The Genetics of *C9orf72* Expansions

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Repeat expansions in the promoter region of *C9orf72* are the most common genetic cause of amyotrophic lateral sclerosis (ALS) and related disorders of the ALS/frontotemporal lobar degeneration (FTLD) spectrum. Remarkable clinical heterogeneity among patients with a repeat expansion has been observed, and genetic anticipation over different generations has been suggested. Genetic factors modifying the clinical phenotype have been proposed, including genetic variation in other known disease genes, the genomic context of the *C9orf72* repeat, and expanded repeat size, which has been estimated between 45 and several thousand units. The role of variability in normal and expanded repeat sizes for disease risk and clinical phenotype is under debate. Different pathogenic mechanisms have been proposed, including loss of function, RNA toxicity, and dipeptide repeat (DPR) protein toxicity resulting from abnormal translation of the expanded repeat, but the major mechanism is yet unclear.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease for which effective therapies aimed at delaying, halting, or preventing the disease are lacking. ALS patients show reduced control of voluntary muscle movement expressed in increased muscle weakness and disturbances in speech, swallowing, or breathing as a result of progressive upper and lower motor-neuron degeneration in the motor cortex, brain stem, and spinal cord. Up to 50% of ALS patients show mild disturbances in executive functions, whereas a minority also develops overt frontotemporal lobar degeneration (FTLD), presenting with either changes in personality and social conduct or language problems (Neary et al. 1998; Lomen-Hoerth et al. 2003; Ringholz et al. 2005). ALS is the most common neurodegenerative motor-neuron dis-

order (Rowland and Shneider 2001), and in the age group of <65 years, FTLD is the second most common neurodegenerative dementia after Alzheimer's disease (Rosso et al. 2003). There are families and individual patients in which both diseases occur (ALS-FTD) (Lomen-Hoerth et al. 2002), and TDP-43 inclusions (Arai et al. 2006; Neumann et al. 2006) in ALS and FTLD patients are indistinguishable (Tsuji et al. 2012), despite the pathological distribution being different for ALS and FTLD patients. There is ample evidence that common disease pathways are involved in ALS and FTLD because their clinical and pathological hallmarks overlap; hence, the pure forms of these diseases are considered the two extremes of one disease continuum (Lillo and Hodges 2009). In 10% of ALS patients and nearly 50%

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of FTLN patients, familial aggregation has been observed, suggesting a strong genetic component. Genetic studies identified mutations in the same genes in FTLN and ALS—for example, *TBK1*, *TARDBP*, *FUS*, *VCP* (Neumann et al. 2006; Kovacs et al. 2009; Johnson et al. 2010; Van Langenhove et al. 2010; Cirulli et al. 2015; Freischmidt et al. 2015; Pottier et al. 2015). The most convincing genetic evidence for a common disease pathomechanism was provided by the identification of the repeat expansion mutations in *C9orf72* in patients with ALS, FTLN, and ALS-FTD (Gijselinck et al. 2010; DeJesus-Hernandez et al. 2011; Renton et al. 2011).

IDENTIFICATION OF *C9orf72* REPEAT EXPANSIONS

In 2006, genetic linkage was reported at chromosome 9p21.3–9p21.1 in families with concomitant ALS and FTLN (Momeni et al. 2006; Morita et al. 2006; Vance et al. 2006). Since then, several families have been reported with conclusive or suggestive linkage to overlapping genomic regions (for review, see Gijselinck et al. 2012a). Despite the relatively small 3.6-Mb genomic segment shared by all conclusively linked families and major gene sequencing efforts by multiple research groups, the genetic defect remained long undetected. When genome-wide association studies in both ALS (van Es et al. 2009; Laaksovirta et al. 2010; Shatunov et al. 2010; Gijselinck et al. 2012b) and FTLN-TDP (Van Deerlin et al. 2010) identified a risk haplotype spanning three genes within the linked region, intensive mutation-detection strategies resulted in the identification of a pathological expansion of a noncoding G₄C₂ repeat in the 5' region of *C9orf72* (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Gijselinck et al. 2012b), which explained both linkage and association (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Gijselinck et al. 2012b).

