



DNA Damage and Repair Deficiency in ALS/FTD-Associated Neurodegeneration: From Molecular Mechanisms to Therapeutic Implication

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Emerging studies reveal that neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), are commonly linked to DNA damage accumulation and repair deficiency. Neurons are particularly vulnerable to DNA damage due to their high metabolic activity, relying primarily on oxidative phosphorylation, which leads to increased reactive oxygen species (ROS) generation and subsequent DNA damage. Efficient and timely repair of such damage is critical for guarding the integrity of genomic DNA and for cell survival. Several genes predominantly associated with RNA/DNA metabolism have been implicated in both ALS and FTD, suggesting that the two diseases share a common underlying pathology with varied clinical manifestations. Recent studies reveal that many of the gene products, including RNA/DNA binding proteins (RBPs) TDP-43 and FUS are involved in diverse DNA repair pathways. A key question in the etiology of the ALS/FTD spectrum of neurodegeneration is the mechanisms and pathways involved in genome instability caused by dysfunctions/mutations of those RBP genes and their consequences in the central nervous system. The understanding of such converging molecular mechanisms provides insights into the underlying etiology of the rapidly progressing neurodegeneration in ALS/FTD, while also revealing novel DNA repair target avenues for therapeutic development. In this review, we summarize the common mechanisms of neurodegeneration in ALS and FTD, with a particular emphasis on the DNA repair defects induced by ALS/FTD causative genes. We also highlight the consequences of DNA repair defects in ALS/FTD and the therapeutic potential of DNA damage repair-targeted amelioration of neurodegeneration.

Keywords: amyotrophic lateral sclerosis, frontotemporal dementia, FUS, DNA damage, DNA repair deficiency

INTRODUCTION

Neurodegeneration is characterized by an age-associated or pathological progression-induced loss of neuronal structures and functions, eventually causing neuronal death. The four most common neurodegenerative disorders are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). In the motor neuron disease ALS,

the degeneration can occur in both upper and lower motor neurons in the brain, brainstem, and spinal cord, which leads to muscle weakness and atrophy (Ragagnin et al., 2019). ALS commonly starts with the early symptoms of weakness in the limb or bulbar muscles and progresses to difficulties in swallowing, speech, and respiration and eventual death due to respiratory failure. ALS is typically late-onset and fatal, with a median incidence of approximately 1–3 cases per hundred thousand persons per year worldwide and a 5–10% survival rate for over 10 years after diagnosis (Vijayakumar et al., 2019; Rojas et al., 2020). Approximately 90% of ALS cases are identified as sporadic (SALS), while ~10% are familial (FALS). In addition to motor dysfunction, up to 50% of ALS patients may also develop cognitive impairments and behavioral deficiencies, and among them, 15–20% are diagnosed with another neurodegenerative disorder, frontotemporal dementia (FTD), one of the most common types of dementia in people under 65 (Zago et al., 2011; Bartoletti et al., 2019; Abramzon et al., 2020). Similarly, up to 30% of FTD patients can develop motor dysfunction (Abramzon et al., 2020).

The etiology of ALS and FTD remains complex. Given the overlap of their clinical and pathological characteristics, the genetic basis of the two disorders has been extensively investigated, and some genes that are highly associated with both diseases have been identified as common genetic causes of ALS and FTD, including chromosome 9 open reading frame 72 (*C9orf72*), fused in sarcoma (*FUS*), and TAR DNA binding protein 43 (*TDP-43*). Among the causative genes, hexanucleotide repeat expansion within a non-coding region of *C9orf72* is one of the most common genetic cause of ALS and FTD (DeJesus-Hernandez et al., 2011; Ling et al., 2013). In this review, we discuss the emerging molecular mechanisms of various subtypes of ALS/FTD, with a particular focus on diverse causative genes and the role of genome damage and repair defects, as well as novel therapeutic avenues.

COMMON MECHANISMS OF NEURODEGENERATION IN ALS AND FTD

While both ALS and FTD share several neuropathological, clinical, and genetic attributes, they are distinguished by the region they affect in the central nervous system (CNS), symptoms, and onset age of the disease. Both diseases have been linked to a diverse set of genes that cumulatively cause loss of critical cellular functions and gain of toxicity. For example, RNA binding proteins (RBPs) *FUS* and *TDP-43*, are predominantly localized to the nucleus in healthy neurons, with a small fraction shuttling between the nucleus and cytoplasm. However, in ALS/FTD, these RBPs show cytoplasmic aggregate formation due to their nucleo-cytosolic mislocalization. The nuclear clearance of *FUS* and *TDP-43* leads to their loss of function in repairing genome damage in addition to their role in RNA processing. Together with the gain of toxicity in the cytosol likely *via* altered stress granule dynamics, this contributes to neuronal death (Lenzi et al., 2015; Wang H. et al., 2018; Mitra et al., 2019; Wang and Hegde, 2019; Zhang et al., 2020; Ding et al.,

2021). A third key genetic factor in ALS/FTD is *C9orf72*, which involves an expanded hexanucleotide repeat (GGGGCC) in its non-coding region (DeJesus-Hernandez et al., 2011). In healthy individuals, the hexanucleotide repeats range from 2 to 24, and expansion above 30 is considered pathogenic. While in ALS/FTD patients, the repeats can range anywhere between 30 and 100 in a small number of cases, and 100–1,000 repeats or even higher in the majority of the cases (DeJesus-Hernandez et al., 2011; Dols-Icardo et al., 2014; Smeyers et al., 2021). Apart from variations in the repeat number among individuals, there is variation in repeat size in the same individual when compared between CNS and blood (Van Mossevelde et al., 2017). Although the actual function of the *C9orf72* encoded protein is not known, the pathogenic repeat expansion in *C9orf72* causes haploinsufficiency as well as a gain of function in the form of aggregating expanded RNAs and dipeptide repeat proteins (Balendra and Isaacs, 2018; Fumagalli et al., 2021).

Disruption of RNA Transactions/Homeostasis

Dysfunctional RNA metabolism has been recognized as a central pathway in the progression of ALS and FTD, and the identification of RBP dysregulation as a major player in ALS-FTD supports this mechanism. RBP gene mutations including *TDP-43*, *FUS*, Ataxin-2 (*ATXN2*), RBP EWS (*EWSR1*), TATA-box binding protein-associated factor 15 (*TAF15*), heterogeneous nuclear ribonucleoprotein A1 (*hnRNPA1*), heterogeneous nuclear ribonucleoprotein A2/B1 (*hnRNPA2/B1*), TIA1 cytotoxic granule associated RNA binding protein (*TIA1*), and matrin3 (*MATR3*) are reported in FALS and FTD cases, which comprises ~10% and 50% of the total cases, respectively (Kabashi et al., 2008; Elden et al., 2010; Ticozzi et al., 2011; Couthouis et al., 2012; Kim et al., 2013; Johnson et al., 2014; Mackenzie et al., 2017). Mutant RBPs may have a diverse effect on RNA homeostasis, including splicing, transport, and editing.

