Chapter 1
An Epigenetic Spin to ALS and FTD

Mark T. W. Ebbert, Rebecca J. Lank, and Veronique V. Belzil

Abstract Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two devastating and lethal neurodegenerative diseases seen comorbidly in up to 15% of patients. Despite several decades of research, no effective treatment or disease-modifying strategies have been developed. We now understand more than before about the genetics and biology behind ALS and FTD, but the genetic etiology for the majority of patients is still unknown and the phenotypic variability observed across patients, even those carrying the same mutation, is enigmatic. Additionally, susceptibility factors leading to neuronal vulnerability in specific central nervous system regions involved in disease are yet to be identified. As the inherited but dynamic epigenome acts as a cell-specific interface between the inherited fixed genome and both cell-intrinsic mechanisms and environmental input, adaptive epigenetic changes might contribute to the ALS/FTD aspects we still struggle to comprehend. This chapter summarizes our current understanding of basic epigenetic mechanisms, how they relate to ALS and FTD, and their potential as therapeutic targets. A clear understanding of the biological mechanisms driving these two currently incurable diseases is urgent—well-needed therapeutic strategies need to be developed soon. Disease-specific epigenetic changes have already been observed in patients and these might be central to this endeavor.

Keywords Amyotrophic lateral sclerosis · Epigenetic modifications · Frontotemporal dementia · Methylation · RNA-mediated regulation

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1.1 Introduction

Amyotrophic lateral sclerosis (ALS) is the most prevalent motor neuron disease, causing progressive degeneration of upper and lower motor neurons in 2–3 individuals per 100,000 worldwide [1, 2]. Clinically, ALS is characterized by rapidly progressing muscle weakness and spasticity leading to paralysis and eventually respiratory failure [3]. Patients present with symptoms at a mean age of 55 years and die within 2–4 years [4].

Patients with ALS are also frequently affected by the second most common form of early-onset dementia known as frontotemporal dementia (FTD). While patient symptoms will start with either ALS or FTD, 13–15% of patients will eventually develop symptoms for the other disease [5–9]. This strong link between ALS and FTD was observed before researchers were able to link them genetically. FTD has a prevalence of 15–22 individuals per 100,000 worldwide [10] and is characterized by disturbances in behavior, personality, and language as a result of neurodegeneration in the frontal and temporal lobes. Cognitive impairments are usually detected around age 58, and it is fatal within 6–10 years after symptoms onset [11].

Historically, researchers have extensively searched for the underlying genetic etiologies for these conditions, yet, despite strong efforts to uncover genetic causes, the vast majority of ALS/FTD patients have no known genetic cause and no family history. The discovery of the *C9orf72* DNA GGGGCC (G_4C_2) repeat expansion in ALS and FTD patients brought to light the possibility of RNA-mediated toxicity in disease which could be controlled by epigenetic mechanisms. In this chapter, we will summarize the major epigenetic regulation mechanisms. Then, we will review the current knowledge of epigenetic regulation as it relates to ALS and FTD (Fig. 1.1).

1.2 Epigenetic Mechanisms

The epigenome is generally defined as the collection of heritable but dynamic mechanisms that largely dictate access to the DNA for templated functions such as gene transcription, DNA synthesis, and repair without altering the primary nucleotide sequence; there is, however, a valid argument that chimeric DNA, which does modify the primary nucleotide sequence, is facilitated by the epigenome. These intricacies regarding how far-reaching the epigenome's involvement is in nucleotide sequence will be continually clarified as the field matures. Here, we will focus on general regulation processes.

Post-transcriptional regulation of RNA function and metabolism—by noncoding RNAs (ncRNAs), such as microRNA, or chemical modifications to ribonucleotides—is now also considered part of epigenetic control. Whereas an individual's genome is mostly established at conception, the individual's inherited epigenome continues to change throughout embryonic, fetal, and postnatal life in response to

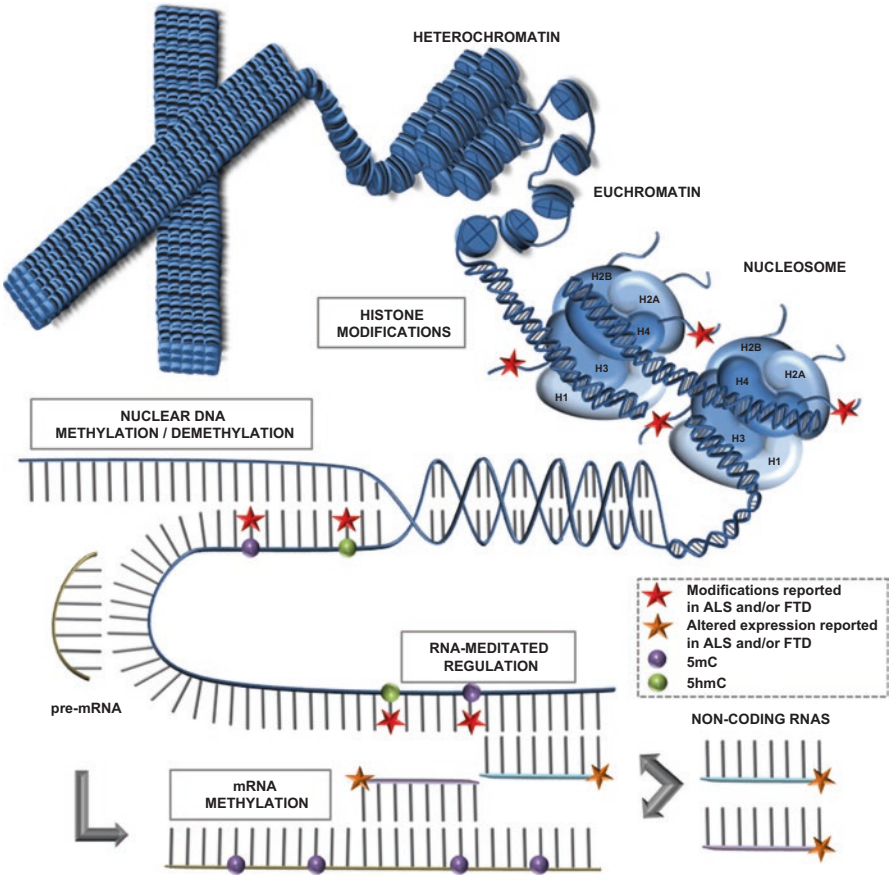

Fig. 1.1 Schematic representation of the major epigenetic regulatory mechanisms and their interactions. The major epigenetic regulatory mechanisms are framed. Red stars mark epigenetic changes known to be involved in ALS/FTD. Orange stars indicate that altered expression of these RNAs has been reported in ALS/FTD. Purple spheres represent 5mC marks while green spheres represent 5hmC marks

cell-intrinsic mechanisms and environmental input. Epigenetic changes are cell-specific and are presumed to be adaptive responses to internal and external stimuli.

