes than SCA3 mice [6]. If a loss of ataxin-3 function underlies SCA3 pathogenesis, it would be expected that ataxin-3 knockout mice present with similar affected pathways as transgenic SCA3 mice. Hence, a gain of toxic protein function over loss of ataxin-3 function seems more likely to be the main source of pathogenicity in SCA3.

Role of toxic polyQ fragments

The aim of the experiments described in chapter 3 was to prevent proteolytic cleavage of ataxin-3, in order to reduce cellular toxicity. The reasoning behind this approach ties in with the toxic gain of protein function, as it has been shown that shorter protein fragments containing the polyQ expansion were in fact more toxic than full-length mutant ataxin-3 [7]. Besides displaying increased cellular toxicity, the polyQ fragments also appear to be central to initiation of the aggregation process and subsequent formation of inclusions in cells [8]. It remains to be determined whether the cellular toxicity can be attributed mostly to the ataxin-3 cleavage fragments themselves, or to their initiation of aggregate formation. The two main families of proteolytic enzymes associated with ataxin-3 cleavage are the caspases and calpains. As we reviewed in chapter 1, a greater body of evidence supports the role of calpain generation of toxic ataxin-3 protein fragments. Both the spontaneous generation of the polyQ fragments as well as the cytotoxic effects appear to be highly cell-type specific. In general, polyQ containing protein fragments are generated in non-neuronal cells only when perturbing the cells by artificially inhibiting autophagy or the proteasome. Using induced pluripotent stem cell (IPSc)-derived neurons, Koch and colleagues were able to show that only active neurons were prone to generation of the toxic ataxin-3 cleavage fragments due to calcium influx induced activation of calpains, with subsequent formation of aggregates [9]. As this process was not observed in fibroblasts or glia cells, these experiments provided a possible explanation as to why predominantly neurons are vulnerable to ataxin-3 aggregate formation. These results were however not replicated in more recent studies and thus require further validation [10]. The latest studies in cell models were however able to show that point mutations of 2 calpain sites abolished ataxin-3 fragmentation [11]. Also, transgenic mouse models were used to further establish the initiating role of the polyQ fragment in aggregate formation [12], and inhibition of calpains was shown to diminish mutant ataxin-3 cleavage, nuclear localisation and aggregation [13]. As such, the toxic fragment hypothesis of ataxin-3 is currently well supported, and a central role for calpain cleavage in this process still appears valid.

Recent findings suggest potential involvement of transcriptional dysregulation, as well as RAN and RNA toxicity in SCA3 pathogenicity

Other emerging disease mechanisms

The mechanisms through which ataxin-3 fragments and aggregates exert their cellular toxicity in the brain are still under investigation. Transcriptional dysregulation due to mutant ataxin-3 was already described over a decade ago in SCA3 cells and brain material, where inflammatory gene upregulation was shown [14]. Since this period, subsequent transcriptomic analysis in mouse models have identified potential involvement of glutamatergic neurotransmission, calcium

signalling and MAP kinase pathways [15]. Further, oligodendrocytes were found particularly affected by the process of transcriptional dysregulation, suggesting white matter vulnerability in SCA3 as well [6]. In our transcriptomic studies of the MJD84.2 mouse (chapter 4), we also found evidence suggestive of affected oligodendrocyte function and altered transcription of genes involved in myelination. Potential involvement of the immune system or calcium signalling was however not strong in our dataset. The observed differences between the studies may reflect distinct SCA3 disease stages, since the mice used for our study can be considered presymptomatic. Indeed, we did not detect signs of neurodegenerative or apoptotic processes taking place in the brain transcriptome of SCA3 mice, and we were unable to detect reduced performance in various motor coordination tests. Validation of the transcriptional changes in brain material of SCA3 patients would thus be useful to determine whether the transcriptional alterations we outline in chapter 4 are consistent with the human condition.

Another disease process associated to repeat disorders is repeat associated non-ATG (RAN) translation [16-19] and ribosomal slippage upon repeat translation [20]. Both these processes can result in translation of the CAG repeat in a different reading frame, generating either polySer or polyAla amino acid stretches, or even polyLeu and polyCys stretches for RAN translation of antisense transcripts [17]. These protein stretches, especially polyLeu [21], were shown to be more toxic and aggregation prone in cells compared to polyQ repeats [17]. Interestingly, the RAN proteins were detected in affected brain regions of HD patients, and polyAla accumulation and polySer aggregation were particularly abundant in cells expressing repeats >52 [17]. These findings are interesting when relating them to SCA3, as the repeat length required for RAN protein accumulation to occur in HD corresponds to the pathogenic repeat threshold for SCA3 (>54 CAGs). A logical next step is therefore to probe SCA3 mouse and patient material for presence of the alternative repeat protein stretches and establish whether they are present at levels that may contribute to neurodegeneration.

The aspect of RNA toxicity is also a disease mechanism that may be involved in SCA3. Ling-Bo and colleagues were the first to discover that interrupting the pure CAG repeat of ataxin-3 with CAA codons mitigated toxicity in *Drosophila* [22]. Since a CAA codon also encodes glutamine and the protein expression levels were similar, the observed neuronal degeneration in this experiment was attributed to an RNA effect. Additionally, it was shown that a pathogenic length of CAGs in an untranslated region also conveyed neuronal toxicity [22]. However, RNA foci typically arise for repeat expansion lengths that are beyond what is normally found in SCA3 patients, and hence involvement of RNA toxicity in SCA3 still remains to be reliably established.