The role of *FUS* and *TDP-43* in RNA splicing is well documented. High-throughput and computational approaches identified ~5,500 RNA targets for *FUS* in human and mouse brains. *FUS* affects expression levels of more than 600 genes, and splicing patterns of more than 350 genes are changed, whereas *TDP-43* affects the expression of over 100 of the same genes (Lagier-Tourenne et al., 2012). The common feature among these genes is the presence of exceptionally long introns with multiple binding sites for both *FUS* and *TDP-43* (Lagier-Tourenne et al., 2012). *FUS* interacts in the spliceosome complex with key splicing factors, including Y box binding protein 1 (YB-1), hnRNP A1, and U1 small nuclear ribonucleoprotein (U1 snRNP; Butti and Patten, 2019). *FUS* is also a part of the minor spliceosome complex and functions in the removal of minor introns. The *FUS* P525L mutation, causing nuclear clearance, leads to an inhibition in splicing minor introns by mis-localizing minor spliceosome components (Reber et al., 2016).

Furthermore, both *FUS* and *TDP-43* are components of cytoplasmic ribonucleoprotein (RNP) transport granules. In mouse neuronal cultures, *FUS* is translocated to dendrites as

an RNA-protein complex in response to the activation of metabotropic glutamate receptor 5 (mGluR5; Fujii et al., 2005). FUS binds to the mRNA of the actin-stabilizing protein nuclear distribution protein nude homolog 1 (Ndl-1), and its transcripts are increased in dendrites following mGluR activation, whereas levels of Ndl-1 are significantly reduced in FUS-null dendrites (Sasagawa et al., 2002; Fujii and Takumi, 2005). In neuronal axons, TDP-43 is co-localized with known transport-associated RBPs, including IGF2 mRNA-binding protein-1 (IMP1), fragile X mental retardation protein (FMRP), and ELAV-like protein 4 (HuD; Fallini et al., 2012; Ferro et al., 2018). Cytoplasmic TDP-43 mRNA granules show bidirectional microtubule-dependent transport in neurons, whereas ALS-causing TDP-43 mutations (M337V, G298S, and A315T) impair this function (Alami et al., 2014). Neurofilament light chain (NEFL) mRNA is transported by TDP-43 mRNPs and is defective in mutant motor neurons (Alami et al., 2014). TDP-43 represses splicing of non-conserved cryptic exons; when TDP-43 is depleted, it leads to splicing of cryptic exons into mRNA and translation disruption causing nonsense-mediated decay of mRNA. In TDP-43 ALS-FTD cases, cryptic exon repression was impaired (Ling et al., 2015). In the case of the Neurofascin gene, TDP-43 binding to its mRNA is important for its expression. In TDP-43 loss of function phenotype, a previously unidentified cryptic exon is retained in the neurofascin gene targeting the mRNA to undergo nonsense-mediated decay (Chang et al., 2021). TDP-43 regulates the expression of stathmin-2, a neuronal growth associated factor. Depletion of TDP-43 reduces its binding to the first intron of stathmin-2 pre-mRNA, which uncovers a cryptic polyadenylation site whose retention produces truncated, nonfunctional mRNA. The depletion of TDP-43 by antisense oligonucleotides causes inhibition in axonal regeneration in induced pluripotent cell (iPSC)-derived motor neuron cells, these cells can be rescued by stathmin-2 expression causing restoration of axonal regenerative capacity (Melamed et al., 2019). UNC13A an ALS/FTD risk gene encodes a neuronal protein that functions in the vesicle priming step before synaptic vesicle fusion, mice lacking the protein exhibit functional deficits in glutamatergic synapses (Augustin et al., 1999; Lipstein et al., 2017). Neuronal loss of TDP-43 causes a reduction in UNC13A expression by the inclusion of cryptic exon in UNC13A mRNA. The cryptic exon was also shown to carry a variant associated with ALS/FTD risk in humans (Ma et al., 2021).

Stress Granules and Protein Inclusions

Stress granules are an important part of RNA metabolism during cellular stress and are composed primarily of RNAs and RBPs. Stress granules can be induced by various cellular stressors, including glucose starvation, oxidative stress, viral infection, and mitochondrial dysfunction (Piotrowska et al., 2010; Fu et al., 2016; Palangi et al., 2017; Moon and Parker, 2018). Once stress is relieved, the stress granule can be either disassembled or degraded by autophagy. Stress granules represent an RNA silencing strategy to conserve energy by shifting translation to produce only essential proteins for survival (Ivanov et al., 2019). Several ALS/FTD associated proteins, including FUS,

TDP-43, TIA-1, Ataxin-2, and hnRNPs, are associated with stress granules. As most of these proteins possess long intrinsically disordered domains, failure to resolve the stress granules can lead to protein aggregate formation (Wolozin and Ivanov, 2019; Baradaran-Heravi et al., 2020; Marcelo et al., 2021). Consistently, defective stress granule assembly and disassembly leading to the formation of pathogenic protein aggregates is also linked to neurodegeneration (Protter and Parker, 2016). Ubiquitin-positive protein aggregates are commonly seen in the neuronal cytoplasm of postmortem ALS/FTD cases (Vanden Broeck et al., 2014). The majority of these aggregated proteins belong to the RBP family of proteins, and mutated RBPs account for the majority of familial cases. TDP-43 pathological inclusions are seen in 97% of ALS and 45% of FTD cases, whereas FUS-positive inclusions are seen in 2% of ALS and 9% of FTD cases (Aulas and Vande Velde, 2015; Prasad et al., 2019).

Autophagy

Due to their postmitotic nature, neurons cannot dilute toxic cell components by cell division, instead, they depend on active protein quality control mechanisms for cell viability and homeostasis. Studies have demonstrated that defects in the induction and clearance of autophagosomes are associated with the pathogenesis of neurodegenerative diseases (Tran and Reddy, 2020). Many ALS-associated genes whose toxicity is related to protein misfolding and aggregation have a connection to the autophagy cascade and function in different stages of autophagy. ALS-associated SOD1 mutations cause defective vesicle nucleation by destabilizing the BECN1-BCL2L1 complex, thereby impacting autophagy stimulation (Nassif et al., 2014). Mutations in p62 (SQSTM1) are reported in the case of FALS and SALS, and Optineurin (OPTN) mutations are seen in rare ALS cases, TANK binding kinase-1 (TBK1) mutations connected to ALS are identified in eight independent genetic screens. Both p62 and OPTN function as autophagy receptors and are important for the initiation step of autophagy (Maruyama et al., 2010; Lattante et al., 2015; Shen et al., 2015; Cui et al., 2018). TBK1 is important for autophagy as it phosphorylates a number of autophagy adaptors, including p62, OPTN, and NDP52, and it is also important for autophagosome maturation (Pilli et al., 2012; Heo et al., 2015). In the maturation stage, an autophagosome fuses with a lysosome. Valosin-containing protein (VCP), an AAA-ATPase whose mutation is associated with ALS, is important for recycling or degradation of ubiquitinated proteins either by the ubiquitin proteasomal or autophagy-mediated lysosomal system. VCP loss leads to the accumulation of immature autophagosomes with ubiquitin-positive substrates (Ju et al., 2009; Johnson et al., 2010; Meyer et al., 2012). FUS and TDP-43 clearance are associated with autophagy, and the pharmacological activation of autophagy leads to increased turnover and early clearance of these proteins from stress granules (Wang et al., 2012; Ryu et al., 2014; Cheng et al., 2015). In the case of C9orf72, wild-type protein interacts with Rab1a preferentially in the GTP-bound state and controls trafficking of ULK1, a kinase involved in autophagosome formation. C9orf72 depletion causes

dysregulation of autophagosome function (Farg et al., 2014; Monahan et al., 2016).