As next-generation sequencing is becoming more commonplace, the epigenome is garnering greater attention in many fields, from developmental biology to environmental epidemiology, because it offers a potential mechanistic link between the environment and phenotype. For ALS/FTD researchers, epigenetics is a particularly interesting field that may explain the clinical variability within families sharing ALS/FTD-causing genetic variants, and the cause of disease in patients affected sporadically.

Epigenetic control of genomic functions occurs by regulating nucleosome position and density via histone chaperones and chromatin remodeling complexes [12].

Nucleosomes are the fundamental repeating unit of chromatin, and consist of nucleoprotein complexes containing two copies each of four core histone proteins (H2A, H2B, H3, and H3) and 147 base pairs of DNA wrapped 1.67 turns counterclockwise around this globular structure [13]. Key signals directing nucleosome positioning and chromatin structure include chemical modifications to the DNA and histones, as well as ncRNA species. Epigenetic regulation is also exerted via post-transcriptional regulation of RNA function and metabolism. This section will briefly review our current understanding of these epigenetic mechanisms.

1.2.1 Nuclear DNA Methylation

DNA methylation is the biochemical addition of a methyl group to a nucleotide, often resulting in altered transcription factor binding, chromatin remodeling, and ultimately altered gene expression. Methylation of the carbon 5 on cytosine (5mC) is the most studied and best understood DNA modification.

5mC most frequently occurs at cytosine-guanine nucleotide sequences (mCG), often referred to as CpG sites. Genomic regions enriched in CpG sites, referred to as CpG islands, are often associated with gene promoters. Whether located near transcription initiation sites or distant from annotated promoters, CpG islands play a central role in destabilizing nucleosomes and recruiting proteins that initiate chromatin remodeling [14–16]. Generally, methylation in a CpG island is associated with the reduced expression of a nearby gene.

Recent research has found 5mC marks at cytosine-adenine sequences (mCA). It is believed that mCA may be particularly important in the central nervous system, as the number of mCA and mCG marks detected in the adult brain are approximately equal [17]. Although both mCA and mCG are associated with transcriptional repression, early evidence suggests mCA may be particularly important for more precise transcription regulation—1–2-fold expression changes compared to 100–1000-fold changes observed with mCG [18].

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) 1, 3A, and 3B. While DNMT3A and DNMT3B are associated with de novo methylation, DNMT1 is mainly responsible for maintaining methylation across cell generations by methylating the daughter strand of DNA during cell division, thus preventing hemimethylation of double-stranded DNA [19]. Because of DNMT1's maintenance function, DNA methylation is considered a fairly stable epigenetic mark. Further research into the differentiating roles of DNMT3A and DNMT3B are warranted, as early evidence suggests DNMT3A may be primarily responsible for mCA methylation. Its proper regulation may be especially critical in the brain [20–22].

Bisulfite conversion is currently the preferred method to detect cytosine methylation. Briefly, bisulfite ion deaminates unmethylated cytosines, which result in its chemical conversion to uracil upon alkaline desulfonation [23]. As bisulfite converts 5mCs much more slowly, there is a selective conversion favoring unmethylated cytosines. After DNA amplification, all unconverted cytosines are considered

methylated. Importantly, standard bisulfite conversion does not differentiate methylated cytosines from cytosines that have been demethylated, which is the topic of the next section.

1.2.2 Nuclear DNA Demethylation

Although DNA methylation is considered a stable epigenetic change, it is now well understood that it is closely and dynamically regulated not only by epigenetic "writers," such as DNMTs, but also by "eraser" mechanisms, which keep CpGs unmethylated. Importantly, demethylation is of particular interest in neurodegeneration research because, among all organs, its footprints are most abundant in the brain and further increase with age [24].

DNA demethylation can occur as a passive or active process. Passive demethylation occurs when the process of copying methylation marks onto the daughter strand of DNA is impaired. As a result, future cell generations will lose methylation marks by dilution. Active demethylation is mediated by TET family of dioxygenases, which catalyze the oxidation of 5mC in a stepwise manner, resulting in 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Oxidation is followed by replication-dependent dilution or thymine DNA glycosylase-mediated base excision and repair, resulting in full demethylation and restoration to unmodified cytosine [25]. 5fC- and 5caC-containing regulatory elements show very limited overlap and are therefore believed to play distinct regulatory roles. 5caC sites are more active than 5fC sites, and both are more active than regions decorated by 5hmC [26].

Our current understanding is that DNA methylation and demethylation processes must be highly orchestrated to appropriately regulate the transcriptome. Further research is necessary to elucidate the exact functions of 5hmC, 5fC and 5caC as it is still difficult to estimate the extent to which these events occur and appreciate their full potential as therapeutic targets.