SCA3 cell and mouse models

Due to the scarcity of human SCA3 brain material and the limitations this tissue presents in investigating molecular disease mechanisms, the vast majority of knowledge on pathogenic mechanisms have been obtained using SCA3 cell and mouse models. Cell models are typically useful for high throughput screening of therapeutics, as well as discovery of novel molecular effects in response to mutant ataxin-3 expression. Variation in cell culture experiments is

typically very low, due to strictly controlled culturing conditions and absence of cell type induced variation. Conversely, modelling of neurodegenerative disorders in cell culture experiments has been notoriously difficult due to complexity of the brain. As a result, predictive validity of cell culture to the *in vivo* situation is often limited. In case of SCA3, a good example of this limitation is aggregate formation, which must be artificially induced in cell culture and yet spontaneously occurs in SCA3 mouse brain. An important step forward in this regard has been the advent of IPS cells, which can be differentiated into neurons and glial cells hence offer a promising opportunity to more accurately study disease mechanisms of neurodegenerative diseases in cell culture [23]. IPS cell lines for SCA3 have been generated [9, 24], and studies with these cells are currently ongoing. In the research described in this thesis, we have typically used SCA3 patient derived fibroblast to screen AON efficacy. So far, results of *in vitro* AON efficacy has correlated well to the effects observed when infused in the mouse brain (chapter 5). However, a clear limitation of the fibroblasts is their inability to predict AON tolerability in the mammalian brain. As we show in chapter 6, intracerebroventricularly infused AONs in mouse brain can result in immune stimulation which is long lasting and can preclude dosing of AONs to therapeutic levels. Though the extent of AON-induced immune stimulation in the human CNS remains to be established, animal models remain imperative to establish pharmacokinetics and tolerability of new potential therapeutics.

Several SCA3 mouse models have been generated that can be useful for this type of research (reviewed by Gould [25]). Most of the mouse models are generated through exogenous gene expression, i.e. through integration of a human *ATXN3* construct in the mouse genome. To promote an early onset, the mice typically have repeat lengths of over 70 CAGs, and have wide-spread expression of mutant ataxin-3. Nonetheless, phenotypes between the mouse lines differ, and mild to severe phenotypes are reported. So far, SCA3 mouse models suggest a correlation between severity of behavioural abnormalities and presence of ataxin-3 cellular inclusions [25]. In our studies investigating the therapeutic potential of AONs, a SCA3 mouse model (MJD84.2) containing the human *ATXN3* gene with 84 repeats was used [26], as the human sequence as well as introns are required to assess splicing effects. In this mouse model, we did not detect the behavioural deficits (chapter 5 and 6) which were reported originally [26]. Correspondingly, we also did not observe nuclear ataxin-3 inclusions in the brain cells of these mice. We did, however, detect insoluble ataxin-3 protein using filter trap assays in all brain regions we have tested thus far (brainstem, cerebellum and cortex) (chapter 5). Further transcriptomic analysis of brainstem, cerebellum, striatum and cortex revealed that various signalling pathways were affected (chapter 4). However, in the transcriptomic analysis we showed larger transcriptional dysregulation in brainstem compared to cerebellum and cortex, whilst the levels of insoluble ataxin-3 protein was comparable between these brain regions. These observations hint toward brain region specific effects of mutant ataxin-3 at the transcript level, which may be unrelated to insoluble ataxin-3 levels. To further establish this relation a recently developed humanized SCA3 mouse model may thus be useful, though the reported behavioural phenotype appears mild [27]. Similarly, a recently developed knock in SCA3 mouse model where 82 CAGs were inserted into the murine *Atxn3* gene also displayed aggregate pathology, but did not present

with a behavioural phenotype [28]. It hence appears that the SCA3 mouse models most closely mimicking the human situation in terms of expression level and CAG repeat length, do not develop reliable motor phenotypes.

ADVANCES IN ANTISENSE OLIGONUCLEOTIDE THERAPEUTICS

In the research described here we have tested AON based therapies for SCA3. AONs have been used in research settings since the 1960's, but rapid degradation of the phosphodiester bonds by nucleases rendered these molecules initially unsuitable for therapeutic use. A solution to this problem was discovered in 1966 by Fritz Eckstein, who introduced the phosphorothioate (PS) backbone. The PS backbone rendered the AON remarkably resistant to nuclease degradation, and additionally conveyed protein binding properties favourable for *in vivo* use [29]. Together, these properties greatly improved therapeutic viability of the AONs, and the PS backbone is still one of the most commonly used AON modification to this day [30]. The second major step forward in AON modifications came in the form of the sugar group modifications, such as the 2'Ome and MOE modifications, which enhance target affinity, improve uptake and simultaneously reduce toxicity [31]. The drawback of these sugar group modifications, however, is that they are incompatible with RNAse-H target degradation [32], thereby impeding desired downregulation of a target transcript. This issue was subsequently circumvented using the "gapmer" strategy, where a few nucleotides in the central region of the AON contained unmodified sugar groups, hence recovering RNAse-H activation [33]. Making use of these modifications, AONs have proven a particularly useful molecule for application in the CNS (reviewed in chapter 2). In the research described in this thesis, we made use of the commonly used phosphorothioate backbone and both 2'Ome and MOE modifications. These chemistries resulted in similar potency in cell culture experiments following transfection, but the 2'Ome modification resulted in more side effects and lower potency when infused in the mouse brain, in line with previous reports [34]. Since completion of these studies, more recent AON modifications such as 2'Fluoro and Cet have gained traction, and may offer benefits for future clinical application. The potential of these latest AON modifications for use in CNS will be discussed below.

> Decades of research has resulted in an ever-expanding arsenal of AON modifications, which together hold exciting potential for clinical use for neurodegenerative disorders.

Bridged sugar ring modifications

Conformational constraints by bridging of the sugar ring of AONs can achieve similar properties as the other sugar group modifications; they result in an RNA-like structure, rendering them insensitive to RNAse-H, and increase nuclease resistance and especially binding affinity [35]. One of the older modifications belonging to this category is the locked nucleic acid (LNA) [36], but

more recently the 'constrained ethyl' (cET) [37] and Tricyclo DNA (tcDNA) [38] were discovered. The LNA modifications were confirmed to have increased potency, but suffered from significant toxicity [37, 39]. This increased toxicity appears to be a by-product of the high binding affinity, since hepatotoxicity was shown to be the result of off-target RNA degradation [40]. Subcutaneous injections of an LNA modified gapmer AON in a human patient confirmed the toxicity, as acute kidney injury occurred [41]. Due to high binding affinity, LNA efficacy typically occurs at short lengths, such as 13-mers [42]. The major advantage of the bridged ring modifications, their improved binding affinity, hence appears to be their downfall as well due to increased off-target effects. Compromises using so-called mixmers, where for instance LNA modifications are alternated with DNA nucleotides have also been designed, and these molecules show promise for splicing modulation [43]. In order to circumvent the reported toxicity, these mixmers may thus prove useful in future clinical application [44]. Clinical use of cET modified AONs for the treatment of lung cancer have shown good tolerability up to 3 mg/kg upon intravenous administration, but encountered dose limiting toxicity at 4 mg/kg likely due to on-target depletion of STAT3 [45]. Whether cET AONs provide additional clinical benefit compared to the other sugar group modifications thus still remains to be determined. Though already under investigation for almost two decades [46], tcDNA was recently demonstrated to have superior uptake in muscle tissue, be better tolerated than LNA, and was shown to be able to cross the blood brain barrier in modest amounts [47]. Future clinical trials should comprehensively establish tolerability and efficacy of these bridged ring AON modifications, as they offer potentially great benefits for AON efficacy [48]. An intriguing possibility is that AONs making use of the older 2'O-modifications can be combined with the newer bridged sugar ring modifications, in order to maximise the benefits of both chemistries in a single molecule.