Mitochondrial Dysfunction

Although it is debatable whether it is a cause or an effect, mitochondrial dysfunction is a constant feature in neurodegeneration. Mitochondrial fragmentation, alteration in mitochondrial morphology, fission, and fusion-associated gene expression variations are seen in ALS, along with altered Ca^{2+} metabolism and reactive oxygen species (ROS) generation (Lin and Beal, 2006). Many proteins associated with ALS, including FUS, TDP-43, and SOD1, also localize in mitochondria and affect their dynamics, including mitochondrial fission, fusion, and localization. Mutations or cellular stress-induced increases in TDP-43 expression lead to mitochondrial unfolded protein response (UPR^{mt}) and downregulation of LonP1, a protease involved in the degradation of mitochondrial TDP-43, which leads to severe mitochondrial damage and causes advancement of disease onset in a TDP-43 expressing fly model (Wang P. et al., 2019). FUS interacts with mitochondrial chaperon HSP60. HSP60 expression increase is seen in two out of three FTD-FUS patients, and knockdown of HSP60 causes a decrease in FUS localization to the mitochondria. In a transgenic FUS fly model, RNAi-mediated downregulation of the HSP60 homolog partially rescued the neurodegenerative FUS phenotype (Deng et al., 2015). A FUS mutation associated with ALS/FTD, FUS R521C, causes mitochondrial dysfunction by preferentially sequestering respiratory chain complex mRNAs leading to reduced expression of those proteins. In comparison to wild-type FUS, mutant FUS expressed in mitochondria binds to the mitochondrial mRNAs ND1, CYTB, COX1, and ATP6 five-fold more strongly (Tsai et al., 2020). FUS interacts with mitochondrial ATP synthase 5 beta and disrupts ATP synthase complex assembly (Deng et al., 2018). ALS-associated FUS mutations and FUS Δ NLS cause mitochondrial shortening and fragmentation due to the mutant FUS binding to mature mRNAs and altering the expression of those genes, including mitochondria-associated genes (Nakaya and Maragkakis, 2018). Both FUS and TDP-43 affect endoplasmic reticulum (ER)-mitochondria interaction dynamics, leading to defective Ca^{2+} metabolism, and both proteins do so by activating GSK-3 β , which disrupts VAPB-PTPIP51 interaction. The alteration in Ca^{2+} levels causes defects in ATP production (Stoica et al., 2014, 2016). Mitochondrial DNA repair is another frontier, which needs further investigation due to emerging findings that show DNA repair roles of ALS-associated proteins, such as FUS and TDP-43 (Kodavati et al., 2020).

Nucleocytoplasmic Transport Defects

The nuclear pore complex (NPC), which is central to nucleocytoplasmic transport, disassemble and reassemble during mitosis. In post-mitotic cells, although some components like Nup153 and Nup50 of the complex are continuously exchanged, the scaffold nucleoporins like Nup107/160 remain in the complex. Aging is associated with deterioration of the NPC integrity (D'Angelo et al., 2009). TDP-43 cytoplasmic inclusions are identified in approximately 97% and 45% of

ALS and FTD cases, aggregate specific interacting partners of TDP-43 include components of NPC and nucleocytoplasmic transport machinery (Ling et al., 2013; Chou et al., 2018). In Human neurons expressing mutant FUS, reduction in nucleocytoplasmic transport and decreased density of nucleoporins (Nups) is observed. FUS and Nups are found to be interacting independent of RNA, FUS-Nup interaction is seen in the nucleus of healthy neurons, whereas in mutant FUS carrying cells, they are seen in the cytoplasm (Lin et al., 2021). In the case of C9orf72 repeat expansion, the toxic dipeptides expressed are shown to be directly binding to the central channel of the nuclear pores and inhibit the transport of macromolecules through the nuclear pore. The binding of these toxic dipeptides to NPC is mediated by polymeric forms of nuclear pore protein and phenylalanine: glycine repeats of dipeptide repeats (Shi et al., 2017).

DNA REPAIR DEFECTS AND RESULTING GENOME INSTABILITY IN ALS/FTD

Genome damage and its defective repair have been etiologically linked to degenerating neurons in various neurodegenerative diseases, including ALS/FTD, however, the specific mechanisms remain enigmatic (Wang et al., 2017). Early studies of ataxia-telangiectasia, a childhood disease affecting the development of the neuron system caused by mutations in ATM, a serine/threonine kinase that plays a critical role in repairing DNA double-strand breaks (DSBs), provided the first linkage between DNA damage and neurodegeneration (Savitsky et al., 1995; Rothblum-Oviatt et al., 2016). Subsequent investigations revealed that several ALS/FTD-linked genes including FUS, TDP-43, SOD1, C9orf72, *Never-in-mitosis A (NIMA) related kinase-1 (NEK1)*, C21orf2, *Senetaxin (SETX)*, and *Valosin containing protein 1 (VCP)* are involved in the DNA damage response (DDR; Acs et al., 2011; Fang et al., 2015; Guerrero et al., 2016; van Rheenen et al., 2016; Higelin et al., 2018; Konopka and Atkin, 2018; Wang H. et al., 2018; Bordoni et al., 2019; Mitra et al., 2019). The mutation, mislocalization, or defects in those genes mediated DNA repair deficiency have been etiologically linked to ALS and FTD (Sun et al., 2020).