STRUCTURE OF THE *C9orf72* GENE

C9orf72 is transcribed in three major transcripts encoding two protein isoforms (C9orf72 a and b) (Fig. 1). A long isoform (481 amino acids) of

the C9orf72 protein, isoform a, is encoded by transcript variants 1 and 3, whereas the shorter isoform b (222 amino acids) is encoded by transcript variant 2. Owing to the use of alternative first noncoding exons, the G₄C₂ repeat is either located in the upstream regulatory region of transcript 2 or in the first intron, following a noncoding exon 1, of transcripts 1 and 3 (Fig. 1). Based on ENCODE data, the repeat sequence is part of the functional core promoter of all three *C9orf72* transcripts (Fig. 1) (Gijselinck et al. 2012b). The function of the gene is currently unclear, but it may play a role in autophagy and endosomal trafficking (Farg et al. 2014), and may be involved in regulating endoplasmic reticulum stress (Zhang et al. 2014).

FREQUENCY OF *C9orf72* REPEAT EXPANSIONS

In both ALS (ALS Online Genetics Database [Abel et al. 2012]) and FTLN (AD and FTLN Mutation Database [Cruts et al. 2012]), *C9orf72* repeat expansions are the most common mutation compared with other known genes. The highest mutation frequencies were recorded in familial patients with combined ALS and FTLN symptoms and a positive family history of these disorders. In the group of individuals with ALS-FTD, *C9orf72* G₄C₂ repeat expansions are, together with *TBK1* mutations, the only known common genetic causes. Numerous *C9orf72* repeat expansion studies worldwide have confirmed that the G₄C₂ repeat expansion is the most common cause of disease in the ALS/FTLN continuum. When comparing mutation rates of known ALS genes, *C9orf72* repeat expansions occurred one to two times more frequently than *SOD1* mutations, together explaining 18%–50% of familial ALS patients (Smith et al. 2013; Stewart et al. 2012). Nevertheless, especially in ALS, in which family history is observed in only 10% of patients, sporadic ALS patients who carry an expanded repeat outnumber familial ALS patients. It is important to note that the frequency of *C9orf72* G₄C₂ hexanucleotide repeat expansions greatly depends on ethnicity and geographical region. Globally, frequencies are the highest in the Caucasian popu-

Figure 1. Genomic organization of *C9orf72* and the G_4C_2 hexanucleotide repeat at chromosome 9p21. (A) The *C9orf72* gene and its major transcript variants numbered according to the National Center for Biotechnology Information (NCBI) RefSeq transcript collection. The red vertical line indicates the location of the noncoding G_4C_2 hexanucleotide repeat. The chromatin state illustrates transcriptional activity with the red bar indicating the active promoter based on combined analysis of histone marks in lymphoblastoid cell line GM12878 (Ernst et al. 2011). Black and blue boxes represent transcribed segments: wide boxes are coding, and narrow boxes are untranslated exonic regions. Horizontal connecting lines represent intronic regions. The black box labeled "B" is shown in detail in B. (B) Detailed view of the genomic context of hexanucleotide repeat sequence. The bar chart indicates relative 11 bp windowed GC content showing the contiguous GC-rich (>55%) low-complexity sequence in dark gray. Red boxes represent individual hexanucleotide repeat units in human reference genome sequence build GRCh37/hg19, with light red areas indicating nucleotide mismatches in the G_4C_2 consensus sequence. The gray box shows the common deletion in the 3' flanking low-complexity sequence (LCS) containing the GTGGT motif, as frequently observed in expansion carriers. The blue box indicates the first non-coding exon of *C9orf72* transcript variant 2, and blue lines indicate intronic regions.

lations of Europe and North America, ranging from 7% to 28% in ALS, 3.5% to 18% in FTL, and 15% to more than 50% in ALS-FTD (Cruts et al. 2013). In European populations, frequencies were markedly elevated in ALS patients from Scandinavian countries (Majounie et al. 2012; Lindquist et al. 2013; Smith et al. 2013; van der Zee et al. 2013). In strong contrast, pathological repeat expansions were rarely observed in Asian populations, with frequencies of 0.4%–4.8% in ALS cases (Majounie et al. 2012; Tsai et al. 2012; Jang et al. 2013; Konno et al. 2013; Zou et al. 2013).

