Scuola Universitaria Superiore IUSS Pavia

ALS-linked FUS mutation reduces DNA Damage Response activation through RNF168 signalling pathway

A Thesis Submitted in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy in

BIOMOLECULAR SCIENCE AND BIOTECHNOLOGY

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April, 2021

In memoriam

Prof dr. Ir. Theo J. Visser

&

Cav. Vincenzo De Chiara

Cover image represents the ability of human mind to explore the infinity of science.

Cover image was conceived and designed by Carmela Farina. The technical implementation was handled by Simone di Segni.

ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by the progressive degeneration of upper and lower motor neurons and it is associated with the progressive paralysis of almost all skeletal muscles leading to death within 3-5 years from the diagnosis. One ALS-linked gene is Fused in Sarcoma (FUS) that encodes for a DNA/RNA binding protein also involved in DNA repair. Common pathological hallmarks in ALS are cytoplasmic neuronal inclusions containing FUS protein in a mutated form. In this regards, the ALS-linked mutation FUS-P525L leads to a severe juvenile onset and immunohistochemistry performed on tissues from ALS patients carrying this mutation reveal that the mutant FUS protein accumulate into cytoplasmic inclusions (CI).
ALS neurons have been shown to accumulate oxidative DNA damage and DNA breaks, hazardous events that healthy cells efficiently counteract by activating a set of molecular mechanisms known as DNA-Damage response (DDR). The activation of this pathway is cytologically detectable at single cell level in the form of nuclear DDR foci, multiprotein complexes at site of damage.

My PhD project aims to understand whether mutant FUS-P525L recruitment into CI could hinder the efficacy of DDR and DNA repair, progressively leading to DNA damage accumulation.

Moreover, FUS P525L mutation has been associated to autophagy impairment and accumulation of the cargo autophagic protein p62. In the context of cancer, p62 interacts with and inhibits the activity of the E3 Ubiquitin ligases RNF168 a key factor in DNA signaling and DNA repair.

The results obtained indicate that the induction of mutant FUS CI is per se genotoxic and induce a nuclear wide accumulation of the γH2AX DNA damage marker. Importantly, mutant FUS CI strongly alters DDR signaling as demonstrated by the loss of DDR foci in cells exposed to DNA damage. Concomitantly, we observed that p62 accumulates in the cytoplasm of cells harboring mutant FUS CI and, unexpectedly, those cells also have reduced nuclear signal of RNF168, which appears in the cytoplasm co-localizing with p62.

As a consequence DDR foci are lost. Indeed, we observed that the overexpression of RNF168 or RNF8, another ubiquitin ligase acting upstream of it in DDR, can restore RNF168 nuclear level and DDR foci, thus reducing γH2AX signal in cells with FUS CI. Importantly, same result can be obtained by p62 inactivation, enhancing the survival of cells with CI.

These results indicate a novel mechanistic link between FUS CI, mis-regulation of the autophagic pathway and DNA damage signalling. Our data pave the-way to consider DDR alterations triggered by FUS CI as a novel, relevant aspect driving ALS pathogenesis and a new pathway to target in the envision of future therapeutic treatment.

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γH2AX: Phosphorylated histone variant H2AX (Ser 139)

1. INTRODUCTION

1.1. DNA DAMAGE AND DNA DAMAGE RESPONSE

1.1.1. TYPE OF DNA DAMAGE

Preservation of genome integrity within DNA structure is vital for living being including humans. Proper DNA functionality ensures genetic information preservation against possible faithful transmission across generations. Both physiological events and environmental factors can cause DNA alterations which arise to genome integrity mis-regulation (Ciccia and Elledge 2010). DNA metabolism leads to many error prone events with a frequency of up to 10^5 spontaneous DNA lesions per day occurrence per cell. DNA lesions may arise from dNTP incorporation during DNA replication, DNA depurination and consequent loss of DNA bases or interconversion of DNA bases due to deamination events and chemical modifications of DNA bases by alkylation (Lindahl and Barnes 2000). Reactive Oxygen Species (ROS) resulting from oxidative respiration pathway, also promote DNA break. Moreover, environmental DNA damage can be induced by physical factors and chemical compounds. Sunlight exposure is the main source of ionizing radiation (IR) and ultraviolet (UV) light which can induce pyrimidine dimers formation up to 10^5 lesions per cell per day (Hoeijmakers 2009). In particular, IR can trigger DNA- bases oxidation and the formation of the two most harmful DNA lesions: single-strand breaks (SSB) and double-strand breaks (DSB) (Hoeijmakers 2009).

In vertebrates, DSBs can be also considered as an essential step causing programmed genome alteration useful for diverse biological functions. The most suitable example is the V(D)J recombination, class switch recombination and somatic hyper-mutation: these events occur during B and T lymphocytes development essential for immunoglobulin and T-cell receptor (TCR) diversity (Bassing and Alt 2004; Schlissel, Kaffer, and Curry 2006). From this point of view this harmful mechanism is also able to ensure the effective recognition of different pathogens by immune system. Furthermore, DSBs modulate genetic diversity generation via sexual reproduction. In particular, during meiosis DSBs are involved in HR, activated by the topoisomerase-II-related enzyme, Spo11, which generates Spo11-bound DSBs (Jackson and Bartek 2009).