Genomic DNA Damages and Their Repair in the Context of Normal Physiology

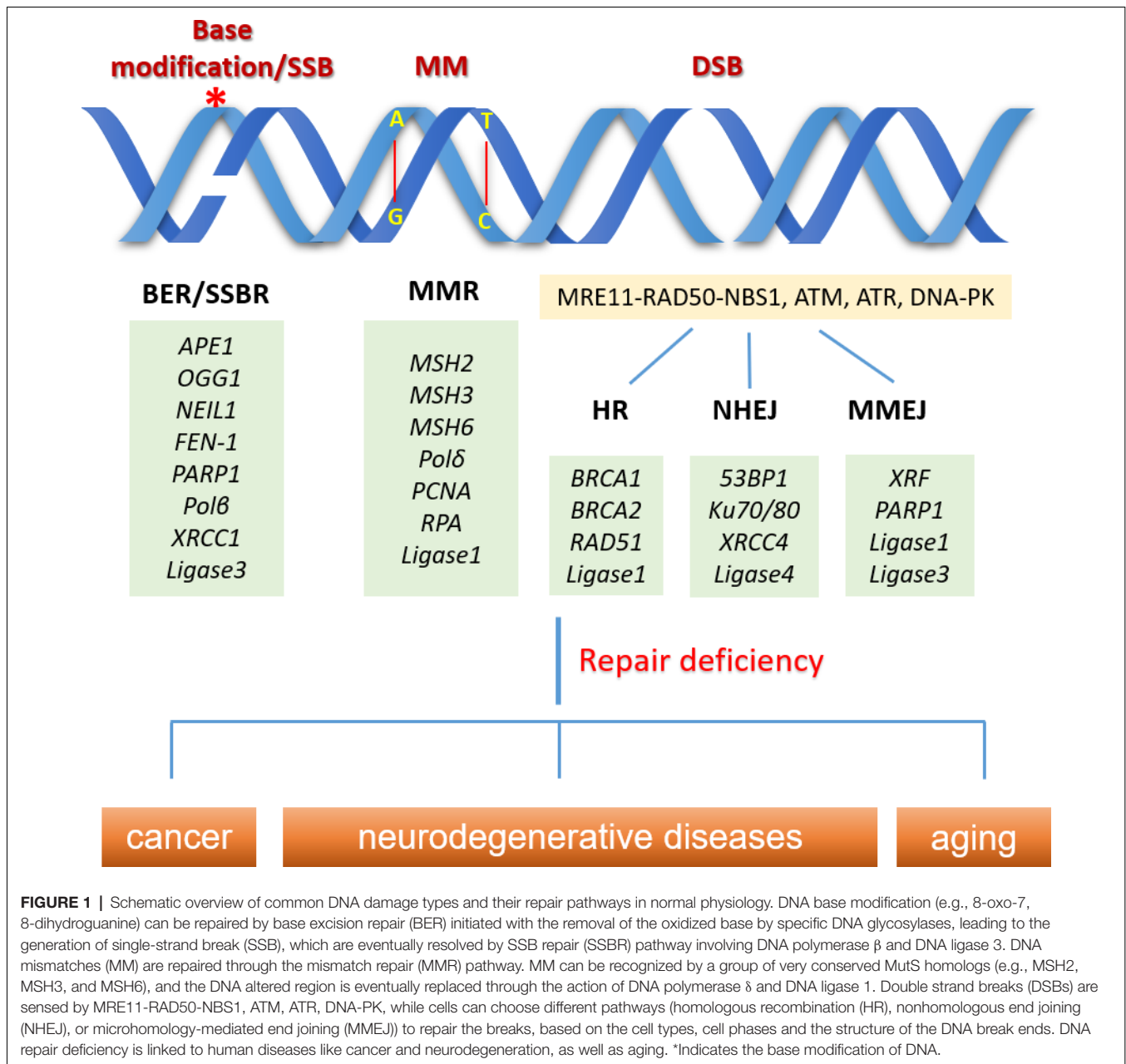
Human genomic DNA is continuously damaged by multiple endogenous or exogenous factors, including DNA replication errors, activities of enzymes like DNA glycosylase and topoisomerase, ROS, ultraviolet (UV) radiation, ionizing radiation (IR), and chemicals (Hegde et al., 2008; Chatterjee and Walker, 2017). Depending on the sources, various types of DNA damage are induced, which include several dozen oxidized DNA bases, base mismatches, DNA interstrand crosslinks, and strand breaks, including single-strand breaks (SSBs) and DSBs. The DSBs are the most lethal form of damage, and a single unrepaired DSB may be sufficient to trigger cell death (Chatterjee and Walker, 2017; Trenner and Sartori, 2019). The endogenous factor, ROS, is continuously generated during the cellular metabolisms like ATP production and inflammation

response, and thus ROS-induced damage is the most critical threat to the genome of normal tissues, especially in the CNS due to the high consumption of oxygen by the brain (around 20% of the body; Lopez-Gonzalez et al., 2016; Wang et al., 2017). The major forms of ROS include superoxide radical anion (O_2^-), hydroxyl radical ($\bullet OH$), as well as hydrogen peroxide (H_2O_2 ; Kowalska et al., 2020). The most commonly observed oxidative DNA damage is the 8-oxo-7, 8-dihydroguanine (8-oxoG), which is a $\bullet OH$ mediated C-8 hydroxylation of guanine and is recognized as oxidative DNA damage marker. Notably, 8-oxoG lesion is higher in mitochondrial DNA than in nuclear DNA, suggesting a susceptibility of mitochondrial DNA to oxidative damage (Guillaumet-Adkins et al., 2017). ROS can induce SSBs directly or indirectly (during oxidized lesion repair), and unrepaired adjacent oxidative damage can result in secondary DSBs. It's worth mentioning that oxidation-related genome damages occur transiently at early-response gene promoters. This neural activity-induced gene expression is essential during the development of the brain and neuron maturation (Madabhushi et al., 2014, 2015; Su et al., 2015). Although the evolutionary significance of such DNA break formations during normal physiological processes is still debated, DNA damage is highly increased in the aging human brain and has been linked to defects in learning, memory, and neuronal survival (Lu et al., 2004).

DNA damage can be both mutagenic and cytotoxic, and the DNA damages that are not repaired efficiently and timely are associated with multiple human diseases, including cancer and neurodegenerations. Oxidative DNA damages are mainly repaired by base excision repair (BER). BER is initiated with the removal of the oxidized base by specific DNA glycosylases, leading to the generation of apurinic/aprimidinic (AP) site that is further cleaved to form an SSB. The DNA breaks are eventually resolved by DNA polymerase β and DNA ligase 3, which share the same steps of the SSB repair (SSBR) pathway. BER repairs non-bulky DNA lesion, while ROS and UV-induced bulky lesion is repaired by nucleotide excision repair (NER). Although BER and NER share some common repair steps like DNA damage recognition, damaged nucleotide excision, and DNA resynthesis, BER repairs fragments having a length of 1–6 bases, whereas NER is capable of repairing DNA fragments with a length of 30 bases (Lee and Kang, 2019; Casal-Mourino et al., 2020). DNA mismatches are usually generated during DNA replication, which is repaired through the mismatch repair (MMR) pathway. DNA mismatches can be recognized by a group of very conserved MutS homologs (e.g., MSH2, MSH3, and MSH6), and the DNA altered region is eventually replaced through the action of DNA polymerase δ and DNA ligase 1 (Jiricny, 2013). DNA DSBs can be repaired by homologous recombination (HR), nonhomologous end joining (NHEJ), or microhomology-mediated end joining (MMEJ). HR is the most accurate pathway for repairing DSB because the repair is processed based on the sister chromatid and thus, it is only available in the S and G2 phases of dividing cells when the DNA is replicated, but not in the neurons, which is one of the major differences in the DNA repair pathways between dividing and non-dividing cells, although some proteins of the HR pathway are still present in non-dividing cells (Iyama

and Wilson, 2013; Fugger and West, 2016). Compared with HR, NHEJ provides an error-prone repair by direct ligating the DSB ends, which exists in all cell cycles and is a major pathway in repairing cellular DSBs. Besides, NHEJ was shown to be critical in maintaining the proliferation of non-dividing cells and is the only active DSB repair pathway in postmitotic cells (Iyama and Wilson, 2013; Baleriola et al., 2016). The alignment of microhomologous sequences between the broken ends differentiates MMEJ from NHEJ (Sfeir and Symington, 2015). MMEJ was initially believed to play a backup role of NHEJ and was less studied, while a recent work showed a more important role of MMEJ as an advantageous choice for cells to quickly repair DSBs to avoid cell death (Truong et al., 2013; Vaidya et al., 2014; Dutta et al., 2017). However, the function of MMEJ in DSB repair in neurons is unclear. DNA interstrand crosslinks are highly toxic due to the covalent connection of the two DNA strands, thereby blocking DNA replication and transcription (Truong et al., 2013). The repair of DNA interstrand crosslink is complicated and involves multiple DNA repair pathways. The interstrand crosslinks occurring outside of S phase are mainly repaired by NER while those occurring in S phase are primarily repaired by Fanconi anemia (FA) pathway, a repair pathway relying on an E3 ubiquitin ligase complex formed by eight FA genes and coordination with HR (McHugh and Sarkar, 2006; Kim and D'Andrea, 2012; Hashimoto et al., 2016; Liu et al., 2020). However, HR was shown to be unessential for DNA interstrand crosslinks repair in quiescent cells (G0/G1 phase), likely because NER plays a dominant role in those cells (Figure 1; Hashimoto et al., 2016).