CLINICOPATHOLOGICAL HETEROGENEITY

Patients with a *C9orf72* repeat expansion clinically present with widely variable symptoms on the ALS/FTLD spectrum, including ALS, FTL, and ALS-FTD. The clinical characteristics of motor neuron disease (MND) associated with expanded *C9orf72* repeats are in almost all instances compatible with a diagnosis of ALS. Spinal symptoms affecting muscles of the limbs

were observed in ~60% of reported repeat-expansion carriers diagnosed with ALS. Bulbar symptoms affecting muscles required for swallowing or speech were predominant in ~40% and were significantly more frequent than the observed 25% in general ALS (Kiernan et al. 2011; Stewart et al. 2012). A generalized spino-bulbar onset was seen in ~1% (Brettschneider et al. 2012b; Millecamps et al. 2012; Smith et al. 2013). Dementia was enriched in ALS patients with a *C9orf72* repeat expansion (Smith et al. 2013), with the dementia type usually being FTL (Stewart et al. 2012). The clinical FTL subtype was most often of the behavioral type, although progressive nonfluent aphasia (PNFA) ALS was also observed (Van Langenhove et al. 2013). Minor behavioral or cognitive abnormalities not meeting diagnostic criteria of FTL were even more common (Byrne et al. 2012). In an extended European study, the fraction of ALS patients with symptoms of FTL tripled from 10% in general ALS to 27% in repeat expansion carriers (Smith et al. 2013).

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Figure 2. Putative, mutually nonexclusive pathological mechanisms associated with the hexanucleotide repeat expansion in *C9orf72*. (1) Haploinsufficiency resulting from reduced expression of the gene copy located on the repeat-containing haplotype (e.g., due to altered binding of transcription factors, DNA methylation, and/or position effects). (2) RNA gain-of-function toxicity due to the formation of aggregates of repeat-containing RNA species and sequestered RNA-binding proteins. (3) Abnormal bidirectional transcription of expanded repeat sequences followed by repeat-associated non-ATG-initiated (RAN) translation into aggregation-prone dipeptide repeat proteins (DPRs).

ALS patients with a *C9orf72* repeat expansion have neuronal cytoplasmic inclusions (NCI) immunoreactive to TDP-43 present in the spinal cord (Al-Sarraj et al. 2011). Compared with noncarriers, repeat expansion carriers showed a tendency toward more extensive microglial pathology in the medulla and motor cortex, which might correlate with the clinical enrichment of bulbar disease onset (Bretschneider et al. 2012a). In ALS patients without clinical signs of FTL, extramotor neuronal TDP-43 pathology was limited (Al-Sarraj et al. 2011; Murray et al. 2011; Stewart et al. 2012). In addition to TDP-43 inclusions, p62 immunoreactive star-like inclusions have been associated with TDP-43 proteinopathies, including ALS-FTD linked to chromosome 9 (Boxer et al. 2011). p62 inclusions have a wider distribution

than TDP-43 inclusions and far exceed them in number as well (Al-Sarraj et al. 2011; King et al. 2011; Troakes et al. 2011; Stewart et al. 2012; Simon-Sanchez et al. 2012). p62 inclusions are composed of dipeptide repeat (DPR) proteins resulting from unconventional repeat-associated non-ATG-initiated (RAN) translation of the expanded *C9orf72* repeat in different reading frames (Fig. 2) (Ash et al. 2013; Mori et al. 2013c). In contrast to TDP-43 pathology, which might fit into different subclasses or even be lacking (Gijselinck et al. 2012b; Snowden et al. 2012a), DPR pathology is highly specific for *C9orf72* positive cases.

Irrespective of the presenting symptoms, onset age is highly variable, ranging from 27 (Majounie et al. 2012; Simon-Sanchez et al. 2012) to 83 (Sabatelli et al. 2012; Simon-San-

chez et al. 2012) years in ALS patients and from 30 to 76 years in FTLN (Majounie et al. 2012) patients. Also, carriers living up to 76 years without signs of disease were reported (Gijssels et al. 2012b; Van Langenhove et al. 2013). Survival time was strongly associated with clinical diagnosis. For ALS, *C9orf72* repeat expansions were associated with a disease duration between 3 and 96 months (Cooper-Knock et al. 2012; Millicamps et al. 2012; Stewart et al. 2012), whereas in FTLN patients, disease duration ranged from 1 to 22 years (DeJesus-Hernandez et al. 2011; Mahoney et al. 2012; Snowden et al. 2012b; Simon-Sanchez et al. 2012; van der Zee et al. 2013; Van Langenhove et al. 2013). As expected, when ALS symptoms became apparent, survival was dramatically compromised to an average of 1.8 years (Van Langenhove et al. 2013).