1.2.3 Mitochondrial DNA Methylation

While nuclear DNA (nDNA) methylation is well recognized, mitochondrial DNA (mtDNA) methylation is still controversial. Using restriction enzyme cleavage, early studies estimated that about 2–5% of mtDNA CCGG sequences are fully methylated whereas the remainder of mtDNA is unmethylated [27]. Mass spectrometry confirmed the presence of methylated bases in human mtDNA [28]. Later, using affinity-based methods, both 5mC and 5hmC modifications were found in the D-loop region of human mtDNA at both CpG and non-CpG dinucleotides [29, 30]. Other studies used bisulfite conversion coupled with sequencing or pyrosequencing and claimed that mtDNA methylation may be as high as 2–18% in the D-loop region

[31, 32]. Recent reports however suggest that mtDNA methylation levels may have been greatly overestimated due to the circular structure of mtDNA, which affects bisulfite conversion efficiency [33–35]. Nonetheless, the reported presence of methyltransferases DNMT1 and DNMT3A inside mitochondria suggests that methylation may take place in mtDNA [30, 36]. Since nDNA and mtDNA interact to modulate the transcriptome [37, 38], future studies need to better characterize mtDNA modifications and their potential role in the regulation of gene transcription.

1.2.4 RNA Methylation

Epigenetic regulation goes beyond transcription. RNA is also under epigenetic regulation and can be methylated or otherwise modified. In fact, methylation occurs in most RNA classes, but the exact function of RNA modifications remains unclear due to limitations in detection and quantification.

Whereas DNA methylation has mainly been observed at cytosines, RNA methylation can occur at cytosine (m^5C), adenine (m^6A), and guanine (m^7G). m^7G (N^7-methylguanine) at the 5′ cap structure is the most widely studied RNA methylation and is necessary for the translation of most messenger RNAs (mRNAs). m^7G cap also mediates nuclear transport of some mRNAs, preserves mRNA stability by protecting it from degradation, and facilitates other processing such as the addition of poly(A) tails to mRNAs [39, 40]. m^7G cap methylation is reversible and can either go through the process of "decapping," where the entire m^7G cap is removed [41], or potentially through m^7G demethylation.

In contrast to the extensively studied 5mC in DNA, the m^5C (N^5-methylcytosine) RNA modification has not been thoroughly explored. While it is still unclear how m^5C is regulated by DNMTs, a study suggested that mouse Dnmt2 RNA methyltransferase may be required for epigenetic heredity [42]. Early studies also demonstrated that m^5C affects interactions of long noncoding RNAs (lncRNAs) with chromatin-associated protein complexes [43].

The most abundant mRNA modification is m^6A (N^6-methyladenine). Dominissini et al. found that silencing m^6A methyltransferase—mostly acting at highly conserved long internal exons, stop codons, and 3′UTRs—alters gene expression and alternative splicing patterns [44]. The same group conducted affinity-based m^6A profiling and identified proteins that bind specifically to m^6A. Their findings suggest that RNA methylation, through the binding of these m^6A-specific binding proteins, may disturb RNA binding proteins' affinity to associate with partner unmethylated RNAs. Importantly, these m^6A-binding proteins recruit additional factors that facilitate functions such as alternative splicing, nuclear export, and mRNA stability [40, 44]. One example is the m^6A-binding protein YTHDC1, which facilitates the export of methylated mRNA from the nucleus to the cytoplasm in vitro. Knockdown of YTHDC1 results in retention of nuclear m^6A-containing mRNA, where transcripts accumulate in the nucleus and are depleted from the cytoplasm [45]. Of interest,

m^6A has been the first reversible modification identified in coding and noncoding RNAs after the discovery that the fat mass and obesity-associated protein (FTO) act as a m(6)A demethylase [46–48], suggesting that, analogous to reversible DNA, reversible RNA methylation may affect gene expression and downstream regulation of RNA-related cellular pathways.

1.2.5 RNA-Mediated Regulation

While 70–90% of the genome is transcribed, only 1–3% of these transcripts encode proteins [49, 50]. As such, the transcriptome is mainly composed of ncRNAs having infrastructural and regulatory functions. These transcripts are gaining increased attention, not only for their recognized roles in transcriptional and post-transcriptional regulation, but also for their targeted effects on gene expression, making them attractive therapeutic targets.

ncRNAs are divided into short (<30 nucleotides) and long ncRNAs (>200 nucleotides), and each subtype of ncRNA fulfills very specific regulatory roles. Specifically, short RNAs (sRNAs) are sub-categorized into micro-RNAs (miRNAs), piwi-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs). lncRNAs are generally sub-classified according to their proximity to protein coding genes—sense, anti-sense, bidirectional, intronic, and intergenic. Apart from their size, sRNAs and lncRNAs have largely different functions in transcription regulation. Two new classes of ncRNAs have also been recently recognized: enhancer RNAs (eRNAs) and promoter-associated RNAs (PARs).

Mature miRNAs are single-stranded 20–24 nucleotide sequences derived from precursor miRNA (pre-miRNAS). These are ~70 nucleotide transcripts with distinct hairpin structures initially derived from nuclear primary miRNA (pri-miRNAs). Mature miRNAs pair with complementary sequences on target mRNA transcripts through the 3′ untranslated region (3′UTR) and repress the targeted mRNA translation after recruiting the RNA-induced silencing complex (RISC) [51, 52]. Mature miRNAs may have several target mRNAs, but their effect on their targets is a modest 1–2-fold change in expression [53]. Although miRNAs seem to initiate modest changes in their targets, changes in expression of miRNAs themselves associate with widespread mRNA expression changes, indicating that miRNAs are central to global expression regulation [53]. In fact, a large screening of the mammalian genome found hundreds of mRNAs with conserved pairing to specific miRNAs, with an enrichment of genes involved in transcriptional regulation [54]. Specifically, several miRNAs target DNMTs that can have far-reaching impact on global methylation levels, which has been implicated in several cancers and autoimmune diseases [55]. The story becomes more complicated when one considers that miRNA expression can in turn be modulated by DNA methylation and histone modifications [55, 56], suggesting that transcriptional regulation needs to be well orchestrated by an epigenetic–ncRNA feedback loop. This highlights the complexity of epigenetics and the interdependence of these mechanisms to regulate gene expression.

piRNAs are 24–31-nucleotide sequences able to form complexes with Piwi proteins of the Argonaute family. They are part of a complex population of small RNAs highly enriched in male gonads [57]. Their main function is to silence transposable elements. These mobile elements are autonomous sequences of DNA that replicate and insert themselves into the genome, potentially introducing detrimental DNA damage. Some results also suggest a role for piRNA in transcriptional regulation and deadenylation-mediated mRNA degradation [58].