Multiple biochemical methods are used to investigate DNA damages. Foci formation assay is a very commonly used tool to visualize DNA damage in cells. The assay is based on the posttranslational modification (e.g., phosphorylation), or physical recruitment and accumulation of certain DDR factors (e.g., H2XA, ATM, and 53BP1) at DNA damage termini in response to a DSB, which makes it possible to stain the proteins with fluorescent antibodies and show bright dots as "foci" under a fluorescence microscope. The foci formation assay provides a straightforward way to observe the DSB, and the disappearance of the foci is closely related to the kinetics of DNA damage repair. However, despite the utilization of DSB detection only, DSB independent foci formation of the DDR proteins is a major challenge to the assay (Wang et al., 2014). Single-cell gel electrophoresis assay (also known as comet assay) is a method to measure DNA strand breaks in eukaryotic cells. Depending on the electrophoresis condition, comet assay is subdivided into neutral and alkaline comet assays. The neutral comet assay is mostly used to detect DSBs, whereas alkaline comet assay reveals a phenotype with multiple types of DNA damages, including both DSBs and SSBs (Collins, 2004; Lu et al., 2017). By comparing neutral and alkaline comet assays, DNA damages other than DSBs can be exhibited. However, comet assay does not directly show the number of specific DNA lesions (Moller et al., 2020). Besides, the severity of the DNA damage is reflected by the measuring of the tail length and tail moment of the comet image by software, the outcome may vary when measured with different software (Lu et al., 2017). Long amplicon-polymerase



chain reaction (LA-PCR) is another widely used way to assess DNA damages. LA-PCR reveals DNA damage by amplifying a long fragment (around 10kb) of a target gene (like housekeeping genes). The PCR product may be reduced due to the damage of the DNA template compared to undamaged control. Parallel amplification of a shorter fragment within the same target gene is needed to normalize the DNA copy number or to compare DNA damage from different DNA sources (Jung et al., 2009). Although LA-PCR doesn't differentiate DNA damage types, since it's a PCR-based measurement, LA-PCR can be directly used to detect DNA damages of cultured cells, fresh or frozen tissues from animal models or patients. LA-PCR can also be used to detect the DNA damage in mitochondria by using mitochondrial

DNA-specific primers. Finally, several DNA damage repair reporter systems have also been well developed and established in living cells to study specific DNA damage repair pathways, including MMR, HR, NHEJ, and MMEJ, although it relies on the detectable expression of the reporter constructed plasmids in cultured cells (Wang et al., 2009, 2013; Zhou et al., 2009; Dutta et al., 2017; Mitra et al., 2019).

ALS/FTD-Associated Proteins Play a Role in DNA Damage Repair

While genome damage has been consistently linked to neurodegeneration, including ALS/FTD, the role of disease-linked genetic factors in causing genome instability was not

clearly established until recently. For example. Significantly up-regulated DDR markers, phosphorylated H2AX (γ -H2AX) and ATM (p-ATM), cleaved PARP1, and 53BP1 were shown in the lumbar spinal cord from C9orf72-positive ALS patients (Farg et al., 2017).

C9orf72

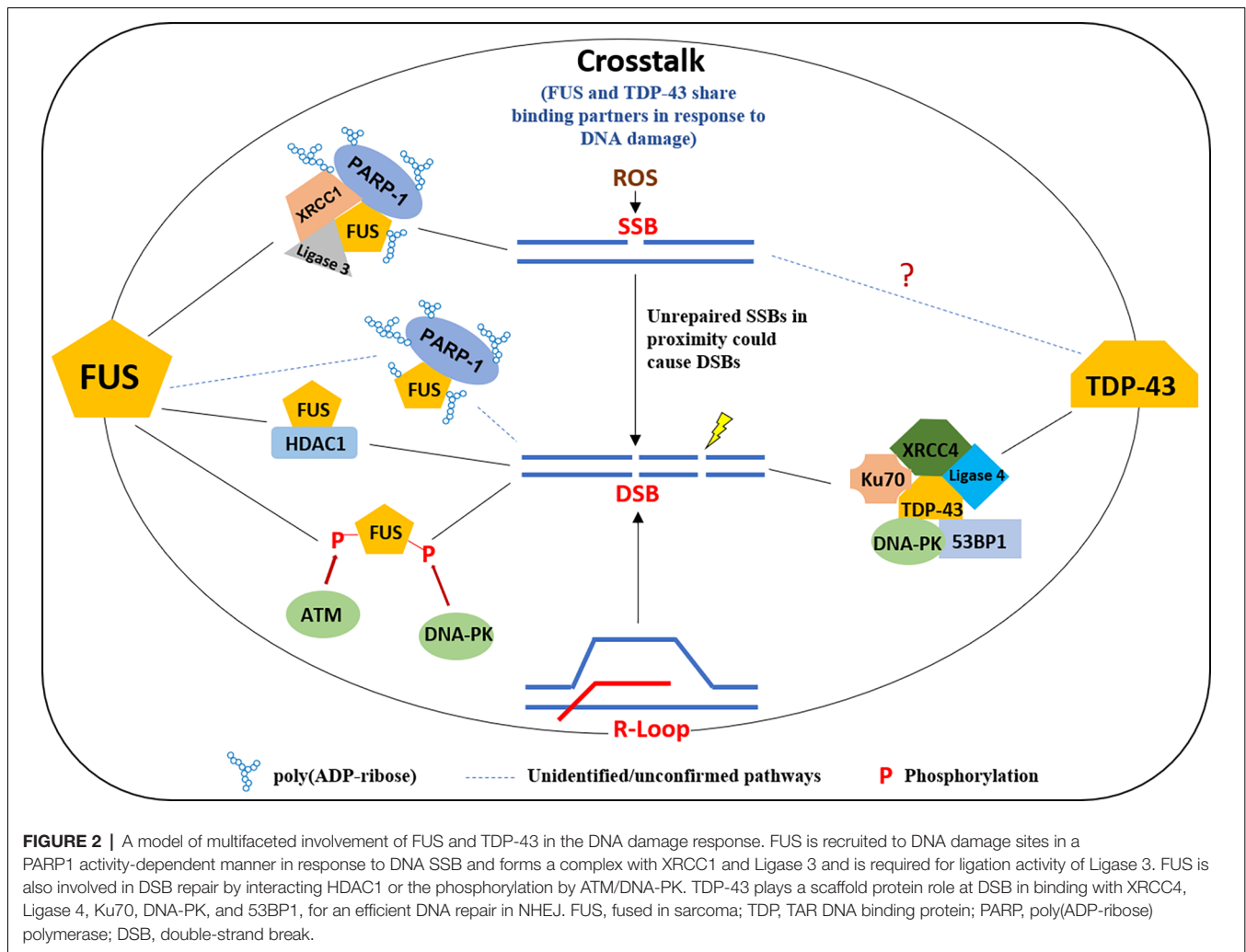
In motor neurons differentiated from ALS/FTD patient-derived iPSCs containing the GGGGCC repeat in *C9orf72*, oxidative stress, and DNA damage were increased compared with healthy controls (Lopez-Gonzalez et al., 2016). The same group subsequently revealed that the expression of the DNA repair protein Ku80, accompanied with the DSB markers phosphorylated ATM and P53, as well as other downstream proapoptotic proteins, are up-regulated in both the *Drosophila* model expressing poly-GR and in C9orf72 iPSC-derived patient neurons (Lopez-Gonzalez et al., 2019). The study also showed that proapoptotic pathways regulated by ATM and P53 could be suppressed by the partial loss of Ku80 in C9orf72 iPSC-derived neurons. Andrade et al. showed that C9orf72 expansion-encoded dipeptide repeat proteins (DPRs) inhibit multiple DNA DSB repair pathways. In addition, single-strand annealing (SSA) repair, a sub-pathway of MMEJ-mediated DSB repair, is impaired partially, which is mediated *via* inhibition of nucleolar protein nucleophosmin (NPM1) by DPRs.