In recent studies of families segregating a G_4C_2 expansion, evidence showed a decreasing onset age of 7–11 years in each younger generation, which suggests possible genetic anticipation of the disease (Arighi et al. 2012; Boeve et al. 2012; Chió et al. 2012a; Ferrari et al. 2012; Gijssels et al. 2012b; Hsiung et al. 2012; Stewart et al. 2012; Van Langenhove et al. 2013).

***C9orf72* MODIFYING FACTORS: REPEAT SIZE VARIABILITY**

The variability in clinical phenotype as described in the previous section is reminiscent of the presence of modifying factors of *C9orf72* expansions and for genetic anticipation within families. Genetic modifiers have been proposed, including *TMEM106B*, which has been associated with a later age at onset and death in patients with FTLN (Gallagher et al. 2014) and with protecting carriers from developing FTLN (van Blitterswijk et al. 2014b), and *ATXN2*, which might predispose patients to the development of ALS rather than FTLN (van Blitterswijk et al. 2014a) and which could lead to the development of clinical signs featuring both FTLN and ALS in the presence of a *C9orf72* repeat expansion (Lattante et al. 2014).

In most repeat expansion disorders, however, the size of a pathological repeat influences

the expression of the clinical phenotype. For example, in myotonic dystrophy type I (Ashizawa et al. 1992; Harley et al. 1993; Gennarelli et al. 1996), the increasing number in successive generations of CTG units, located in the non-coding region of *DMPK*, correlates with more severe symptoms and an earlier age at onset. Also, in Friedreich's ataxia (FRDA), the onset age is influenced by the size of a GAA expansion within the first intron of *FXN* (Filla et al. 1996).

The size of the *C9orf72* hexanucleotide repeats ranges from two repeat units to more than 4000 (DeJesus-Hernandez et al. 2011; Ishiura et al. 2012; Beck et al. 2013; Buchman et al. 2013; Dobson-Stone et al. 2013; Dols-Icardo et al. 2013; van Blitterswijk et al. 2013; Hubers et al. 2014; Waite et al. 2014; Gijssels et al., 2015). Normal repeat sizes range between 2 and 24 units (Majounie et al. 2012; van der Zee et al. 2013), with those of 7–24 (van der Zee et al. 2013), 20–22 (Gomez-Tortosa et al. 2013), 12–21 (Benussi et al. 2014), 20–29 (Xi et al. 2012), 7–30 (Pamphlett et al. 2012), or 20–30 units (Nuytemans et al. 2013) considered as intermediate alleles. The pathogenic nature of the repeat depends on its size, but setting a sharp size cutoff between normal and pathogenic alleles is complicated. Some studies consider repeats of >30 units as pathogenic (Renton et al. 2011), whereas others use a cutoff of 60 units (Gijssels et al. 2012b) depending on the upper limit of the repeat-primed polymerase chain reaction (PCR) detection method, which allows the detection of an expanded repeat allele as an allele being larger than about 30–60 repeat units in size, without further indication of the exact repeat length.

Exact sizing of the expanded G_4C_2 repeat has been limited because of its 100% GC content (Fig. 1), its large size, and the repetitive nature of its flanking sequences. Southern blot hybridization studies visualized the expanded alleles and estimated that the size of most repeat expansions ranged between several hundred and several thousand repeat units (DeJesus-Hernandez et al. 2011; Ishiura et al. 2012; Beck et al. 2013; Buchman et al. 2013; Dobson-Stone et al. 2013; Dols-Icardo et al. 2013; van Blitterswijk et al. 2013; Hubers et al. 2014; Waite et al. 2014;