Other strategies to improve AON efficacy

New strategies to improve the drug like properties of AONs are still being discovered. One well investigated way to improve the cellular delivery of AONs is by linkage to peptides [49]. In spite of the already favorable distribution and cellular uptake of naked AONs in the CNS, conjugation to peptides may still provide useful properties that improve AON efficacy. Peptides may for instance be of added value in improving endosomal escape of AONs [50], potentially providing better access to the nucleus of brain cells. The endosomal escape of the peptides themselves is currently still a challenging task, however [51]. Also, the added benefit of this strategy is still unclear, since the mechanisms underlying the favorable *in vivo* cellular uptake mechanisms of AONs in the brain are still largely unclear. A second intriguing application of the peptide conjugates may lie in assisting in the crossing of the blood brain barrier by AONs. Currently, AONs for neurodegenerative disorders are injected intrathecally in order to reach the brain [52, 53]. The invasive nature of this procedure as well as the required hospital visits are a burden for the patients, and efforts are therefore made to generate peptide conjugated AONs that can cross the blood brain barrier. For instance, a peptide linked splice modulating PMO for ataxia-telangiectasia showed uptake throughout the mouse brain following intravenous

injection [54]. Similarly, an internalizing peptide linked PMO (pip6a-PMO) was recently generated for potential treatment of spinal muscular atrophy (SMA). Intravenous administration of the pip6a-PMO showed effective splicing modulation of the SMN2 transcript in both peripheral as well as CNS tissue of SMA mice [55]. At present, peptide linked AONs show greater promise for improving uptake in peripheral tissues, such as muscle, rather than in effective crossing of the blood brain barrier. Nonetheless, optimization of peptides may in the future yield molecules capable of crossing the blood brain barrier with better efficiency, potentially warranting systemic administration of AONs targeting the CNS.

Recently 6 alternative genetic polymers, termed xeno-nucleic acids (XNAs), were generated based on nucleic acid architectures not found in nature [56]. These new synthetic polymers can be applied to other nucleic acid architectures and open new possibilities for incorporation of novel physicochemical properties of AONs [56]. Since their discovery XNAs have however not been studied extensively, and their therapeutic potential is thus unclear. Another interesting recent development is the control of AON chirality. For PS-modified siRNAs, a bias in stereochemistry during synthesis was noticed, which modulated siRNA activity [57]. An interesting feature of the PS backbone is that one isomer apparently provides better T_m and RNAse H activation, whilst the other isomer is better at improving nuclease stability [58]. When closely examined for AON gapmers, controlling PS chirality did not appear to provide benefits over stereo-random PS AONs obtained in conventional AON synthesis [59]. In contrast, a recent study did report a more durable *in vivo* response for a specific stereopure gapmer AON [60]. A phase 1b/2a clinical trial (NCT03225833) for HD using a intrathecally injected stereopure AON against a SNP in huntingtin was started in 2017 by Wave Life Sciences, with the expected completion date in September 2019.

In 2014, a strategy to incorporate neutral phosphotriester groups into the phosphate backbone of siRNAs was devised in order to create neutrally charged siRNAs [61]. Potential benefit of these molecules include increased serum stability and absence of innate immune response [61]. Together, the strategies listed above provide new avenues to improve AON drug like properties through a variety of chemical modifications and synthesis strategies. Benefits for CNS application of these strategies will first have to be determined in pre-clinical assessments in rodent and non-human primate experiments.

Recent clinical successes with use of AONs in the central nervous system pave the way for development of AON based treatments for other neurodegenerative disorders.

Lessons from clinical trials with AONs

The past few years have been a stimulating period in AON research for neurodegenerative disorders. Synthesis of AONs on a kilogram scale has become affordable, paving the way for

large clinical application of AONs. The forerunner of AON clinical development in the context of the CNS is undoubtedly the neuromuscular disorder SMA. An AON based splice modulating strategy to increase expression of survival of motor neuron (SMN) protein was devised as a therapeutic strategy for SMA [62]. Since then, increasing preclinical success in SMA mouse models have proved the validity of the strategy, and increasingly efficient AONs were developed to increase SMN protein expression in the CNS [63]. Approximately 10 years after being devised, the AON based therapy (Nusinersen) was successfully tested through intrathecal injection in the first phase 1 study with SMA affected children [64]. An encouraging safety profile and AON half-life in the CSF of 4-6 months warranted further clinical development of Nusinersen [64]. Subsequent phase 2 [65] and phase 3 [66] trials have shown unprecedented success in motor improvement and survival of Nusinersen treated SMA patients. The next landmark event was approval of the AON, now termed Spinraza, by the food and drug administration (FDA) at the end of 2016 [67] and is currently also approved by the European Medicines Agency (EMA). The story of Spinraza has underlined the fact that AONs are a very promising therapeutic tool for use in the CNS, and that meticulous preclinical research on AONs and their design can culminate in clinical success and benefit for patients. The successes obtained in SMA research open the door for other AON based therapies targeting the CNS to continue development, and provide hope for patients of several neurodegenerative disorders currently without treatment.

AON AND GENOME EDITING BASED THERAPY FOR SCA3

Following recent clinical successes with AONs for neuromuscular and CNS disorders, it is useful to consider the possibility of AON based treatment for SCA3 and other polyQ disorders. However, there are also other gene therapy strategies that can be potentially used for SCA3 treatment. Below, the potential of AONs and other gene therapies for the treatment of SCA3 will be discussed.