Mature siRNAs are 20–24 nucleotide sequences that modulate a given gene's expression after binding to its complementary nucleotide sequence through a process called RNA interference (RNAi). siRNAs mediate post-transcriptional silencing in a way similar to miRNAs, and may also suppress transposon activity in a way similar to piRNAs [59, 60].

lncRNAs are sequences longer than 200 nucleotides characterized by low nuclear expression and low conservation across species. One exception is highly conserved large intergenic noncoding RNAs (lincRNAs), which recruit histone-modifying complexes and transcription factors to transcriptionally modulate targeted chromosomal regions [61, 62]. The majority of lncRNAs are transcribed as complex networks of sense and anti-sense transcripts overlapping protein-coding genes.

eRNAs are sequences of about 800 nucleotides on average that are derived from regions enriched in RNA Polymerase II and transcriptional co-regulators. What makes the genomic sites encoding eRNAs different from those of other lncRNAs is their specific histone methylation signature at histone 3 lysine 4 (H3K4), which is typical of enhancer sites. Similar to lincRNAs, eRNAs are evolutionarily conserved but have a short half-life. eRNA expression levels correlate with the expression of nearby genes, and is thus dynamically regulated upon signaling [63, 64]. eRNAs are believed to act as transcriptional activators.

PARs are short half-life transcripts that can be classified as short or long ncRNAs—their size range from 16 to over 200 nucleotides. PARS are either expressed near transcription start sites or from elements of the promoter [65], from both strands and in divergent orientation with respect to the transcription start site. Most PARS associate with highly expressed genes while being weakly expressed themselves. Increasing evidence demonstrates that PARs may associate with both transcriptional activation and repression [66–69].

1.2.6 Histone Modifications

As discussed above, the density of nucleosomes determines the accessibility of DNA to protein complexes performing templated functions. Heterochromatin contains tightly wound DNA, where gene transcription is repressed. Euchromatin is a looser conformation of the DNA, which is conducive to gene transcription. Chromatin conformation is dynamic and is regulated in part by covalent posttranslational histone modifications, which mainly occur at amino acid residues within unstructured histone "tails." These modifications include acetylation and

methylation of lysines, methylation of arginines, and phosphorylation of serines and threonines, among others. Specific functions have been assigned to several modifications, which led to the formulation of the histone code hypothesis [70]. It is our current understanding of the roles of histone modifications that their combinations and association with transcription factors and other chromatin regulators define epigenetic states, which can be discovered and assigned to genomic regions by machine learning [71].

Enzymes such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs), and kinases facilitate the addition of chemicals on amino acid residues (writers). For instance, HATs brings in a negative charge that neutralizes the positive charge on the histones. This chemical change decreases the interaction of the N termini of histones with the negatively charged phosphate groups of DNA, resulting in a more relaxed structure. This structure allows transcription factors to access DNA and initiate gene transcription. Other enzymes such as histone deacetylases (HDACs), lysine demethylases (KDMs), phosphatases, and deubiquitylases catalyze the removal of these epigenetic marks (erasers). As such, the process of histone acetylation is reversed by HDACs. Acetyl group removal leads to a more condensed chromatin state and transcriptional gene silencing. Proteins containing DNA methyl-binding domains, chromodomains, bromodomains, and Tudor domains recognize histone modifications and recruit other chromatin modifiers and remodeling proteins to ultimately regulate DNA-dependent processes (readers).

Functional groups affixed to histones can also initiate chromatin remodeling through cis or trans effects. This way, histone marks can modulate the chromatin by directly affecting histone-histone and histone-DNA interactions, or by recruiting non-histone proteins via specific binding domains that recognize particular modifications [72].

1.3 Epigenetic Changes in Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD)

The 2011 discovery that an expanded hexanucleotide (G_4C_2) repeat within a noncoding region of the *C9orf72* gene causes both ALS and FTD highlighted the genetic link between these two diseases and recognized this mutation as the most common genetic cause of ALS and FTD identified to date [73–75], yet more than 80% of all ALS and FTD cases remain genetically unexplained [76]. While other unknown genetic mutations are certainly at play, more researchers are recognizing that epigenetic changes may contribute to these two diseases based on their potential to (1) explain the phenotypic variability observed across family members carrying the same ALS/FTD-associated mutation, and (2) explain why the majority of patients develop disease without any family history of ALS or FTD. Very few epigenetic studies have been conducted on ALS and FTD before the finding of the

pathogenic *C9orf72* G_4C_2 repeat expansion in 2011. This section summarizes the current knowledge on the epigenetics of ALS and FTD. A full summary can also be found in Table 1.1.

1.3.1 ALS/FTD Epigenetic Studies

1.3.1.1 Nuclear DNA methylation/demethylation

Approximately a decade ago, researchers interested in better understanding the etiology of ALS and FTD started interrogating epigenetic mechanisms using cell models, animal models, and biospecimen obtained from patients.

Early studies on ALS evaluated blood and brain promoter DNA methylation status of a few genes known to be implicated in disease including *SOD1*, *VEGF*, and *SLC1A2* [136, 137], but no changes in methylation were detected.

The methylation status of *SOD1*, *FUS*, *TARDBP*, and *C9orf72* promoters has also been evaluated from the blood of ALS patients carrying not fully penetrant *SOD1* mutations, but again, no methylation variations have been detected at these specific regulatory regions [77]. Of interest, Coppede et al. used an enzyme-linked immunosorbent assay (ELISA) to also evaluate global methylation levels in ALS patients carrying not fully penetrant *SOD1* mutations and observed a significant overall DNA methylation increase [77].

Similarly, others compared brain methylation levels of sporadic ALS (sALS) patients to control cases using Affymetrix GeneChip Human Tiling 2.0R Arrays and identified 38 differentially methylated genomic regions in patients. Further analysis of these 38 regions shed light on specifically altered biological pathways involved in calcium homeostasis, neurotransmission, and oxidative stress [78].