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Gijselinck et al. 2016). The shortest pathological G₄C₂ expansion outside this pathogenic size range remains elusive. G₄C₂ hexanucleotide repeat sizes of 25–60 repeat units were rarely observed in ALS, FTLN, and related disorders (Dobson-Stone et al. 2012, 2013; Ratti et al. 2012; Simon-Sanchez et al. 2012; Xi et al. 2012; Beck et al. 2013; Garcia-Redondo et al. 2013; van Blitterswijk et al. 2013; Waite et al. 2014; Nordin et al. 2015); however, in most of the studies, cosegregation with disease or other arguments for pathogenicity were not observed in families, and older, healthy individuals heterozygous for alleles in the same size range have been reported (Xi et al. 2012). One study using Southern blot and a PCR assay to detect the size of short expansions up to 80 units identified short expansion sizes between 45 and 78 units in blood of 5.1% of expansion carriers cosegregating with disease and showing the same FTLN-TDP and DPR pathology as those with a long expansion of at least a few hundred units (Gijselinck et al. 2016). This indicates that short expansions might have the same pathogenic effect as long expansions. However, in different brain regions of the short expansion carrier, a pool of short and long expansion sizes was apparent, pointing to somatic mosaicism (Gijselinck et al. 2016). Several studies reported instability of the repeat from 16 units on due to somatic mosaicism across different tissues (Beck et al. 2013; van Blitterswijk et al. 2013; Nordin et al. 2015), which complicates determination of the repeat pathogenicity based on repeat sizing in blood-derived DNA. However, intra-individual variation of repeat number between tissues was higher than the variation within each tissue group (Nordin et al. 2015).

Nevertheless, none of the published Southern blot hybridization techniques are suitable to detect all repeat expansion sizes accurately (DeJesus-Hernandez et al. 2011; Ishiura et al. 2012; Beck et al. 2013; Buchman et al. 2013; Dobson-Stone et al. 2013; van Blitterswijk et al. 2013; Nordin et al. 2015; Gijselinck et al. 2016). Often, because of somatic mosaicism, repeat expansions present as smears or as several distinct bands instead of single discrete bands. In addition, estimating repeat expansion sizes of

>15 kb could not be determined accurately on normal agarose gel because of the lack of resolution in this size range. Furthermore, not all protocols could detect short expansion sizes (Beck et al. 2013). Therefore, no consensus could be established on the exact correlation between repeat size and onset age (Beck et al. 2013; Dols-Icardo et al. 2013; van Blitterswijk et al. 2013; Hubers et al. 2014; Waite et al. 2014; Nordin et al. 2015; Gijselinck et al. 2016). Three of these studies suggested a positive correlation of onset age with repeat size in the frontal cortex of FTLN patients (van Blitterswijk et al. 2013), in the cerebellum and parietal lobe of ALS patients (Nordin et al. 2015), or in the blood of expansion carriers with diverse neurodegenerative diseases (Beck et al. 2013). In contrast, one study compared two groups of short (45–80 units) and long (>80 units) expansion carriers and found a significant association between longer repeat expansion size and earlier onset age (Gijselinck et al. 2016). The use of a higher resolution gel electrophoresis technique (e.g., pulsed field gel electrophoresis) or more advanced genome-mapping technologies (e.g., the Irys technology of BioNano Genomics) would be useful to perform more detailed sizing of the very large expanded repeats and to detect expansions with higher throughput and sensitivity. Furthermore, implementation of single-molecule DNA sequencing technologies producing long sequence reads (e.g., Pacific Biosciences, Oxford Nanopore Technologies) will hopefully unravel the sequence of repeat expansions because interruptions in the repeat sequence might influence its stability, pathogenicity, and clinical manifestation in the patient.

Studies of repeat sizes between generations in *C9orf72* families are almost lacking. One study identified a family with suggestive evidence for genetic anticipation over three generations (Gijselinck et al. 2016). Also, in several informative *C9orf72* parent–child transmissions, Gijselinck et al. identified earlier onset ages, increasing expansion sizes, and/or increasing the methylation state of the CpG island 5′ flanking of the G₄C₂ repeat across two generations in 90% of parent–offspring pairs. These data were reminiscent of genetic anticipation in

C9orf72 families because, next to the association between expansion size and onset age, they also found a positive association between methylation state of the 5' flanking CpG island and expansion size (see the following section).