Potential of CRISPR/Cas treatment for SCA3

Without a doubt the most revolutionary discovery in genome editing in recent years has been the clustered, regularly interspaced, short palindromic repeat (CRISPR) technology. This technology allows RNA-guided nucleases, such as Cas9, to be targeted to specific DNA sequences. The flexibility in genome editing provided by CRISPR/Cas is a major improvement over previous technologies, as only a protospacer adjacent motif (PAM) consisting of NGG nucleotides is required in the target sequence [68]. A guide RNA (gRNA) can be designed to target the nuclease to the gene of interest, and depending on the specific nuclease or nickase used, a double stranded break can be introduced to induce non-homologous end joining or activate the homology directed repair pathways [69]. The system can hence be used to introduce insertions, deletions or frameshifts to induce gene knockout. In the case of homologous recombination, a repair template can be provided to introduce a sequence of interest in the target gene. In recent years a vast number of publications implementing CRISPR/Cas technology in research settings

have appeared [69]. An important question is hence whether this technology can be implemented in a clinical setting for treatment of hereditary neurodegenerative disorders such as SCA3. Since CRISPR/Cas targets the DNA, an important advantage of this technology over AONs is that one time treatment is sufficient to achieve a permanent treatment effect. One immediate difficulty for CRISPR/Cas in the context of the brain is that homology-directed repair is inefficient in G1 state cells [70], and hence in neurons. Non-homologous end joining based DNA repair is however active in post-mitotic cells, and AAV based delivery of Cas9 and guide RNAs in the mouse brain was successfully used to induce frame-shifts and subsequent protein depletion of several targets [71]. It is hence possible to achieve gene knockout *in vivo* in brain cells using this approach. Interestingly, it was recently shown that non-homologous end joining is a viable strategy for transgene insertion in neurons as well, opening the way for insertion strategies in neurons [72]. In the case of SCA3, CRISPR/Cas could most easily be implemented to knock down *ATXN3* expression. Non-allele specific downregulation of huntingtin was recently achieved through CRISPR/Cas in a Huntington mouse model, and successfully alleviated symptoms [73]. Targeting of the expanded CAG repeat of *ATXN3* directly through CRISPR/Cas does not seem possible at this time. It may however be possible to target SNPs associated with the mutant *ATXN3* allele, as SNP specific knockout through CRISPR/Cas is feasible [74]. Apart from similar complications associated with viral delivery as some of the RNAi strategies, the biggest hurdle for clinical application of CRISPR/Cas is currently the risk of off-target effects. In contrast to AONs, genome editing is permanent, and the risk of off-target effects is thus an important concern. Off-target effects of CRISPR/Cas has sparked an ongoing debate in the scientific community, with contradictive reports appearing [75, 76]. Nonetheless, improvements in CRIPR/Cas technology as well as the required delivery methods are continuously being developed, and the technology undoubtedly shows great promise for future therapeutic use.

AON based downregulation versus exon skipping

In this thesis, we have investigated two exon skipping based treatment strategies for SCA3. In the first strategy, we attempted to alleviate cleavage induced toxicity by removal of exons encoding known cleavage sites associated with mutant ataxin-3 toxicity (chapter 3). In the second strategy, we continued on a previously described exon skipping strategy [77] to remove the CAG repeat containing exon from *ATXN3* pre-mRNA. In the latter strategy, ataxin-3 is truncated just upstream of the polyQ repeat, resulting in a theoretically non-toxic protein (chapter 5). Among these two exon skipping strategies, we have concluded that skipping of the repeat containing exon is undoubtedly the preferred strategy for a number of reasons. Firstly, the cleavage sites within ataxin-3 that are associated with toxicity are encoded by the two exons that also encode the ubiquitin interacting motifs (exon 8) as well as the VCP binding site (exon 9). In contrast, skipping of exon 10 retains ataxin-3 ubiquitin binding function. Secondly, exon skipping of the CAG repeat containing exon was exceedingly more efficient than skipping of exons encoding the proteolytic cleavage sites. Thirdly, it is currently still unclear whether removal of the cleavage sites within mutant ataxin-3 truly alleviates toxicity *in vivo*. Lastly, in light of future clinical

application, the use of a single AON against exon 10 is more favourable than the two separate AON sequences required for removal of the cleavage sites from ataxin-3.

From our studies it is therefore clear that *ATXN3* exon 10 skipping is the preferred therapeutic approach. However, other research groups have shown successful downregulation of *ATXN3* transcripts *in vivo* by making use of gapmer AONs [78] or through lentivirus delivered shRNA [79]. A comprehensive evaluation of differences between AONs and RNAi based on viral delivery is beyond the scope of this thesis, but in brief, AONs have an advantage in their delivery and distribution [34], whereas lentiviral or adeno-associated virus (AAV) based delivery of RNAi molecules have the major advantage of requiring only one time or very infrequent treatments [80], though immune stimulation may be an concern [81]. Choosing the best RNAi based therapeutic strategy for SCA3 largely depends on a currently still unanswered question: is downregulation of ataxin-3 tolerated in the human brain for a prolonged period of time? As described in chapter 1, ataxin-3 downregulation or knockout is tolerated in mice, though protein ubiquitination is increased [82, 83]. On the other hand, intracellular signalling and cytoskeletal organisation was affected in human cell culture experiments upon ataxin-3 downregulation [84, 85]. Therefore, exon skipping based removal of the toxic polyQ tract in ataxin-3 is an interesting treatment option. Conversely, *in vivo* exon skipping occurred at higher AON dosages (chapter 5) than those required for downregulation through gapmer AONs [78]. Given these considerations, allele specific downregulation of mutant ataxin-3 may be a preferable therapeutic approach at this time, as in this case wild type ataxin-3 remains available to perform cellular functions, whereas the toxic entity is specifically removed. In this regard, perhaps the most interesting and safest possibility could be to induce preferential exon skipping of exon 10 of mutant *ATXN3* allele by targeting the CAG repeat, as was serendipitously shown in a large AON screening by Liu and colleagues [86]. A potential caveat to this strategy is that the human transcriptome contains many CAG repeat sequences, and off-target effects may thus occur.