Using ELISA assays, Figueroa-Romero et al. identified global 5mC and 5hmC changes in sALS patients' spinal cords—these changes were not observed in blood [79]. Then, using high-throughput microarrays, the same group conducted genome-wide 5mC and expression profiling and identified loci-specific differentially methylated and expressed genes. The 112 genes identified were highly associated with immune and inflammation responses [79].

In the hope of finding differently methylated regions that might act as modifiers of age of onset in ALS, Tremolizzo et al. evaluated DNA methylation levels in both early onset (<55 years) and late onset (>74 years) ALS patients. They found a global 25–30% increase in DNA methylation levels in whole blood that was independent of age of onset [80]. While no significant difference was found between early onset and late onset ALS, the methylation increase detected in blood is consistent with previous observation in the central nervous system (CNS) of ALS patients [36, 99].

Early epigenetic studies on FTD analyzed the progranulin-encoding gene (*GRN*) known to be mutated in 5–20% of familial FTD and 1–5% of sporadic FTD patients [138–140]. Two groups independently reported that increased *GRN* promoter methylation negatively correlates with *GRN* mRNA levels in FTD subjects [81, 82], a

Table 1.1 Overview of ALS and FTD epigenetic studies

Epigenetic Modification	Overall Findings	Implications for disease	References
Nuclear DNA methylation/ demethylation	Overall DNA methylation is increased in ALS patients carrying not fully penetrant *SOD1* mutations.	Patients with *SOD1* mutations may have unique methylation profiles.	[32]
	Differentially methylated regions are found in sporadic ALS subjects.	Affected genes are associated with calcium homeostasis, neurotransmission and oxidative stress, suggesting a role for these mechanisms in ALS pathogenesis.	[125]
	Global changes in 5mC and 5hmC levels are found in ALS postmortem spinal cords.	Expression changes in 112 genes associate with immune and inflammatory responses.	[51]
	Increased DNA methylation levels are found in ALS blood, independently from age of onset.	While not a modifier of age of onset, DNA methylation levels may act as an ALS marker of epigenetic dysfunction.	[165]
	Increased *GRN* promoter methylation negatively correlates with *GRN* mRNA levels in FTD subjects.	Altered methylation may contribute to *GRN* haploinsufficiency.	[56,11]
	Specific DNA methylation signatures in FTD peripheral blood associate with tauopathy.	Specific DNA methylation signature may be a risk factor for neurodegeneration.	[103]
	DNMT1 and DNMT3A protein levels are increased in motor neurons of ALS patients.	Increased DNMT1 and DNMT3A protein levels may explain the global increase in methylation observed.	[24]
	DNMT3A is present in human cerebral cortex pure mitochondria. Mitochondrial Dnmt3a protein levels are significantly reduced in mice skeletal muscles and spinal cords.	Reduced mitochondria Dnmt3a protein levels may associate with a loss of mtDNA methylation.	[172]
RNA-mediated regulation through miRNAs	Expressing mutant *SOD1* (p.G39A) in mouse muscles alters expression of both miRNAs and genes associated with myelin homeostasis in spinal cords.	Results suggest an epigenetic regulation interplay between muscle cells and neighboring neurons.	[41]
	miRNAs are aberrantly regulated in ALS spinal cords.	Mature miRNAs are globally reduced and miRNA processing is altered.	[52]
	Altered microRNAs have RNA targets part of pathways previously associated with ALS.	Altered miRNA expression may result in altered regulation of key pathological pathways involved in ALS.	[52]
	ALS-associated miRNA expression changes in response to nuclear clearance and cytoplasmic aggregation of TDP-43.	Re-localization of TDP-43 alters RNA-mediated regulation.	[52]
	Altered expression of specific miRNAs in *SOD1* p.G93A mice and patients carrying *TARDBP* p.A90V or p.M337V mutations.	miRNAs and/or their targets may potentially serve as biomarkers or therapeutic targets.	[164,113,183]
	miR-29b regulates human progranulin.	miR-29b may be therapeutically targeted to rescue FTD-associated *GRN*-haploinsufficiency.	[88]
	Expression of FTD-associated *CHMP2B* in mouse brain decreases miR-124 expression.	Altered regulation of miR-124 may lead to altered regulation of AMPAR receptor subunits.	[57]

(continued)

Table 1.1 (continued)

***C9orf72* Epigenetic Studies**	DNA methylation of the *C9orf72* promoter and expanded repeat region in c9FTD/ALS.	Hypermethylation of the *C9orf72 genomic region may contribute* to C9ORF72 loss of function. Evidence for neuroprotective effects and reduced *C9orf72*-associated pathology.	[174,107,12,177,176]
	5mC and 5hmC are both present at the *C9orf72* promoter in *C9orf72*-associated brain tissues.	Estimates of *C9orf72* promoter methylation includes both 5mC and 5hmC modifications.	[49]
	Repressive histone marks at the *C9orf72* locus in c9FTD/ALS.	Reduced expression of *C9orf72*.	[14]
	Differentially methylated regions are abundant in c9ALS and sALS patients and affect many genes and biological pathways involved in ALS.	Altered methylation may result in altered regulation of key pathological pathways involved in ALS.	[45]
	c9ALS and sALS patients have distinct but overlapping brain DNA methylation profiles affecting genes with similar biological functions.	Suggests a conserved pathobiology between c9ALS and sALS.	[45]
Potential drivers of epigenetic changes	Increased incidence of ALS in Chamorro indigenous people of Guam.	Dietary consumption of neurotoxins may induce epigenetic changes through ROS and increase the risk for ALS.	[10]
	Associations between ALS and diet, cycad neurotoxins and ROS.	Neurotoxins may initiate epigenetic changes and increase the risk for ALS.	[16,37,27,134,142]
	Monozygotic twins discordant for ALS.	Supports evidence for environmental contribution to disease onset.	[175,120,180]
	C9orf72 positive identical twins discordant for ALS.		[175,120]
	Stress-induced histone modifications repress transposable elements and other coding and non-coding RNAs.	Acute stress impairs genomic stability and may initiate cognitive impairments.	[82,80,141,48,90,117]
	Exposure to heavy metals such as aluminum sulfate, mercury, lead, and selenium associates with ALS. Heavy metals cause cellular stress and toxicity through ROS.	May drive epigenetic changes, protein denaturation and aggregation and prevent proteasomes from eliminating dysfunctional proteins.	[122,9,30,89,28,50,133]
	Increased risk for ALS in workers exposed to low frequency magnetic fields.	Electromagnetic fields may cause epigenetic changes leading to neurodegeneration.	[70]
	Increased risk for ALS in people exposed to pesticides and herbicides containing organophosphates.	Exposure to organophosphates may initiate epigenetic changes and increase toxicity through ROS.	[115,33,154,124,167,40,89,121,157]
	Increased ALS incidence in athletes and war veterans.	Drug use and ischemia from head injuries may increase ROS and drive ALS.	[25,78,123,137,163]

