Fused in sarcoma-amyotrophic lateral sclerosis as a novel member of DNA single strand break diseases with pure neurological phenotypes

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Accumulation of DNA damage and genomic instability are believed to have crucial effects in neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, Huntington's disease, premature aging diseases as well as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Until recently these studies were largely correlative in nature, though raising the possibility that defects in the DNA damage response (DDR) underlie neurodegenerative diseases. However, more light needs to be shed on (a) the identification of specific lesions, if existing, and their propensity to accumulate in the affected neurons of a given neurodegenerative disease; (b) the underlying mechanisms that impede the repair of these lesions; and based on that (c) the development of animal model systems harboring these identified lesions that are central to progression of neurodegenerative disease in order to see, if interventional strategies that promote DNA repair can alleviate these effects and lead to novel mechanism-driven approaches in drug development to combat neurodegenerative diseases (Madabhushi et al., 2014). It is established that the nervous system requires the largest part of the oxygen consumption producing higher levels of reactive oxygen species (ROS) as a side product, which are known for their oxidative stress on cells in general. Focussing on neurons, ROS are one of the major sources for genotoxic stress producing more than 100 different oxidative base modifications with strong mutagenic potential, which can easily be converted to single strand breaks (SSB) (Madabhushi et al., 2014). Therefore, neurons are particularly dependent on efficient base excision repair and single strand repair processes (**Figure 1**), which is reflected by the volume of neurological disorders caused by defects in these repair pathways (Madabhushi et al., 2014). This highlights the pivotal role of DNA damage and the biological response mechanisms to it as a presumably early event in the process of neurodegeneration. Pathophysiological studies on ALS have particularly drawn attention on its relevance in the recent years.

Signs of increased DNA damage in ALS patient material were reported more than 20 years ago (Tandan et al., 1987). However, it is yet unclear whether this is a specific (up-stream) mechanism within the pathophysiology or rather a secondary event e.g., due to enhanced ROS production. About 10% of ALS patients report a positive family history, which resulted in the discovery of > 50 possible published ALS genes (Taylor et al., 2016) with C9ORF72, superoxide dismutase 1, fused in sarcoma (FUS) and TAR DNA-binding protein 43 being the most prevalent (Taylor et al., 2016). Interestingly, a series of ALS associated genes/proteins have also been implicated in the pathology of FTD including C9orf72, FUS and TAR DNA-binding protein 43. (Taylor et al., 2016). However, FUS-FTD patients do not harbour heterozygous germline mutations in FUS as it is present in ALS even though both syndromes share cytoplasmic FUS mislocalization and aggregation, which has become a pathological hallmark.

Most of the known ALS causing *FUS* mutations are localized within the nuclear localization sequence domain interfering with its proper nuclear shuttling leading to increased cytoplasmic mislocalisation and eventually protein aggregation. Even though a number of N-terminal mutations in *FUS* were reported to be associated with motor neurons disease (Taylor et al., 2016), their prevalence is much lower (Naumann et al., 2019). FUS has been characterized as one of the proteins capable of undergoing liquid-to-liquid phase separation. This is a form of membrane-less liquid droplet formation allowing higher order structures, which are believed to be highly advantageous for the temporal and spatial control of a number of biological processes like transcription or DNA damage repair (Taylor et al., 2016). The presence of so-called low complexity domains within the protein sequence is required for this assembly, which is found in the N-terminal region of FUS (amino acids 1–165). Both N-terminal and C-terminal mutations structurally change FUS droplets *in vitro* into poorly soluble aggregates (liquid-to-solid) suggesting a relevant involvement in disease pathology (Murakami et al., 2015). Interestingly, FUS was observed at sites of artificially induced DNA damage secondary to the recruitment of poly-(ADP-ribose) polymerase 1 (PARP1), which, spatially defined, produces the nucleic acid-like poly-(ADP-ribose) (PAR) biopolymer acting as a dynamic seed to enhance phase separation of FUS, and thereby its local concentration (Altmeyer et al., 2015). Although the RGG domains of FUS were found to be the primary binding site to PAR, it is presumably the N-terminal low complexity domain, which allows very high local FUS concentrations by liquid demixing. If this process is perturbed by clinically relevant N-terminal FUS mutations is not known, but considering the previously found liquid-to-solid phase transition under this condition *in vitro* suggests a possible dominant-negative phenotype of DDR impairment.