Moreover, levels of the SSA component RAD52 are significantly increased in postmortem brain tissues from ALS/FTD samples with C9orf72 pathology as compared to controls (Andrade et al., 2020). A study from Nihei et al. (2020) revealed that heterogeneous ribonucleoprotein (hnRNP) A3 is depleted from the nucleus and partially mislocalizes to cytoplasmic poly-GA inclusions in C9orf72 patients, and the mislocalization caused by poly-GA leads to increased poly-GA production, which partially depletes pATM and consequently enhances DSB accumulation. R-loop is a structure of hybridized nascent RNA and DNA template strands formed during DNA replication, transcription, and DNA repair. The transient formation and disassembly of R-loop structures are tightly regulated. R-loops can be formed by the collision of replication and transcription machinery or due to the absence of RBP's covering the nascent RNA, making them available to bind ssDNA. R-loop formation can cause replication stalling and replication fork collapse leading to activation of ATR, when DSB is induced after R-loops, it leads to activation of ataxia telangiectasia mutated (ATM). Failure to resolve R-loops in a timely manner may induce the formation of DSBs, which involves severe genome instability (Niehrs and Luke, 2020). Given that R-loops primarily occur at GC-rich transcription sites and are likely in the hexanucleotide repeat expansion (GGGGCC) of *C9orf72*, it is proposed that C9orf72 promotes persistent R-loop dysfunction (Haeusler et al., 2014), which was eventually demonstrated by studies conducted by Walker et al., showing elevated R-loop levels and R-loop-driven DSBs in C9orf72 expansion-expressing cells (Walker et al., 2017).

FUS and TDP-43

There is growing evidence that DNA damage is accumulated in FUS and TDP-43 related ALS and FTD patients. γ H2AX

was found to be significantly increased in brain tissues from patients with neuronal intermediate filament inclusion disease (NIFID), a subtype of FTD-FUS, and in the motor cortex of FALS cases harboring R521C and P525L mutations in FUS (Wang et al., 2013; Higelin et al., 2016). Similarly, increased DNA damage was seen in the frontal cortex sections from patients with FTLT-TDP-43, the spinal cord of ALS patients carrying the TDP43 Q331K mutation, as well as in fibroblasts derived from a TDP-43 ALS patient with M337V mutation (Guerrero et al., 2019; Konopka et al., 2020; Wu et al., 2020). Both FUS and TDP-43 play important roles in DNA damage repair (**Figure 2**). FUS can be multi-phosphorylated by the DDR kinases ATM and DNA-PK in response to DNA damage (Gardiner et al., 2008; Deng et al., 2014; Rhoads et al., 2018). FALS patients with FUS R521C and P525L mutations show increased DNA damage and the impairment of HR and NHEJ, and a reduced DDR was found in FUS knockdown cells (Wang et al., 2013). An RT² PCR array-based screening reveals distinct perturbations in DDR signaling in FUS-associated motor neuron disease (Wang H. et al., 2019). A number of studies have explored the molecular mechanisms of FUS involving DDR. Coordination between FUS and Poly (ADP-ribose) polymerase (PARP1) is critical for the DNA repair function of FUS. FUS interacts with Poly (ADP-ribose; PAR) and can be PARylated by PARP1, and the inhibition of PARP1 activity prevents the recruitment of FUS onto micro-laser-induced DNA damage tracks (Mastrocola et al., 2013; Rulten et al., 2014). The direct interaction between FUS, PARP1, XRCC1, and Ligase 3 is important for optimal SSB repair (Wang H. et al., 2018). FUS facilitates PARP1-dependent recruitment of the XRCC1-Ligase 3 complex to oxidized genome sites and activates Ligase 3 through direct interaction (Wang H. et al., 2018). Liquid-liquid phase separation is a phenomenon of forming subcellular non-membrane-bound organelles, such as stress granules, as part of the cellular stress response signaling (Wheeler et al., 2016). FUS forms liquid compartments at DNA damage sites and in the cytoplasm upon stress in a PAR interaction-dependent manner, while the ALS-FUS mutant can promote the aggregation of FUS protein-mediated liquid droplets, a common hallmark of FUS pathology in ALS and FTD (Patel et al., 2015). Singatulina et al. (2019) showed that, following PARP-1 activation, FUS binds to PAR and facilitates the formation of damaged DNA-rich compartments to facilitate DNA damage repair, which can be dissociated by Poly(ADP-ribose) glycohydrolase (PARG). Levone et al. (2021) found that liquid-liquid phase separation depends on FUS, which is required for activation of DDR and DSB repair complex assembly. Our and other recent studies demonstrated that TDP-43 also plays an important role in DDR, primarily for the repair of DSBs (Mitra and Hegde, 2019; Mitra et al., 2019; Singatulina et al., 2019). In healthy neurons, TDP-43 is required for the optimal repair of DSBs *via* NHEJ by facilitating the recruitment of the XRCC4-DNA Ligase 4 complex to damage sites. TDP-43 also forms a complex with several DSB repair and response factors, including Ku, DNA-PKcs, 53BP1, and XRCC4-DNA Ligase 4. An ALS-associated TDP-43 mutation prevents nuclear translocation of XRCC4-DNA Ligase 4 complex and is linked to DNA damage mediated neuronal apoptosis (Guerrero et al.,



2019). Subsequent studies confirmed the role of TDP-43 in DSB repair pathways in cellular models (Mitra et al., 2019; Konopka et al., 2020). Both FUS and TDP-43 are likely involved in R-loop associated DDR. FUS localizes to the sites of transcription-associated DNA damage and function in the prevention/repair of R-loops (Hill et al., 2016), while TDP-43 mutation was shown to disturb R-loop homeostasis and contributes to R-loop-mediated DNA damage (Giannini et al., 2020). Furthermore, FUS and TDP-43 may crosstalk in DDR. A study showed that TDP-43 and FUS share binding partners after treatment with a DNA damage agent, etoposide, and gene ontology revealed a complex network of interactors with both TDP-43 and FUS. The largest group of interactors shared by FUS and TDP-43 was that of ribosomal proteins, including mitochondria-specific ribosomes (Kawaguchi et al., 2020).