Considerations for clinical implementation of SCA3 AONs

Recent successes in clinical trials for HD and especially SMA point to a promising future for clinical use of AONs for treatment of a range of neurodegenerative disorders. There are currently, however, still a few considerations regarding the therapeutic use of AONs. Firstly, clinical trials with AONs have shown good distribution and efficacy in the brains of SMA affected children, but no AON distribution data is as of yet available for the adult human brain. However, given that brain size and CSF flow does not differ greatly between children and adults [87], it is reasonable to assume comparable AON distribution and stability in the adult human brain. A second concern is that the long term effects of exposure of the CNS to AONs are as of yet unclear. Preclinical studies in animals are always comparatively short in duration, whilst treatment of SCA3 patients would in principle require repeated AON dosing during several decades. Currently, children have been treated with the AON drug Spinraza for a period of 15 months, and only adverse events related to the procedure of intrathecal injection were reported [66]. The safety profile of AONs in the brain thus is currently very promising, but long term side effects of continuous

treatment cannot be ruled out yet. Similarly, the actual on-target treatment effect of AONs, such as ataxin-3 protein depletion or truncation through exon skipping, may also induce side effects in the long term. With regards to exon skipping, the ataxin-3 polyQ domain was recently shown to be important in regulation of autophagy [88], and AON mediated removal could thus potentially affect cellular function as well. One advantage of AON mediated therapy in this regard is the fact that an "antidote" complementary AON can be designed in order to bind and inactivate the treatment AON that is already present in cells [89]. Given the reported AON half-life of months, this strategy can be useful if side effects related to AON functional effect arise, and is an advantage over other RNAi and CRISPR/Cas strategies. A last consideration specific to AON based therapy is the high cost of treatment. Treatment for disorders such as SCA3 should arguably be started before onset of symptoms, and would thus require repeated dosing during several decades. With reported costs of Spinraza currently being estimated around €600.000 for the first year and €300.000 for each subsequent year for each patient, insurance companies may not be capable of covering AON based treatments. Nonetheless, treatment prices will likely decline in the future and the many advantages of AONs for use in the CNS will likely assure a place for these molecules in treatment for neurodegenerative disorders.

REFERENCES

1. Paulson, HL, Perez, MK, Trottier, Y, Trojanowski, JQ, Subramony, SH, Das, SS, *et al.* (1997). Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron* **19**: 333-344.

2. Zhong, X, and Pittman, RN (2006). Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates. *Human molecular genetics* **15**: 2409-2420.

3. Sutton, JR, Blount, JR, Libohova, K, Tsou, WL, Joshi, GS, Paulson, HL, *et al.* (2017). Interaction of the polyglutamine protein ataxin-3 with Rad23 regulates toxicity in Drosophila models of Spinocerebellar Ataxia Type 3. *Human molecular genetics* **26**: 1419-1431.

4. Chatterjee, A, Saha, S, Chakraborty, A, Silva-Fernandes, A, Mandal, SM, Neves-Carvalho, A, *et al.* (2015). The role of the mammalian DNA end-processing enzyme polynucleotide kinase 3'-phosphatase in spinocerebellar ataxia type 3 pathogenesis. *PLoS genetics* **11**: e1004749.

5. Gao, R, Liu, Y, Silva-Fernandes, A, Fang, X, Paulucci-Holthauzen, A, Chatterjee, A, *et al.* (2015). Inactivation of PNKP by mutant ATXN3 triggers apoptosis by activating the DNA damage-response pathway in SCA3. *PLoS genetics* **11**: e1004834.

6. Ramani, B, Panwar, B, Moore, LR, Wang, B, Huang, R, Guan, Y, *et al.* (2017). Comparison of spinocerebellar ataxia type 3 mouse models identifies early gain-of-function, cell-autonomous transcriptional changes in oligodendrocytes. *Human molecular genetics* **26**: 3362-3374.

7. Goti, D, Katzen, SM, Mez, J, Kurtis, N, Kiluk, J, Ben-Haiem, L, *et al.* (2004). A mutant ataxin-3 putative-cleavage fragment in brains of Machado-Joseph disease patients and transgenic mice is cytotoxic above a critical concentration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**: 10266-10279.

8. Haacke, A, Broadley, SA, Boteva, R, Tzvetkov, N, Hartl, FU, and Breuer, P (2006). Proteolytic cleavage of polyglutamine-expanded ataxin-3 is critical for aggregation and sequestration of non-expanded ataxin-3. *Human molecular genetics* **15**: 555-568.

9. Koch, P, Breuer, P, Peitz, M, Jungverdorben, J, Kesavan, J, Poppe, D, *et al.* (2011). Excitation-induced ataxin-3 aggregation in neurons from patients with Machado-Joseph disease. *Nature* **480**: 543-546.

10. Hansen, SK, Stummann, TC, Borland, H, Hasholt, LF, Tumer, Z, Nielsen, JE, *et al.* (2016). Induced pluripotent stem cell - derived neurons for the study of spinocerebellar ataxia type 3. *Stem Cell Res* **17**: 306-317.

11. Weber, JJ, Golla, M, Guaitoli, G, Wanichawan, P, Hayer, SN, Hauser, S, *et al.* (2017). A combinatorial approach to identify calpain cleavage sites in the Machado-Joseph disease protein ataxin-3. *Brain : a journal of neurology*.

12. Hsu, JY, Jhang, YL, Cheng, PH, Chang, YF, Mao, SH, Yang, HI, *et al.* (2017). The Truncated C-terminal Fragment of Mutant ATXN3 Disrupts Mitochondria Dynamics in Spinocerebellar Ataxia Type 3 Models. *Frontiers in molecular neuroscience* **10**: 196.

13. Simoes, AT, Goncalves, N, Koeppen, A, Deglon, N, Kugler, S, Duarte, CB, *et al.* (2012). Calpastatin-mediated inhibition of calpains in the mouse brain prevents mutant ataxin 3 proteolysis, nuclear localization and aggregation, relieving Machado-Joseph disease. *Brain : a journal of neurology* **135**: 2428-2439.