The importance of FUS in the DDR has been clearly established, however, the exact mechanisms of which have been reported differently. For instance, by physically interacting with the Histondeacetylase 1 (HDAC1) FUS was demonstrated to play an important role in both, homologous recombination and non-homologous end joining (NHEJ) as the two major pathways of double-strand break (DSB) repair (Wang et al., 2013). Interestingly, *FUS*-nuclear localization sequence mutations abolished this interaction leading to diminished DSB repair and accumulation of DNA damage in post mortem neuronal tissue of FUS-ALS patients. Whether this was mediated by abrogated epigenetic modulation due to HDAC1 functional insufficiency in the surroundings of the DNA damage site remains speculative. However, using chromatin immunoprecipitation in U2OS cells transfected with a series of *FUS* mutant constructs they could also show a diminished interaction of other essential homologous recombination repair factors with the DSB site including HDAC1 suggesting that FUS might be essential for their primary recruitment (**Figure 1**).

On the other hand, a recent publication proposes a mechanism of FUS within SSB repair (Wang et al., 2018; **Figure 1**). Wang et al. (2013) identified its role in promoting adherence of the X-ray repair cross-complementing protein 1 (XRCC1)/DNA ligase 3 (LIG3) complex to the SSB site activating LIG3 downstream of PARP1 activity in an hiPSC based human motor neuron model. They showed that *FUS* mutations

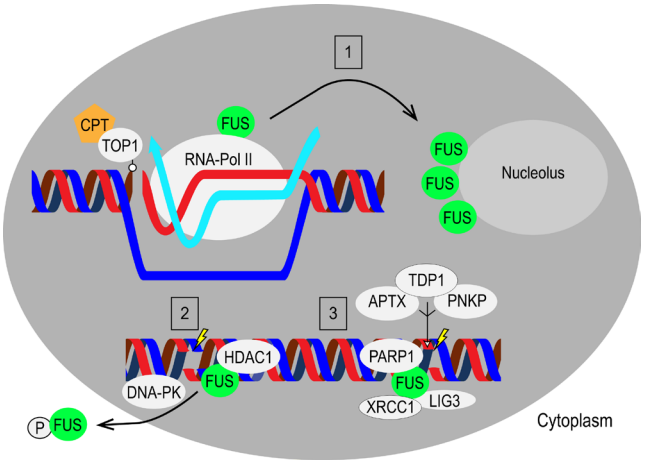

Figure 1 | **The involvement of FUS in transcription and DNA damage repair.**
(1) Transcription associated DNA can arise from abortive TOP1 functioning, which is mimicked by Camptothecin treatment blocking re-ligation. Under this condition a relocation of FUS normally bound to the C-terminal domain of RNA polymerase II to the nucleolus occurs for an unknown reason (Martinez-Macias et al., 2019). (2) Its importance in the DSB repair by allowing recruitment of HDAC1 and other DSB repair factors was found by examining primary neurons and U2OS cells, which was impaired by FUS mutations (Wang et al., 2013). Furthermore, upon induction of DNA damage FUS was demonstrated to be phosphorylated by DNA-PK, which is a member of the classical NHEJ pathway. This N-terminal phosphorylation in its low complexity domain resulted in an egress from the nucleus to the cytoplasm. (3) In human neurons FUS acts downstream of PARP1 and tethers the XRCC1/LIG3 complex to the SSB site enabling proper ligation of the nicks. Of note, SSB nick processing is essential and for instance ensured by the enzymes TDP1, APTX and PNKP. Mutations in these were associated with the neurological syndromes SCAN1, ataxia oculomotor apraxia 1 and MCSZ, respectively. APTX: Aprataxin; DNA-PK: DNA-dependent protein kinase; DSB: double strand break; FUS: fused in sarcoma; HDAC1: histone deacetylase 1; LIG3: DNA ligase 3; MCSZ: microcephaly, early-onset, intractable seizures and developmental delay; NHEJ: non-homologous end joining; PARP1: poly [ADP-ribose] polymerase 1; PNKP: polynucleotide kinase 3′-phosphatase; SCAN1: spinocerebellar ataxia with axonal neuropathy type 1; SSB: single strand break; TDP1: tyrosyl-DNA phosphodiesterase 1; TOP1: topoisomerase 1; XRCC1: X-ray repair cross-complementing protein 1.