SOD1

As the byproduct of normal cellular respiration, ROS is the major source of oxidative stress contributing to DNA damage in neurons. Common ROS include superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which are removed/reused by mechanisms involving superoxide dismutases (SOD), catalases, thioredoxin,

and glutathione (Tsang et al., 2014). SOD1 is one of three superoxide dismutase family members. SOD1 pathological inclusions are seen in both ALS and FTD cases. SOD1 mutations are only associated with familial ALS but not FTD cases (Vance et al., 2006). In a motor neuron injury mouse model, the human FALS SOD1 G93A mutant accumulates greater DNA damage in the injured motor neurons compared with control, accelerates injury-induced degeneration of the motor neurons, and converts the apoptotic phenotype to a necrotic phenotype, a phenotype showing the pathological process of cell death (Martin et al., 2005). SOD1 is a nuclear transcription factor that regulates oxidative stress resistance. Studies revealed that SOD1 is translocated to the nucleus in an ATM/CDS1-dependent way to regulate gene transcription that is involved in DDR or ROS defense (Tsang et al., 2014; Sun et al., 2020). Dysfunction of SOD1 in ALS not only causes the accumulation of ROS but also affects DDR signaling, a double hit in destabilizing genome integrity.

Crosstalk between SOD1 and other ALS-linked genes has been explored by a number of studies. Abundant inclusions containing misfolded wild-type SOD1 were found in spinal and cortical motor neurons in ALS patients carrying other

ALS-causing genes, including the mutations of *C9orf72* and *FUS* (Forsberg et al., 2019). Pokrishevsky et al. observed misfolded SOD1 in perikarya and motor axons of SOD1-FALS and in motor axons of FUS FALS and SALS with TDP-43 pathology, and the overexpression of both wild-type or mutant TDP-43 or the mutant FUS in the cytoplasm can cause misfolding of wild-type SOD1 (Pokrishevsky et al., 2012, 2016). The DNA damage accumulation/DNA repair deficiency in ALS is likely a consequence of interference in DNA damage repair pathways by a combination of factors. Although the detailed mechanisms of this crosstalk require extensive future investigation, Li et al. have demonstrated that cytoplasmic restriction of the mutant SOD1 G93A, which inhibits the nucleic translocation of wild-type SOD1, was directly related to the elevation of DNA damage, partially due to inhibition of the localization of FUS (but not TDP-43) and its related DNA repair enzymes, including HDAC1 and APEX1, in the nuclei (Li et al., 2019).

NEK1

NEK1 is a mitotic protein kinase whose heterozygous mutations are associated with ALS. NEK1 localizes to sites of IR-induced DNA damage foci and is a key factor in the early DNA damage repair pathway. NEK1 is important for activation of checkpoint kinases 1 and 2 (CHK1 and CHK2), NEK1 deficiency causes severe alterations in checkpoint control and DNA damage accumulation leading to genome instability (Higelin et al., 2018). NEK1-ALS iPSC motor neurons showed increased γ H2AX in comparison to controls, ALS-associated mutations in NEK1 cause haploinsufficiency leading to elevated DNA damage and motor neuron death (Chen et al., 2008; Higelin et al., 2018). NEK1 is also shown to phosphorylate and activate HR repair protein RAD54, which leads to Rad51 removal from chromatin and causes degradation of stalled replication forks (Spies et al., 2016).