C9orf72 PROMOTER METHYLATION

Hypermethylation of CpG-rich promoters has been associated with transcriptional silencing in noncoding repeat expansion disorders like, for example, fragile X syndrome (Oberle et al. 1991; Sutcliffe et al. 1992; Knight et al. 1993; Willemssen et al. 2011), and the CpG methylation state has been directly correlated with repeat expansion size in FRDA (Castaldo et al., 2008). The *C9orf72* G₄C₂ repeat is located in a region with high GC content (Fig. 1). Upstream of the G₄C₂ repeat, a CpG island is located showing hypermethylation in expansion carriers compared with normal repeat carriers in the blood, brain, and spinal cord (Xi et al. 2013, 2014; Belzil et al. 2014; Liu et al. 2014; Russ et al. 2014; Gijselinck et al. 2016). One study found a marginally significant association between hypermethylation and shorter repeat expansion size in the blood only (Russ et al. 2014), whereas another study showed a significant association between increased methylation and longer repeat expansion sizes in the blood and the brain, the latter finding correlating with an earlier disease onset (Gijselinck et al. 2016). They also suggested an association between hypermethylation of the G₄C₂ repeat itself and an increasing repeat length, which was confirmed by others using a qualitative assay (Xi et al. 2015). In addition, trimethylation of lysine residues on H3 and H4 histones was found in expansion carriers (Belzil et al. 2014).

Further investigation and replication studies of correlations of age at onset and methylation with repeat size in large cohorts are needed.

INCREASING RISK ASSOCIATED WITH INTERMEDIATE REPEAT ALLELES AND SMALL INDELS IN FLANKING REGION

The risk of carrying a normal intermediate repeat allele is yet unclear. Several studies in ALS

and FTLN patients found no association between repeat number on nonexpanded alleles and disease risk and/or clinical presentation (Rutherford et al. 2012; Garcia-Redondo et al. 2013). In contrast, a 10-repeat allele was associated with four different neurodegenerative diseases including ALS and FTLN (Xi et al. 2012). Also, in Parkinson's disease, intermediate alleles between 20 and 30 units were associated with disease risk in a Caucasian study (Nuytemans et al. 2013); however, this finding could not be replicated in a global study (Theuns et al. 2014). In addition, homozygous carriers of intermediate repeat alleles (7–24 units) showed a significantly increased risk for ALS and ALS-FTD (Gijselinck et al. 2016). These observations might be explained by the significant gradual decrease of *C9orf72* transcriptional activity in vitro with an increasing number of G₄C₂ intermediate repeat units compared with the normal reference allele of 2 repeat units in human kidney and neuroblastoma cell lines (van der Zee et al. 2013; Gijselinck et al. 2016). In accordance, intermediate repeat carriers showed a significantly higher methylation degree of the flanking CpG island and the repeat itself compared with normal short repeats.

Small insertion and deletion polymorphisms (indels) were identified in the GC-rich low-complexity sequence (LCS) downstream from the G₄C₂ repeat (van der Zee et al. 2013; Rollinson et al. 2015), showing an increased frequency in G₄C₂ expansion carriers compared with normal repeat carriers (Fig. 1) (van der Zee et al. 2013). Moreover, in a Belgian ALS, ALS-FTD, and FTLN patient cohort without *C9orf72* expansion, the frequency of a flanking indel was significantly higher than in the control cohort, suggesting the risk increasing effect on disease (Gijselinck et al. 2016). Indeed, a GTGGT deletion and a CGGGCGGGCCCG GGGCGGGCC deletion showed a highly significant decrease in *C9orf72* promoter activity in vitro, thereby possibly affecting essential core promoter elements (Gijselinck et al. 2016).

ORIGIN OF *C9orf72* EXPANSIONS

It is yet unclear by which mechanism a normal G₄C₂ repeat expands to a pathological size. Be-

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cause of the tight genetic association of repeat expansions with a certain SNP-haplotype, it was hypothesized that all *C9orf72* expansions are derived from a single common founder expansion on this risk haplotype (Majounie et al. 2012; Mok et al. 2012; Pliner et al. 2014). However, several lines of evidence suggest that the genomic context of the haplotype itself might render the repeat unstable, resulting in many expansion events on a predisposing haplotype. First, the appearance of pathological G_4C_2 expansions in apparently sporadic patients supports this hypothesis. Also, the absence of shared microsatellite haplotypes in small geographical regions implicated no recent shared ancestry of a large proportion of *C9orf72* carriers (Beck et al. 2013). Furthermore, G_4C_2 intermediate repeats are significantly overrepresented on the same risk haplotype (DeJesus-Hernandez et al. 2011; van der Zee et al. 2013), suggesting that intermediate repeats are more prone to replication slippage triggering pathological expansions. Also, the overrepresentation of small indels in the 3' flanking LCS of expansion carriers containing a deletion of a GTGGT motif in most cases possibly makes the repeat more prone to pathological expansion because of the formation of a longer imperfect G_4C_2 repeat (Fig. 1) (van der Zee et al. 2013).