led to ligation defects and increased SSB abundance. However, using single cell electrophoresis assay and short hairpin knockdown they didn't observe an obvious influence of FUS for DSB repair as it was reported by Wang et al. (2013) in detail. Importantly, abrogated interaction with the XRCC1/LIG3 complex was demonstrated to be the common pathological downstream event, but the upstream mechanisms of various *FUS* mutations were shown to be different. This is of special importance since it addresses the fact that especially amino acid changes at the R521 site in *FUS* only mildly shift the physiological nuclear dominance of FUS, but still gives rise to motor neuron degeneration. In contrast to the P525L mutation, which leads to strong cytoplasmic FUS accumulation, thereby explaining the diminished physical interaction with the nuclear protein complex, the R521C and R521H changes exhibited primarily weakened interaction to XRCC1/LIG3 in a dominant-negative way also corrupting the functioning of the endogenous WT FUS. The biomolecular reason for this diminished interaction should be subject of prospective studies considering that the R521 mutation site is most prevalent among all FUS-ALS patients (Naumann et al., 2019).

In line with this we recently showed that FUS is a downstream effector of PAR in patient-derived motor neurons (Naumann et al., 2018) and that a loss of nuclear function of mutant FUS triggered an axonal retraction reminiscent of a dying back mechanism. The disturbed nucleocytoplasmic shuttling of the P525L mutant FUS protein led to abrogated PARP1-dependent FUS recruitment to DNA damage sites. Accumulation of unrepaired SSBs can potentially advance to the formation of lethal DSB. The most likely DSB repair mechanism in postmitotic neurons comprises the classical NHEJ pathway including DNA-dependent proteinkinase (DNA-PK) activity. While PARP1 inhibition mimicked all FUS phenoytpes in wild-type human motor neurons, the protein level of NHEJ or alternative end joining pathway factors was unimpaired in the *FUS* mutants. This would be in line with the findings that impaired SSB rather than DSB repair might primarily be the main driver of motor neuron demise in FUS-ALS (Naumann et al., 2018). However, DNA-PK was reported to mediate phosphorylation of FUS leading to the exclusion of FUS from the nucleus (Deng et al., 2014). Surprisingly, DNA-PK inhibition did not increase neurodegeneration, but rescued FUS associated-DDR and neurodegeneration in *FUS* mutant motor neurons suggesting that the DSB repair response should also be taken into account, but probably as a secondary event. We could show that this cellular reaction to DNA damage mediated by DNA-PK activity induces a vicious cycle in *FUS* mutant spinal motoneurons furthermore depleting the nuclear FUS reservoir.

As previously suggested by Deng et al. (2014), the mechanism of DNA-PK driven exclusion of nuclear FUS also gives rise to plausible explanations for the phenomenon of aggregate formation of WT-FUS in FUS-FTD as they did show FUS cytoplasmic mislocalization and aggregation formation upon DNA damage induction. Interestingly, it was shown that these FUS accumulations do co-accumulate other FET proteins as typically seen in FTD-FUS. Why FUS-ALS causing germline mutations do not cause FTD-FUS remains however elusive.