14. Evert, BO, Vogt, IR, Vieira-Saecker, AM, Ozimek, L, de Vos, RA, Brunt, ER, *et al.* (2003). Gene expression profiling in ataxin-3 expressing cell lines reveals distinct effects of normal and mutant ataxin-3. *Journal of neuropathology and experimental neurology* **62**: 1006-1018.

15. Chou, AH, Yeh, TH, Ouyang, P, Chen, YL, Chen, SY, and Wang, HL (2008). Polyglutamine-expanded ataxin-3 causes cerebellar dysfunction of SCA3 transgenic mice by inducing transcriptional dysregulation. *Neurobiology of disease* **31**: 89-101.

16. Zu, T, Gibbens, B, Doty, NS, Gomes-Pereira, M, Huguet, A, Stone, MD, *et al.* (2011). Non-ATG-initiated translation directed by microsatellite expansions. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 260-265.

17. Banez-Coronel, M, Ayhan, F, Tarabochia, AD, Zu, T, Perez, BA, Tusi, SK, *et al.* (2015). RAN Translation in Huntington Disease. *Neuron* **88**: 667-677.

18. Buijsen, R (2016). Fragile X-associated Tremor/Ataxia Syndrome: RNA or RAN? Erasmus University Rotterdam.

19. Cleary, JD, and Ranum, LP (2013). Repeat-associated non-ATG (RAN) translation in neurological disease. *Human molecular genetics* **22**: R45-51.

20. Toulouse, A, Au-Yeung, F, Gaspar, C, Roussel, J, Dion, P, and Rouleau, GA (2005). Ribosomal frameshifting on MJD-1 transcripts with long CAG tracts. *Human molecular genetics* **14**: 2649-2660.

21. Dorsman, JC, Pepers, B, Langenberg, D, Kerkdijk, H, Ijszenga, M, den Dunnen, JT, *et al.* (2002). Strong aggregation and increased toxicity of polyleucine over polyglutamine stretches in mammalian cells. *Human molecular genetics* **11**: 1487-1496.

22. Li, LB, Yu, Z, Teng, X, and Bonini, NM (2008). RNA toxicity is a component of ataxin-3 degeneration in Drosophila. *Nature* **453**: 1107-1111.

23. Dolmetsch, R, and Geschwind, DH (2011). The human brain in a dish: the promise of iPSC-derived neurons. *Cell* **145**: 831-834.

24. Hansen, SK, Borland, H, Hasholt, LF, Tumer, Z, Nielsen, JE, Rasmussen, MA, *et al.* (2016). Generation of spinocerebellar ataxia type 3 patient-derived induced pluripotent stem cell line SCA3.A11. *Stem Cell Res* **16**: 553-556.

25. Colomer Gould, VF (2012). Mouse models of spinocerebellar ataxia type 3 (Machado-Joseph disease). *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* **9**: 285-296.

26. Cemal, CK, Carroll, CJ, Lawrence, L, Lowrie, MB, Ruddle, P, Al-Mahdawi, S, *et al.* (2002). YAC transgenic mice carrying pathological alleles of the MJD1 locus exhibit a mild and slowly progressive cerebellar deficit. *Human molecular genetics* **11**: 1075-1094.

27. Switonski, PM, Szlachcic, WJ, Krzyzosiak, WJ, and Figiel, M (2015). A new humanized ataxin-3 knock-in mouse model combines the genetic features, pathogenesis of neurons and glia and late disease onset of SCA3/MJD. *Neurobiology of disease* **73**: 174-188.

28. Ramani, B, Harris, GM, Huang, R, Seki, T, Murphy, GG, Costa Mdo, C, *et al.* (2015). A knockin mouse model of spinocerebellar ataxia type 3 exhibits prominent aggregate pathology and aberrant splicing of the disease gene transcript. *Human molecular genetics* **24**: 1211-1224.

29. Eckstein, F (1985). Nucleoside phosphorothioates. *Annual review of biochemistry* **54**: 367-402.

30. Eckstein, F (2014). Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic acid therapeutics* **24**: 374-387.

31. Agrawal, S, Jiang, Z, Zhao, Q, Shaw, D, Cai, Q, Roskey, A, *et al.* (1997). Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: in vitro and in vivo studies. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 2620-2625.

32. Sproat, BS, Lamond, AI, Beijer, B, Neuner, P, and Ryder, U (1989). Highly efficient chemical synthesis of 2'-O-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases. *Nucleic acids research* **17**: 3373-3386.

33. Monia, BP, Lesnik, EA, Gonzalez, C, Lima, WF, McGee, D, Guinosso, CJ, *et al.* (1993). Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *The Journal of biological chemistry* **268**: 14514-14522.

34. Rigo, F, Chun, SJ, Norris, DA, Hung, G, Lee, S, Matson, J, *et al.* (2014). Pharmacology of a central nervous system delivered 2'-O-methoxyethyl-modified survival of motor neuron splicing oligonucleotide in mice and nonhuman primates. *The Journal of pharmacology and experimental therapeutics* **350**: 46-55.

35. Owczarzy, R, You, Y, Groth, CL, and Tataurov, AV (2011). Stability and mismatch discrimination of locked nucleic acid-DNA duplexes. *Biochemistry* **50**: 9352-9367.

36. Braasch, DA, and Corey, DR (2001). Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chemistry & Biology* **8**: 1-7.

37. Seth, PP, Siwkowski, A, Allerson, CR, Vasquez, G, Lee, S, Prakash, TP, *et al.* (2009). Short antisense oligonucleotides with novel 2'-4' conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. *Journal of medicinal chemistry* **52**: 10-13.

38. Ittig, D, Liu, S, Renneberg, D, Schumperli, D, and Leumann, CJ (2004). Nuclear antisense effects in cyclophilin A pre-mRNA splicing by oligonucleotides: a comparison of tricyclo-DNA with LNA. *Nucleic acids research* **32**: 346-353.

39. Swayze, EE, Siwkowski, AM, Wancewicz, EV, Migawa, MT, Wyrzykiewicz, TK, Hung, G, *et al.* (2007). Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic acids research* **35**: 687-700.

40. Burel, SA, Hart, CE, Cauntay, P, Hsiao, J, Machemer, T, Katz, M, *et al.* (2016). Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic acids research* **44**: 2093-2109.