very interesting finding since *GRN* haploinsufficiency has been recognized as a major cause of FTD [141].

Global DNA methylation levels in blood were also assessed in tau-associated progressive supranuclear palsy (PSP) and FTD patients by Li et al. The major known risk locus for PSP and other neurodegenerative diseases is the H1 haplotype at 17q21.31, a genomic region in linkage disequilibrium with an inverted chromosomal sequence of about 970 kb [142–144]. It was shown that this disequilibrium resulted from an inversion at the H2 haplotype relative to the H1 human reference allele and from a lack of recombination between inverted and non-inverted chromosomes [145]. Li et al. found that the H1/H2 locus may affect the risk for tauopathies through methylation alterations not only at the *MAPT* locus, a region known to be mutated in FTD, but also in at least three neighboring genes. The 17q21.31-associated DNA methylation signature Li et al. identified was unique to tau-associated PSP patients and, to a lesser extent, to tau-associated FTD [83].

As DNA methyltransferases catalyze DNA methylation, researchers have been interested in assessing their expression in disease. Chestnut et al. found that protein levels of DNMT1 and DNMT3A were increased in the motor cortex of ALS patients [36]. Wong et al. went further and confirmed the presence of both Dnmt3a isoform in pure mitochondria of human cerebral cortex and mouse CNS, and 5mcs in mouse mitochondria of neurons and skeletal muscle myofibers, supporting an epigenetic regulation of brain mtDNA [84]. Wong et al. then evaluated mitochondrial Dnmt3a isoform levels in the CNS of different *SOD1* transgenic mouse models including a line hemizygous for a low copy number of hSOD1 -p.G37R mutant allele, a line that expressed high levels of normal wild-type human *SOD1* gene, and a line with skeletal muscle-restricted expression of hSOD1 -p.G37R, -p.G93A, and -wild-type variants. After studying all the lines at presymptomatic or early to middle stages of disease, they found that Dnmt3a protein levels in mouse skeletal muscle and spinal cord mitochondria were significantly reduced early in disease, this even before symptoms' onset. They also found Dnmt1 bound to the outer mitochondrial membrane of the same mice. They observed that 5mC immunoreactivity became aggregated and sequestered into autophagosomes of transgenic mice motor neurons [84].

1.3.1.2 RNA-mediated regulation through miRNAs

It is well established that ncRNAs such as miRNAs are key regulators of RNA functions and metabolism. As such, many have been interested in assessing their potential contribution to ALS/FTD pathogenesis.

Dobrowolny et al. found that selective expression of the human *SOD1* mutation p.G39A after injecting mouse muscles not only leads to hypomyelination in the sciatic nerve, it also alters spinal cord expression of miRNAs and mRNAs known to be involved in myelin homeostasis. This finding suggests that RNA and epigenetic alterations observed in motor neurons may result from changes initiated in neighboring non-neuronal cells [85].

Figueroa-Romero et al. then interrogated spinal cord tissues obtained from post-mortem ALS patients and found that mature miRNA levels are globally reduced. They also identified altered microRNAs having RNA targets part of pathways previously associated with ALS. Knowing that TDP-43 is central to ALS and FTD pathogenesis—TDP-43 pathological signature is observed in about 97% of ALS and 50% of FTD patients [146, 147]—and that TDP-43 plays a central role in miRNA biogenesis [89, 148–152], the same group used transfected cells to determine whether TDP-43 mediates miRNA-induced regulation. They observed ALS-associated miRNA expression changes in response to nuclear clearance and cytoplasmic aggregation of TDP-43, suggesting that TDP-43 pathology may alter the expression or function of endogenous miRNAs and their downstream targets [86].

Several studies have identified alternatively expressed miRNAs in ALS and FTD. Marcuzzo et al. studied the brain of pre-symptomatic and late stage *SOD1* p.G93A transgenic mice and found that expression levels of miR-9, miR-124a, miR-19a, and miR-19b were all altered in late stages of disease. Moreover, the expression analysis they conducted identified miRNA/target gene pairs that were differentially expressed in this mouse model [87]. Toivonen et al. found miR-206 altered in the blood of both *SOD1* p.G93A transgenic mice and ALS patients [88], whereas Zhang et al. found levels of miR-9 decreased in induced pluripotent stem cells (iPSCs)-derived neurons of patients carrying either p.A90V or p.M337V *TARDBP* mutations [89].

Of interest, Jiao et al. found that miR-29b regulates human progranulin (hPGRN, *GRN*) through 3′UTR binding. They demonstrated in vitro that ectopic expression of miR-29b decreased hPGRN expression and knockdown of endogenous miR-29b increased it. Their findings suggest that miR-29b may possibly be therapeutically targeted to rescue the haploinsufficiency observed in FTD patients carrying a *GRN* mutation [90]. Moreover, expression of FTD-associated mutant *CHMP2B* in cerebral cortices of mice has initiated a decrease in miR-124 expression—brain-enriched miR-124 is especially important for the proper regulation of AMPA receptor (AMPAR) subunits. Ectopic expression of miR-124 in the prefrontal cortex of these mice restored AMPAR levels and rescued the behavioral deficits previously observed in the animals [91].