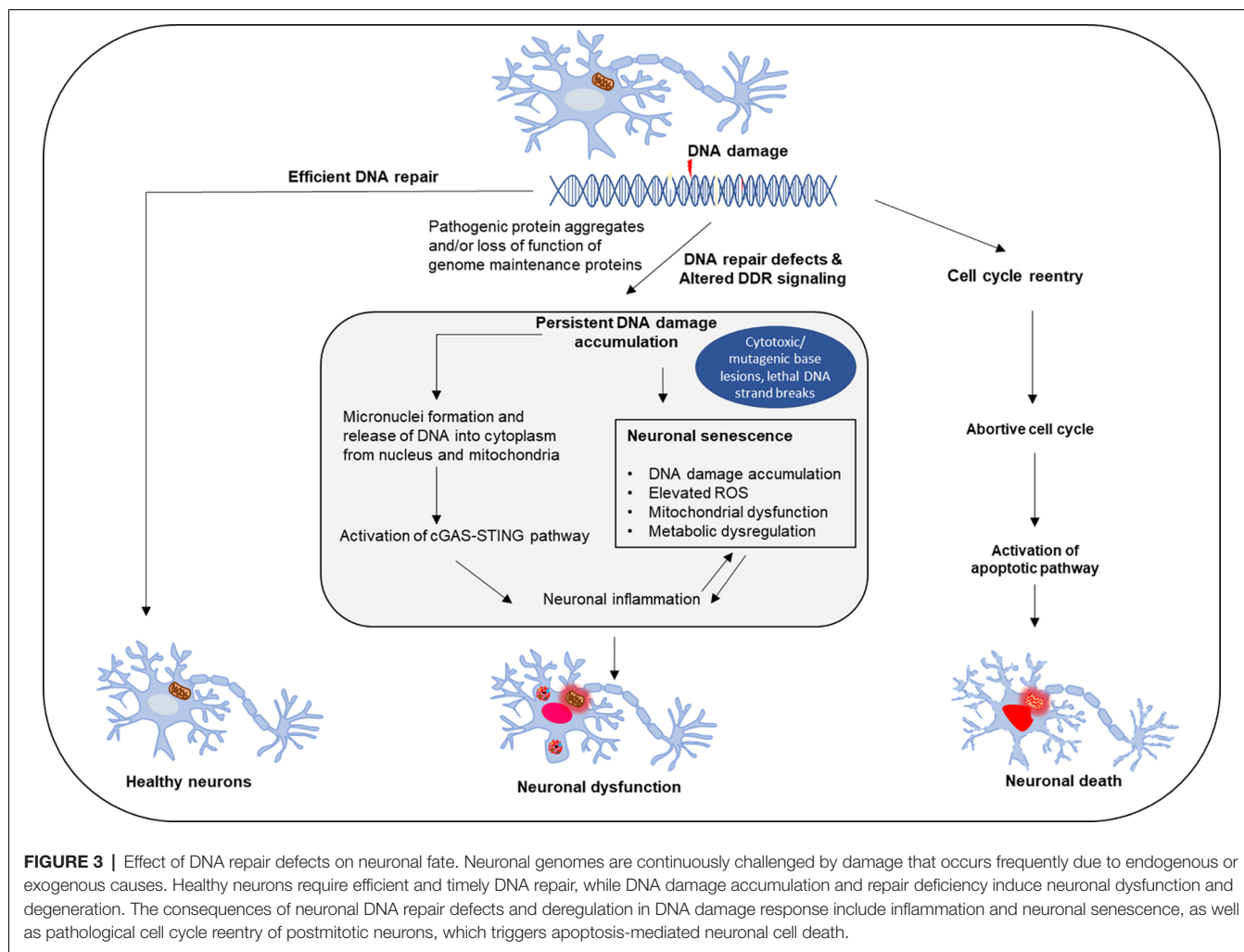
Other ALS/FTD-Associated Proteins

VCP is a central component of the ubiquitin proteasome system, and mutations in VCP are shown to be associated with ALS. VCP functions in the removal of lysine 48 ubiquitin conjugates from DNA damage sites, making them available for DNA repair factors recruitment (Johnson et al., 2010). In response to DNA damage along with ubiquitin ligase RNF8, VCP facilitates recruitment of 53BP1, BRCA1, and RAD51 factors critical for DNA repair and survival (Meerang et al., 2011). In *C9ORF72* ALS, depletion of VCP impairs 53BP1 recruitment to DNA damage sites (Walker and El-Khamisy, 2018). *C21orf2* is identified as an ALS-associated gene by genome-wide association study and meta-analysis, it is known to form a functional complex with NEK1, and both proteins stabilize each other (van Rheenen et al., 2016; Watanabe et al., 2020). *C21orf2* deficient cells show less efficient DNA repair than control cells, NEK1 overexpression can complement *C21orf2* depletion. Only the HR pathway requires *C21orf2*/NEK1 complex, NHEJ repair can function without this complex (Fang et al., 2015). Mutations in the SETX gene are responsible for a dominantly inherited form of ALS, which has early onset (Groh et al., 2017). SETX gene encodes an RNA/DNA helicase involved in DNA damaged response, SETX is recruited to DNA DSBs at transcriptionally active

sites. Upon binding, SETX promotes Rad51 recruitment and prevents mutagenic NHEJ (Cohen et al., 2018; Rawal et al., 2020). Exome sequencing analysis identified mutations in *Matrin3*, an RNA binding protein associated with familial ALS (Boehringer et al., 2017). ALS associated *Matrin3* mutation causes its altered re-distribution in the nucleus and alters mRNA nuclear export, *Matrin3* knockdown causes a reduction in RAD51 protein levels leading to HR disturbances and increased radiation sensitivity (Boehringer et al., 2017). *Matrin3* interacts with PSF, a splicing factor that interacts with RAD51 and modulates its activity in HR depletion of *Matrin3* causes prolonged retention of PSF at DNA damage sites (Salton et al., 2010; Boehringer et al., 2017). ALS-associated mutations are identified in low complexity domains of hnRNPA1, it plays an important role in maintaining the integrity of telomeres (Sui et al., 2015). Phosphorylation of hnRNPA1 promotes a switch from replication protein A (RPA) to the protection of telomeres 1 (POT1) at newly replicated telomeric overhangs (Sui et al., 2015).

Potential Consequences of DNA Repair Defects in ALS/FTD

DDR and DNA repair deficiency are widely recognized as common premises in many neurodegenerative disorders. Mutations in DNA repair and DDR genes associated with neurodegenerative diseases including mutations in NER genes associated with Cockayne syndrome, Xeroderma Pigmentosum, and Trichothiodystrophy, mutations in BER genes associated with ataxia oculomotor apraxia, and DSB repair gene mutations causing ataxia telangiectasia, XLF syndrome, and Ligase 4 syndrome (Le Ber et al., 2003; Kraemer et al., 2007; Chistiakov, 2010; Keimling et al., 2011). Although the link between DNA damage and neurodegeneration is generally accepted, mechanisms of neuronal DNA repair and implications of such repair defects are still not fully understood, which is a roadblock for developing effective DNA repair-based therapeutics. Notably, there could be subtle differences in the preferences for various DNA repair pathways and vulnerability to the DNA damage sources at different stages of neuronal development and maturity. One consequence of the robust increase in the unrepaired genome in postmitotic neurons is accidental cell cycle re-entry, presumably to activate cell cycle-associated DNA repair pathways, but with a catastrophic outcome, as this can cause neuronal cell death by apoptosis (Folch et al., 2012; **Figure 3**). Furthermore, ALS-affected neurons show many classical features of cellular senescence, including senescence-associated neuroinflammation, increased DNA damage, activated DDR, metabolic dysregulation, mitochondrial dysfunction, and elevated ROS levels (Martinez-Cue and Rueda, 2020). Recent studies have identified the cGAS-STING pathway as a link between DNA damage and inflammation (Decout et al., 2021). The formation of micronuclei is associated with DNA damage, and the nuclear envelope of micronuclei is ruptured to reveal DNA in the cytoplasm, which causes activation of cGAS (Kwon et al., 2020). In TDP-43 mutant mice and iPSC-derived motor neurons, recruitment of TDP-43 to mitochondria causes the release of DNA through the permeability transition pore leading to activation of the cGAS-STING pathway (Yu et al.,



2020). Elevated levels of cGMP, a secondary messenger in the cGAS-STING pathway, are seen in patients' spinal cords and activation of this pathway can be a critical determinant of TDP-43 pathology (Yu et al., 2020). Thus, DNA damage defects could directly contribute to neuronal degeneration or disturb other mechanisms that maintain normal cellular functions (Figure 3).

FUTURE DIRECTION: TARGETING DNA DAMAGE FOR ALS-FTD TREATMENT

Although the available treatments can transiently slow down the progression of the ALS-FTD or can help manage some symptoms, there is currently no cure for these diseases. Identification of the involvement of several ALS-FTD causative genes in DDR raises the possibility of targeting DNA damage as a therapeutic strategy. Growing evidence has shown that neurodegeneration can be prevented by rescuing DNA damage repair deficiency, as supported by studies in cultured cells or animal models. Lopez-Gonzalez et al. (2019) found that the expression of protein Ku80, required for NHEJ-mediated DSB repair, was greatly elevated in *Drosophila* expressing

poly-GR and in C9orf72 iPSC-derived FTD patient neurons, and partial inhibition of Ku80 suppressed the cell death of the neurons. In another study, Walker et al. (2017) revealed that defective ATM activation induced by the expression of C9orf72 expansions is a consequence of the accumulation of p62, and the depletion of p62 can restore the defective ATM-mediated DNA repair which suggests a therapeutic opportunity for C9orf72-related neuropathologies. Decreased ligation activity of Ligase 3 and Ligase 4 were seen in FUS mutant, and TDP-43 depleted cells, respectively (Wang H. et al., 2018; Mitra et al., 2019). Identifying ways to target and rescue the DNA ligation activity of these ligases in FUS- and TDP-43-associated ALS-FTD patients could lead to new treatment avenues for these diseases. In response to DNA damage, PARP1 is activated by its auto-PARYlation, which then PARYlates a number of target proteins to activate or recruit DDR factors to repair the damage. In a study conducted by McGurk et al. (2018), significantly elevated levels of nuclear PAR were seen in the postmortem spinal cord from ALS patients with phosphorylated TDP-43 inclusions, indicating that the PARPs are highly activated. They further found that in the cytoplasm, the activity of PARP 1/2 regulates stress granule

formation and stress-induced TDP-43 aggregation, which can be suppressed by the PARP 1/2 inhibitor veliparib. Finally, they found that veliparib mitigates TDP-43 toxicity in primary spinal cord neurons isolated from rat embryos. Neurodegeneration in ALS/FTD could be a consequence of increased DNA damage, DNA damage repair deficiency, or a cumulative effect of both. A therapeutic strategy targeting DNA damage for ALS/FTD treatment likely can be reached by either improving DNA damage repair efficiency or alleviating the toxic events that induce DNA damage in neurons.

AUTHOR CONTRIBUTIONS

HW and MK prepared the manuscript with inputs from MH, who provided supervision. GB provided critical comments and discussions. All authors contributed to the article and approved the submitted version.

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