41. van Poelgeest, EP, Swart, RM, Betjes, MGH, Moerland, M, Weening, JJ, Tessier, Y, *et al.* (2013). Acute Kidney Injury During Therapy With an Antisense Oligonucleotide Directed Against PCSK9. *American Journal of Kidney Diseases* **62**: 796-800.

42. Shimo, T, Tachibana, K, Saito, K, Yoshida, T, Tomita, E, Waki, R, *et al.* (2014). Design and evaluation of locked nucleic acid-based splice-switching oligonucleotides in vitro. *Nucleic acids research* **42**: 8174-8187.

43. Touznik, A, Maruyama, R, Hosoki, K, Echigoya, Y, and Yokota, T (2017). LNA/DNA mixmer-based antisense oligonucleotides correct alternative splicing of the SMN2 gene and restore SMN protein expression in type 1 SMA fibroblasts. *Scientific Reports* **7**: 3672.

44. Bianchini, D, Omlin, A, Pezaro, C, Lorente, D, Ferraldeschi, R, Mukherji, D, *et al.* (2013). First-in-human Phase I study of EZN-4176, a locked nucleic acid antisense oligonucleotide to exon 4 of the androgen receptor mRNA in patients with castration-resistant prostate cancer. *British journal of cancer* **109**: 2579-2586.

45. Hong, D, Kurzrock, R, Kim, Y, Woessner, R, Younes, A, Nemunaitis, J, *et al.* (2015). AZD9150, a next-generation antisense oligonucleotide inhibitor of STAT3 with early evidence of clinical activity in lymphoma and lung cancer. *Sci Transl Med* **7**: 314ra185.

46. Steffens, R, and Leumann, CJ (1999). Synthesis and Thermodynamic and Biophysical Properties of Tricyclo-DNA. *Journal of the American Chemical Society* **121**: 3249-3255.

47. Goyenvalle, A, Griffith, G, Babbs, A, El Andaloussi, S, Ezzat, K, Avril, A, *et al.* (2015). Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. *Nature medicine* **21**: 270-275.

48. Goyenvalle, A, Leumann, C, and Garcia, L (2016). Therapeutic Potential of Tricyclo-DNA antisense oligonucleotides. *Journal of neuromuscular diseases* **3**: 157-167.

49. Zou, LL, Ma, JL, Wang, T, Yang, TB, and Liu, CB (2013). Cell-penetrating Peptide-mediated therapeutic molecule delivery into the central nervous system. *Current neuropharmacology* **11**: 197-208.

50. Boisguerin, P, Deshayes, S, Gait, MJ, O'Donovan, L, Godfrey, C, Betts, CA, *et al.* (2015). Delivery of therapeutic oligonucleotides with cell penetrating peptides. *Advanced drug delivery reviews* **87**: 52-67.

51. Erazo-Oliveras, A, Muthukrishnan, N, Baker, R, Wang, TY, and Pellois, JP (2012). Improving the endosomal escape of cell-penetrating peptides and their cargos: strategies and challenges. *Pharmaceuticals (Basel, Switzerland)* **5**: 1177-1209.

52. Hache, M, Swoboda, KJ, Sethna, N, Farrow-Gillespie, A, Khandji, A, Xia, S, *et al.* (2016). Intrathecal Injections in Children With Spinal Muscular Atrophy: Nusinersen Clinical Trial Experience. *Journal of child neurology* **31**: 899-906.

53. Miller, TM, Pestronk, A, David, W, Rothstein, J, Simpson, E, Appel, SH, *et al.* (2013). An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. *Lancet Neurol* **12**: 435-442.

54. Du, L, Kayali, R, Bertoni, C, Fike, F, Hu, H, Iversen, PL, *et al.* (2011). Arginine-rich cell-penetrating peptide dramatically enhances AMO-mediated ATM aberrant splicing correction and enables delivery to brain and cerebellum. *Human molecular genetics* **20**: 3151-3160.

55. Hammond, SM, Hazell, G, Shabanpoor, F, Saleh, AF, Bowerman, M, Sleigh, JN, *et al.* (2016). Systemic peptide-mediated oligonucleotide therapy improves long-term survival in spinal muscular atrophy. *Proceedings of the National Academy of Sciences of the United States of America* **113**: 10962-10967.

56. Pinheiro, VB, Taylor, AI, Cozens, C, Abramov, M, Renders, M, Zhang, S, *et al.* (2012). Synthetic genetic polymers capable of heredity and evolution. *Science* **336**: 341-344.

57. Jahns, H, Roos, M, Imig, J, Baumann, F, Wang, Y, Gilmour, R, *et al.* (2015). Stereochemical bias introduced during RNA synthesis modulates the activity of phosphorothioate siRNAs **6**: 6317.

58. Koziolkiewicz, M, Krakowiak, A, Kwinkowski, M, Boczkowska, M, and Stec, WJ (1995). Stereodifferentiation--the effect of P chirality of oligo(nucleoside phosphorothioates) on the activity of bacterial RNase H. *Nucleic acids research* **23**: 5000-5005.

59. Wan, WB, Migawa, MT, Vasquez, G, Murray, HM, Nichols, JG, Gaus, H, *et al.* (2014). Synthesis, biophysical properties and biological activity of second generation antisense oligonucleotides containing chiral phosphorothioate linkages. *Nucleic acids research* **42**: 13456-13468.

60. Iwamoto, N, Butler, DCD, Svrzikapa, N, Mohapatra, S, Zlatev, I, Sah, DWY, *et al.* (2017). Control of phosphorothioate stereochemistry substantially increases the efficacy of antisense oligonucleotides. *Nat Biotechnol* **35**: 845-851.

61. Meade, BR, Gogoi, K, Hamil, AS, Palm-Apergi, C, van den Berg, A, Hagopian, JC, *et al.* (2014). Efficient delivery of RNAi prodrugs containing reversible charge-neutralizing phosphotriester backbone modifications. *Nat Biotechnol* **32**: 1256-1261.

62. Singh, NK, Singh, NN, Androphy, EJ, and Singh, RN (2006). Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron. *Molecular and cellular biology* **26**: 1333-1346.

63. Burghes, AH, and McGovern, VL (2010). Antisense oligonucleotides and spinal muscular atrophy: skipping along. *Genes & development* **24**: 1574-1579.