1.3.2 C9orf72 *Epigenetic Studies*

Decreased expression of one or multiple *C9orf72* transcript variants has been observed in various human biospecimen carrying the pathological *C9orf72* G_4C_2 repeat expansion. These biospecimen include frontal cortex, motor cortex, cerebellum, cervical spinal cord, lymphoblastoid cell lines, iPSCs and neurons differentiated from iPSCs, all obtained or derived from ALS and FTD patients [98, 153–159]. In an attempt to better understand the biological mechanism underlying the reduced *C9orf72* expression in *C9orf72*-associated ALS and FTD (c9FTD/ALS) patients,

methylation status of the regions flanking or encompassing the repeat expansion has been evaluated.

Using bisulfite sequencing and restriction enzyme assays, Rogaeva's group found that the CpG island upstream of the G_4C_2 repeat expansion is hypermethylated in the brain and blood of about 36% of ALS and 17% of FTD cases [94, 96]. Lee's group not only confirmed these results, but also uncovered that hypermethylation associates with reduced accumulation of intronic *C9orf72* RNA and reduced burden of *C9orf72*-associated pathological signature (RNA foci and dipeptide repeat accumulation). They also found that demethylation increases cell vulnerability to oxidative and autophagic stress, suggesting that *C9orf72* promoter hypermethylation may mitigate downstream molecular aberrations associated with the pathological G_4C_2 repeat expansion [93]. As the methods initially used to estimate DNA methylation in c9FTD/ALS cases could not differentiate 5mC from 5hmC, Esanov et al. were able to confirm the presence of 5hmC within the *C9orf72* promoter in post-mortem brain tissues of hypermethylated patients [97]. This finding suggests that the previous estimates by Rogaeva's and Lee's groups included both 5mC and 5hmC modifications.

However, considering that all c9FTD/ALS patients show a 50% reduction in total *C9orf72* RNA expression [73] and only approximately one third of patients are found with a hypermethylated CpG island, many details were missing. As such, Rogaeva's group attempted to assess whether it is the repeat expansion that is hypermethylated in patients and consequently drives the reduced expression. For this purpose, they developed a new qualitative assay that was independently validated by a methylation-sensitive restriction enzyme assay, and found that the *C9orf72* repeat expansion was indeed hypermethylated in all ALS and FTD cases carrying more than 50 G_4C_2 copies [95]. This finding was later confirmed by Bauer [92]. In addition, Belzil et al. investigated the brain of c9FTD/ALS patients and found that all patients carried repressive histone marks at the *C9orf72* locus [98]. As such, methylation of the repeat expansion together with repressive histone marks at the *C9orf72* locus likely explains the 50% *C9orf72* reduced expression observed in c9FTD/ALS patients.

A recent multi-omic study aimed to better understand the molecular mechanisms initiating RNA misregulation in *C9orf72*-associated c9ALS and sALS combined RNA and DNA methylation data obtained from brain next-generation RNA sequencing (RNAseq) and reduced representation bisulfite sequencing (RRBS). They found an abundance of differentially methylated cytosines in c9ALS and sALS patients, including changes in many genes and biological pathways known to be involved in ALS. They also observed that c9ALS and sALS patients have generally distinct but overlapping brain DNA methylation profiles that differ from control individuals. Of importance, they found that the c9ALS- and sALS-affected genes and biological pathways have very similar biological functions, suggesting a conserved pathobiology between c9ALS and sALS [99].

Several studies aimed to assess whether DNA methylation is a clinical modifier of ALS and FTD but so far, few correlations have been identified. Among these, hypermethylation of the CpG island upstream of the *C9orf72* repeat expansion has

been found to correlate with shorter disease duration in ALS [96], but was found associated with longer disease duration and later age of death in FTD [160].

1.3.3 Potential Drivers of Epigenetic Changes

The epigenome is a dynamic, complex machinery that plays a critical role in coordinating cellular functions. It is constantly changing to address cellular needs or to react to environmental threats, such as infections. A prime example is heat-shock proteins. These proteins were discovered in the early 1960s by Ferrucio Ritossa when he noticed a "puffing" pattern—now known to be a sudden increase in RNA transcription—in *Drosophila* cells when one of his lab mates increased his incubator's temperature [161]. Many discoveries have resulted from this unintended finding, but one of the most intriguing discoveries was that the "puff" Ritossa described was observable within 2–3 min of heat exposure, demonstrating how agile the epigenomic machinery is. Understanding not only which epigenetic modifications affect disease, but what drives these epigenetic changes is critical to better understanding human health and disease.

As demonstrated by Ritossa's landmark discovery that "heat shock" can induce an immediate response from the epigenetic machinery, environmental influences are a clear driver. "Environment" has broad implications, however, and can include a cell's internal or external influences, such as neighboring cells, as research has shown that epigenetic changes can be transmitted from cell to cell [162]. Many factors affect the dynamic interaction between environmental influences and the epigenome, including exercise, age, diet, and toxic exposures.

Researchers observed a clear example of environmental factors driving ALS in the indigenous Chamorro people of Guam, who experience high ALS incidence because their diet is enriched in cycad neurotoxins. The Chamorro diet includes the flying fox, which has high levels of cycad neurotoxins because it feeds on cycad seeds [100–102]. Additional studies have found associations between ALS and cycad neurotoxins or reactive oxygen species (ROS) [102–104, 163–165]. Exactly how these neurotoxins are driving disease is unknown, but epigenetic modifications are a primary suspect. Importantly, diet has also been shown to induce epigenetic changes across other diseases [105].

The most striking support for epigenetic contribution is perhaps the reports of ALS-discordant monozygotic twins (monozygotic twins where one has disease and the other is unaffected), implicating environmental and epigenetic factors in disease [106–108]. One study identified monozygotic twins that both carry the *C9orf72* repeat expansion, but only one has developed disease. The other study could not find a clear genetic factor that caused disease. A third study by Young et al. identified thousands of large between-twin differences at CpG sites in five monozygotic twin pairs. Young et al. conducted biological pathway analysis, which revealed that impairments in GABA signaling were common to all ALS individuals. Other altered pathways were also identified, including some relevant to ALS such as glutamate

metabolism and the Golgi apparatus [108]. Importantly, Young et al. applied to their 450K data the Horvath algorithm of epigenetic age [166]—an aging clock of chromatin states derived from the characterization of 353 CpG sites—and found that ALS-affected twins were epigenetically older than their unaffected co-twins, confirming previous findings that ALS is characterized by accelerated brain aging [108, 167]. In all cases, the other twin may develop disease in time, but the question would still remain regarding why a significant time gap in onset exists.