DISEASE MECHANISMS

Different pathogenic mechanisms were proposed including loss of function, RNA toxicity, and DPR toxicity resulting from abnormal translation of the expanded repeat, but their relative contribution is yet unclear (Fig. 2).

In patients carrying a G_4C_2 expansion, allele-specific reduction of *C9orf72* expression in brain tissue (DeJesus-Hernandez et al. 2011; Gijssels et al. 2012b; Belzil et al. 2013; Ciura et al. 2013; Fratta et al. 2013; Waite et al. 2014), hypermethylation of the CpG island flanking the G_4C_2 repeat, and the G_4C_2 repeat itself in intermediate and expanded repeat carriers (Xi et al. 2013, 2014, 2015; Belzil et al. 2014; Liu et al. 2014; Russ et al. 2014; Gijssels et al. 2016), reduced promoter activity of intermediate alleles (van der Zee et al. 2013; Gijssels et al.

2016), and histone trimethylation (Belzil et al. 2013) were observed, suggesting a loss-of-function mechanism through transcriptional silencing of the promoter, as seen in other repeat expansion disorders like fragile X syndrome (Fig. 2) (Oberle et al. 1991; Sutcliffe et al. 1992; Knight et al. 1993; Gecz et al. 1996; Gu et al. 1996). An association between hypermethylation of a CpG island flanking an expanded repeat and repeat expansion size has also been found in the first intron of the *FRDA* gene *FXN*, which could possibly be correlated with reduced mRNA levels (Castaldo et al. 2008; Evans-Galea et al. 2012). In favor of this loss-of-function hypothesis are two *C9orf72* loss-of-function models: a zebra fish knock-down model showing axonal degeneration of motor neurons (Ciura et al. 2013) and a *Caenorhabditis elegans* knockout model displaying age-dependent paralysis and neurodegeneration of GABA-ergic motor neurons (Therrien et al. 2013).

On the other hand, a toxic gain-of-function mechanism is supported by studies identifying sense and antisense RNA foci and DPR protein aggregates produced by the expanded *C9orf72* G_4C_2 repeat in human neurons of different tissues involved in FTL and ALS in vivo (DeJesus-Hernandez et al. 2011; Ash et al. 2013; Donnelly et al. 2013; Gendron et al. 2013; Lagier-Tourenne et al. 2013; Lee et al. 2013; Mizielinska et al. 2013; Mori et al. 2013a,c; Zu et al. 2013) and RNA foci in induced pluripotent stem-cell (iPSC)-derived human neurons (Fig. 2) (Almeida et al. 2013; Donnelly et al. 2013; Sareen et al. 2013). In addition, RNA-binding proteins were sequestered in the pathological deposits of repeat expansion carriers like hnRNPA3 (Donnelly et al. 2013; Lee et al. 2013; Mori et al. 2013b; Xu et al. 2013). Moreover, RNA foci burden in the frontal cortex showed a significant inverse correlation with onset age (Mizielinska et al. 2013) and repeat length (Mizielinska et al. 2014). Also, a later onset age was observed in short repeat expansion carriers than in long repeat expansion carriers (Gijssels et al. 2016), which might be in favor of a toxicity mechanism as unstable, growing repeat lengths will result in a gradually more harmful effect, as seen in other repeat expansion diseases

like myotonic dystrophy type I (Ashizawa et al. 1992; Harley et al. 1993; Gennarelli et al. 1996).

Some observations might be benign side effects, but, more likely, different mechanisms acting on specific transcripts together are involved in the disease process (reviewed in Gendron et al. 2014; see also Gendron and Petrucelli 2017). Alternatively, hypermethylation might be a rescue mechanism to prevent the formation of RNA foci (Liu et al. 2014) and might therefore be neuroprotective (Russ et al. 2014; McMillan et al. 2015).