64. Chiriboga, CA, Swoboda, KJ, Darras, BT, Iannaccone, ST, Montes, J, De Vivo, DC, *et al.* (2016). Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. *Neurology* **86**: 890-897.

65. Finkel, RS, Chiriboga, CA, Vajsar, J, Day, JW, Montes, J, De Vivo, DC, *et al.* (2016). Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. *Lancet* **388**: 3017-3026.

66. Finkel, R, Kuntz, N, Mercuri, E, Chiriboga, CA, Darras, B, Topaloglu, H, *et al.* (2017). Efficacy and safety of nusinersen in infants with spinal muscular atrophy (SMA): Final results from the phase 3 ENDEAR study. *European Journal of Paediatric Neurology* **21**: e14-e15.

67. Ottesen, EW (2017). ISS-N1 makes the First FDA-approved Drug for Spinal Muscular Atrophy. *Translational neuroscience* **8**: 1-6.

68. Mojica, FJ, Diez-Villasenor, C, Garcia-Martinez, J, and Almendros, C (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology (Reading, England)* **155**: 733-740.

69. Sander, JD, and Joung, JK (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotech* **32**: 347-355.

70. Orthwein, A, Noordermeer, SM, Wilson, MD, Landry, S, Enchev, RI, Sherker, A, *et al.* (2015). A mechanism for the suppression of homologous recombination in G1 cells. *Nature* **528**: 422-426.

71. Swiech, L, Heidenreich, M, Banerjee, A, Habib, N, Li, Y, Trombetta, J, *et al.* (2015). In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotech* **33**: 102-106.

72. Suzuki, K, Tsunekawa, Y, Hernandez-Benitez, R, Wu, J, Zhu, J, Kim, EJ, *et al.* (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* **540**: 144-149.

73. Yang, S, Chang, R, Yang, H, Zhao, T, Hong, Y, Kong, HE, *et al.* (2017). CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease. *The Journal of Clinical Investigation* **127**: 2719-2724.

74. Yoshimi, K, Kaneko, T, Voigt, B, and Mashimo, T (2014). Allele-specific genome editing and correction of disease-associated phenotypes in rats using the CRISPR–Cas platform **5**: 4240.

75. Cho, SW, Kim, S, Kim, Y, Kweon, J, Kim, HS, Bae, S, *et al.* (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome research* **24**: 132-141.

76. Schaefer, KA, Wu, W-H, Colgan, DF, Tsang, SH, Bassuk, AG, and Mahajan, VB (2017). Unexpected mutations after CRISPR-Cas9 editing in vivo. *Nat Meth* **14**: 547-548.

77. Evers, MM, Tran, HD, Zalachoras, I, Pepers, BA, Meijer, OC, den Dunnen, JT, *et al.* (2013). Ataxin-3 protein modification as a treatment strategy for spinocerebellar ataxia type 3: removal of the CAG containing exon. *Neurobiology of disease* **58**: 49-56.

78. Moore, LR, Rajpal, G, Dillingham, IT, Qutob, M, Blumenstein, KG, Gattis, D, *et al.* (2017). Evaluation of Antisense Oligonucleotides Targeting ATXN3 in SCA3 Mouse Models. *Molecular therapy Nucleic acids* **7**: 200-210.

79. Alves, S, Nascimento-Ferreira, I, Auregan, G, Hassig, R, Dufour, N, Brouillet, E, *et al.* (2008). Allele-specific RNA silencing of mutant ataxin-3 mediates neuroprotection in a rat model of Machado-Joseph disease. *PloS one* **3**: e3341.

80. Boudreau, RL, Rodriguez-Lebron, E, and Davidson, BL (2011). RNAi medicine for the brain: progresses and challenges. *Human molecular genetics* **20**: R21-27.

81. Hareendran, S, Balakrishnan, B, Sen, D, Kumar, S, Srivastava, A, and Jayandharan, GR (2013). Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. *Reviews in medical virology* **23**: 399-413.

82. Schmitt, I, Linden, M, Khazneh, H, Evert, BO, Breuer, P, Klockgether, T, *et al.* (2007). Inactivation of the mouse Atxn3 (ataxin-3) gene increases protein ubiquitination. *Biochemical and biophysical research communications* **362**: 734-739.

83. Switonski, PM, Fiszer, A, Kazmierska, K, Kurpisz, M, Krzyzosiak, WJ, and Figiel, M (2011). Mouse ataxin-3 functional knock-out model. *Neuromolecular Med* **13**: 54-65.

84. Rodrigues, A-J, do Carmo Costa, M, Silva, T-L, Ferreira, D, Bajanca, F, Logarinho, E, *et al.* (2010). Absence of ataxin-3 leads to cytoskeletal disorganization and increased cell death. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1803**: 1154-1163.

85. Neves-Carvalho, A, Logarinho, E, Freitas, A, Duarte-Silva, S, Costa Mdo, C, Silva-Fernandes, A, *et al.* (2015). Dominant negative effect of polyglutamine expansion perturbs normal function of ataxin-3 in neuronal cells. *Human molecular genetics* **24**: 100-117.

86. Liu, J, Yu, D, Aiba, Y, Pendergraff, H, Swayze, EE, Lima, WF, *et al.* (2013). ss-siRNAs allele selectively inhibit ataxin-3 expression: multiple mechanisms for an alternative gene silencing strategy. *Nucleic acids research* **41**: 9570-9583.

87. Cutler, RW, Page, L, Galicich, J, and Watters, GV (1968). Formation and absorption of cerebrospinal fluid in man. *Brain : a journal of neurology* **91**: 707-720.

88. Ashkenazi, A, Bento, CF, Ricketts, T, Vicinanza, M, Siddiqi, F, Pavel, M, *et al.* (2017). Polyglutamine tracts regulate beclin 1-dependent autophagy. *Nature* **545**: 108-111.

89. Crosby, JR, Zhao, C, Zhang, H, MacLeod, AR, Guo, S, and Monia, BP (2015). Reversing Antisense Oligonucleotide Activity with a Sense Oligonucleotide Antidote: Proof of Concept Targeting Prothrombin. *Nucleic acid therapeutics* **25**: 297-305.