Stress is also an environmental variable that has received increased attention in recent years. Both histone methylation and acetylation modifications have been observed in rodents because of stress after social defeat [168]—acute and chronic stress has been shown to activate and repress genes through histone modifications [169]. Interestingly, transposable elements are repressed during acute stress, as are hippocampal coding and noncoding RNA as a result of stress-induced histone modifications. These expression changes have been suggested to impair genomic stability and give rise to cognitive impairments [109–114].

ROS, a species of free radical, can be induced through environmental signals, causing oxidative stress and, ultimately, cause a range of epigenetic modifications altering gene expression [162, 170–172]. Heavy metals are believed to cause cellular stress and toxicity through ROS, driving protein denaturation and aggregation and preventing proteasomes from eliminating dysfunctional proteins [120]. One study used carboxy-DCFDA (5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate) to quantify stress-induced ROS production from metal sulfates in human neurons [121] and found aluminum sulfate induced the most ROS.

Repetitive electromagnetic field exposure is also believed to trigger epigenetic changes, including DNA methylation and histone modifications, as was suggested by a study of a large cohort of workers [122]. Resistance welders had a higher incidence of Alzheimer's Disease and ALS, potentially because they are exposed regularly to low frequency magnetic fields [122]. Other studies have suggested that exposure to other heavy metals such as mercury, lead, and selenium, plus pesticides and herbicides containing organophosphate may increase risk for ALS, though no clear association has been found [115–119, 123–130].

As science continues to demonstrate the environmental effects of some high-contact sports on mental health, additional studies to explore the increased ALS incidence in athletes that play American football and soccer [131, 173], and in war veterans [132, 133] are needed. These data further suggest that some ALS cases arise from environmental exposures, potentially from epigenetic consequences of violent jarring in the brain [134]. While various methods, including illicit drug use and ischemia from head injuries, have been proposed to increase ROS production and drive dementia [135], the exact molecular mechanism leading to ALS and FTD needs to be clearly mapped through future rigorous studies.

1.3.4 Therapeutic Potential

The ultimate goal in ALS and FTD research is to develop therapeutic interventions for these diseases, allowing those who are affected to live a long and healthy life. Here, we discuss potential effects of epigenetic therapeutics.

Targeted epigenetic modifiers capable of regulating expression of both the normal and mutant alleles are an exciting possibility. Although significant work must be done in this area, this concept was successfully demonstrated in 1994, suggesting epigenetic therapy is feasible [174]. Since that discovery, other research has been performed in neurodegeneration and cancer in an effort to translate this for clinical use [175, 176]. A recent ALS-specific study utilized bromodomain small molecule inhibitors to increase mRNA and pre-mRNA expression for the normal *C9orf72* allele without destroying the epigenetic markers that repress expression of the expanded allele [177]. Increasing expression of the normal allele without increasing expression for the disease-causing allele is a significant achievement and demonstrates the reality of epigenetic therapy.

DNA methylation effects have been extensively evaluated across many diseases. One study found DNA methylation changes may be good ALS biomarkers for disease and potentially future therapeutic targets [79]. DNMTS have been shown to promote apoptosis and increase 5mC levels in motor neurons, and administering Dnmt inhibitors in a motor neuron-degenerative mouse model mitigated both apoptosis and 5mC levels in the motor neurons [36].

The therapeutic potential of oligonucleotides targeting miRNAs has also been evaluated by researchers using mouse models of ALS. Two groups showed that oligonucleotides able to inhibit either miR-155 or miR-29a extended the lifespan of *SOD1* p.G93A transgenic mice [178, 179]. Similarly, Morel et al. found that injecting oligonucleotides targeting miR-124a in the same transgenic mouse model prevented the pathological loss of EAAT2/GLT1 (encoded by human *SLC1A2*), an astroglial glutamate transporter known to be implicated in ALS [180].

Researchers have shown that histone marks at the *C9orf72* locus are associated with reduced gene expression in ALS and FTD patients carrying a repeat expansion when compared to controls [98]. They were then able to increase *C9orf72* mRNA expression by treating patient-derived fibroblasts with 5-aza-2-deoxycytidine (a demethylating agent). While this study demonstrates a proof of concept, increasing the mutated allele might not be a good therapeutic approach for c9ALS/FTD, as results from others suggest that *C9orf72*-associated hypermethylation may actually be neuroprotective in patients [92, 93]. Nonetheless, similar epigenetic strategies have been developed for cancer therapy, where HDAC inhibitors have reversed the effects of cancer-induced epigenetic changes [181]. These techniques have also been applied in ALS both in vitro and in an animal model, and later proceeded to clinical trials. Specifically, sodium phenylbutyrate (NaPB) prolonged *SOD1* p.G93A mouse survival [182], and was subsequently tested in a phase 2 clinical trial. The participants of this clinical trial tolerated this treatment well, and increased histone

acetylation in participant blood samples [183]. These results present an exciting and realistic opportunity to treat ALS and FTD using targeted epigenetic therapeutics.

Given the epigenome's dynamic and targetable nature combined with its apparent involvement in disease, it is a primary target for additional therapeutic efforts. As a field, researchers studying epigenetics in neurodegenerative diseases have made significant progress characterizing their involvement, but it is unclear whether the observed epigenetic modifications are driving disease or whether they are just a consequence. For example, researchers recently observed clear transcriptomic and epigenetic differences between c9ALS and sALS brains [99, 184], but it has not been shown whether reversing them would rescue neuronal health. If researchers can establish that the epigenetic dysregulation is driving these changes and that reversing them rescues neuronal behavior, epigenetic therapeutics would revolutionize ALS and FTD treatment.

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