Another major source of DNA damage in postmitotic cells is transcription associated stress by abortive topoisomerase 1 functioning. Recently, an important role of FUS in this setting was identified (Martinez-Macias et al., 2019). Topoisomerase 1 (TOP1) induced DNA damage was shown to lead to a stalling of the RNA polymerase II and relocalisation of FUS from this point to the nucleolus (**Figure 1**). This was accomplished by application of Camptothecin, which is an established inhibitor of TOP1 resulting in the formation of SSB by preventing proper re-ligation of the transient SSB-TOP1 nick. We could show that *FUS*-nuclear localization sequence-mutant patient fibroblasts are hypersensitive to TOP1-induced DNA breakage, suggesting either a relevant susceptibility to prominent transcriptional stress or to the secondarily appearing SSBs (Martinez-Macias et al., 2019). Furthermore, the biological meaning of FUS moving from the mRNA transcription site to the nucleolus in the situation of TOP1 induced SSB requires further clarification.

One intriguing question arises from the fact, that $FUS^{-/-}$ mouse models do neither show ALS nor FTD phenotypes. The main reason for this might be that FUS-ALS is an autosomal-dominant disease with heterozygous mutations. Thus, nuclear loss of function by FUS-ALS mutations is either incomplete or an additional toxic effect of cytoplasmic FUS does exist. The latter seems more likely as it was for instance demonstrated that injection of engineered mutant FUS into retinal ganglion cells compromised axonal protein synthesis (Murakami et al., 2015). This is clearly different in Ataxia teleangiectatica, which is caused by autosomal-recessive,

biallelic mutations in *Ataxia-telangiectasia mutated* (*ATM*) leading to genomic instability and a general higher malignancy rate and increased radiation sensitivity (Madabhushi et al., 2014). The latter, however, fits to the data from *FUS*$^{-/-}$ mice which – in case of homozygous loss of function – do also show genomic instability with much more tissues being affected (Hicks et al., 2000).

Therefore we performed a cross-sectional clinical study assessing the prevalence of malignant neoplasias in FUS-ALS patients (Naumann et al., 2019). We identified one in 40 patients who suffered from a malignancy prior to ALS which resulted in a prevalence for malignancies of 2.5%. This was not higher than the cancer prevalence within a German control population (1.6%) provided by data from the German cancer statistics from 2004.

How do these preclinical data fit to a pure neurological phenotype without obvious involvement of an altered immune status or enhanced predisposition to cancer (Madabhushi et al., 2014; Naumann et al., 2019)? The same clinical description is evident for a group of neurological diseases caused by mutations of factors relevant for SSB nick repair, namely ataxia oculomotor apraxia 1, spinocerebellar ataxia with axonal neuropathy, Microcephaly, early-onset, intractable seizures and developmental delay (Madabhushi et al., 2014; **Figure 1**). This is molecularly underpinned by the finding that FUS might be centrally implicated in the PARP1-XRCC1-LIG3 mediated SSB ligation (Wang et al., 2018). The pathomechanistic pathways explaining why distinct human neurons are affected differentially by impairment of distinct SSB or DSB repair factors are still speculative. One possible explanation for the reason we and others did not find signs of increased malignant tumor risk in neurological diseases associated with SSB nick repair like FUS-ALS might be that in non-neuronal cells sufficient cell cycle control and p53 mediated apoptosis mechanisms are preserved by regular ATM activity probably diminishing the influence of accumulated DNA damage. Since its various functions in DSB repair and downstream events, the loss of ATM might not be so easily to compensate for, mirroring the complex clinical phenotype in Ataxia teleangiectatica.

However, it implies that neurons are especially vulnerable for defects in SSB repair probably by higher ROS production in the brain exposing them to the risk of occurring DSB, which are a rare, but perilous fate in non-cycling cells lacking the homologous recombination process. If not directly resulting in apoptosis, the error-prone NHEJ allows DSB repair in neurons instead by direct ligation of broken DNA ends (Madabhushi et al., 2014), but thereby increases the risk of genomic errors to occur. In summary, recent data imply that FUS is crucial in SSB repair at least in postmitotic neurons thereby adding FUS-ALS to the spectrum of SSB repair disorders, a class of pure neurological syndromes. However, many questions are remaining, including loss-of-nuclear-function versus cytoplasmic gain-of-function, crosstalk to DSB repair pathways, the cascade leading to (axonal) neurodegeneration as well as cell type/tissue selectivity.

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