Furthermore, increasing evidence suggests the role of DPR proteins in neurodegeneration. DPR toxicity, in particular of arginine-rich DPRs, was shown in a *Drosophila* model (Mizielinska et al. 2014), and DPR neurotoxicity was observed in primary neurons (May et al. 2014; Wen et al. 2014). Modifier screens of the DPR toxicity in *Drosophila* and yeast suggested involvement of nucleocytoplasmic transport defects (Jovičić et al. 2015; Boeynaems et al. 2016).

DOUBLE GENE HITS

A pathological *C9orf72* repeat expansion was repeatedly detected in patients who also carry a mutation in another Mendelian disease gene. Especially in ALS, occurrences of double gene hits is not uncommon: mutations in *TARDBP* (Chió et al. 2012b; Cooper-Knock et al. 2012; van Blitterswijk et al. 2012), *TBK1* (Van Mossevelde et al. 2016), *FUS* (Millecamps et al. 2012; van Blitterswijk et al. 2012), *SOD1* (Millecamps et al. 2012; van Blitterswijk et al. 2012), *OPTN* (Cooper-Knock et al. 2012; Millecamps et al. 2012), *ANG* (Millecamps et al. 2012), *UBQLN2* (Millecamps et al. 2012), *DAO* (Millecamps et al. 2012), *GRN* (Ferrari et al. 2012), *SQSTM1* (Almeida et al. 2016), and *PSEN2* (Ferrari et al. 2012b) have been reported, including mutations of proven clinical significance. One study has noted an exceptionally early onset age of 37 years in an ALS patient carrying a pathological *C9orf72* repeat expansion and an established *TARDBP* Ala321Val mutation, suggesting a synergistic effect of both genetic changes on the disease pathogenesis (Cooper-Knock et al. 2012). Similarly, in a family segregating the

TBK1 Glu643del mutation and a *C9orf72* repeat expansion, two siblings carrying both mutations had a remarkably early onset age of 41 and 51 years, respectively, whereas a sibling carrying only the repeat expansion was clinically asymptomatic at age 62 (Van Mossevelde et al. 2016). Further research of the pathogenic nature of these double mutations not only will provide information on the contribution of *C9orf72* to disease in these patients, but also will shed new light on the impact of specific mutations in the previously established ALS and FTLN genes.

BIOMARKERS AND THERAPEUTIC STRATEGIES

Sensitive and specific biomarkers are extremely helpful in earlier diagnosis, evaluation of disease progression, and assessment of drug efficacy during clinical trials aimed at treating the disease. To date, no biomarkers for *C9orf72* expansions have been validated. One study specifically detected the GP DPR protein in the cerebrospinal fluid of *C9orf72* expansion carriers, which could potentially serve as a biomarker for therapies targeting RNA foci or DPRs (Su et al. 2014). In addition, a functional magnetic resonance imaging study revealed promising data in detecting early stage altered network connectivity in FTLN patients with a *C9orf72* expansion (Lee et al. 2014). Also, the use of antisense oligonucleotides targeting and decreasing RNA foci will be promising as a potential therapeutic approach (Donnelly et al. 2013; Lagier-Tourenne et al. 2013; Sareen et al. 2013). Furthermore, the correlation of repeat size with onset age and methylation state of the *C9orf72* region indicates that methylation might serve as a potential biomarker. Aberrant DNA methylation is becoming a promising therapeutic target in FTLN and ALS since hypermethylation of the *GRN* promoter was shown to be involved in FTLN (Banzhaf-Strathmann et al. 2013), and several reports have suggested that abnormal DNA methylation might be involved in ALS disease mechanisms (Morahan et al. 2009; Martin 2010; Chestnut et al. 2011).

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CONCLUDING REMARKS

In conclusion, repeat expansions in the promoter region of *C9orf72* are the most common genetic cause of the ALS/FTLD spectrum of diseases. Understanding the effect of the variability in normal and expanded repeat size and repeat sequence content on disease risk and clinical phenotype is of major importance in assessing clinical presentation, disease risk, and severity, and in providing better diagnostic guidelines for molecular genetic testing and counseling. Elucidating the exact disease mechanism(s) will have a major impact on understanding the underlying biology of ALS, FTLD, and related disorders and will contribute to the development of new therapeutic targets leading to relevant therapies for halting or preventing the disease.

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The Genetics of *C9orf72* Expansions

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