Cancer therapies involve the treatment with chemical agents, which induce severe DNA lesions. In particular, crosslinking agents, including cisplatin and psoralen, introduce covalent links between the strands, while topoisomerase inhibitor I or II (camptothecin and etoposide, respectively) cause formation of SSBs or DSBs (Ciccia and Elledge 2010). It was predicted that in the human body, every single cells receives tens of thousands of the above mentioned DNA lesions per day (Lindahl and Barnes 2000), thus if not promptly corrected may lead to a large number of genome aberrations which in turn may compromise cell and organism viability (Jackson and Bartek 2009). For this reasons, DNA damage is intimately linked to severe human diseases including cancer and neurodegeneration. Both liquid and solid tumours are characterized by the presence of chromosomal instability especially in nascent tumours where telomeres become shorter and more prone to dangerous chromosomal fusions (Jackson and Bartek 2009). Moreover, both oncogene activation or tumour suppressors inactivation trigger DNA damage formation causing aberrant cell proliferation (Jackson and Bartek 2009). Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD) and Amyotrophic Lateral Sclerosis (ALS) have been associated with high DNA lesions accumulation in neurons (Coppede 2011; Kulkarni and Wilson 2008). Although the mechanism is still unclear, one possible explanation may be due to the high mitochondrial respiration typical of neurons and the concomitant production of ROS species that can induce both nuclear and mitochondrial DNA damage (Pignataro et al. 2017; Weissman et al. 2007). On the other hand, neuronal tissues are arrested in G0 cell phase, thus have limited capacity to replace cells, augmenting the irreversible state of DNA damage accumulation. This cell phase leads neurons to predominantly repair DSBs via the major error-prone repair mechanism Non Homologous End Joining (NHEJ) at the expanse of the error-free pathway Homologous Recombination (HR), mainly active in S-G2 phase (Jackson and Bartek 2009; Kulkarni and Wilson 2008). Furthermore, scatter evidences suggest that DNA damage accumulation predispose to ageing. In healthy individuals, different endogenous lesions in DNA accumulate with age in both nuclear and mitochondrial DNA and this may be due to reduced DNA-repair efficiency together with ongoing accumulation of DNA-damage (Jackson and Bartek 2009). Collectively, these evidences support the concept that DNA damage accumulation represents a critical event strictly related to mammalian disease onset. In order to counteract DNA damage and prevent its accumulation, cells have developed a signalling cascade pathway named as DNA Damage Response (DDR) able to immediately sense the lesion and activate a coordinated and interconnected events that ultimate in DNA repair (Jackson and Bartek 2009).

1.1.2 DNA damage response pathways

The wide range of DNA lesions that may threaten genome stability requires a well established DDR signalling which involves hundreds of different key factors. As soon as the lesion is sensed, some factors are directly recruited at site of damage while others act as scaffold proteins necessary for protein-protein interaction. These events promote the increased local recruitment of protein with enzymatic activity such as polyADP rybosilases, kinases, phosphatases and ubiquitin ligases, which modify hundreds of downstream targets ensuring the signalling cascade continuation (Chatterjee and Walker 2017; Lukas, Lukas, and Bartek 2011). Thus, starting from local DNA lesion, the resulting chromatin modifications together with continuous recruitment of proteins, causes the formation of cytological detectable nuclear foci which represent a visible proof of the proper DDR activation (Lukas, Lukas, and Bartek 2011).

DDR activation involves the apical recruitment of the poly(ADP)ribose polymerases 1 and 2 (PARP1 and PARP2) belonging to PARP family, which in turn catalyse the addition of poly ADP-ribose (PAR) chains on local histone tails and on PARP1 itself thus acting as a molecular sensor of SSBs and DSBs and recruit other DDR key factors and chromatin modifying complexes (Schreiber et al. 2006). The MRN complex, composed by MRE11-RAD50-NBS1 proteins, together with KU70/KU80 (KU) represent another apical DDR sensors that stimulate the localization at damage sites of phosphatidylinositol 3-kinase-like protein kinases (PIKKs) including ataxia telangiectasia-mutated (ATM) and DNA-dependent protein kinase (DNA-PK)(Ciccia and Elledge 2010). This event is crucial for the proper activation of DDR signalling since it drives the activation of DNA-PK and ATM by KU and MRN complex respectively. Particularly, ATM is required for both HR and NHEJ pathways activation (Bredemeyer et al. 2006; Lavin 2008) while DNA-PK strictly modulates NHEJ mechanism (Meek, Dang, and Lees-Miller 2008). Differently, resected DSBs and stalled replication forks trigger the formation of RPA-coated ssDNA complex which represent the primary signal for ATM Rad3-related (ATR) activation (Cimprich and Cortez 2008). ATM/ATR activation causes different downstream effects including modulation of cell cycle phosphatase CDC25 through checkpoint kinases CHK1/CHK2 phosphorylation and p53 modulation which in turn activate the transcription of CDK inhibitor 21 (Branzei and Foiani 2008; Riley et al. 2008)›. Finally, CDKs inhibition promotes cell-cycle arrest at G1-S, intra-S and G2-M phases essential to ensure enough time for proper DNA repair before replication and mitosis. As mentioned before, ATM/ATR phosphorylation at DNA lesions stimulates DNA repair by regulating post-transcriptional modifications of DNA repair proteins as well as modulating the recruitment of many others key factors at sites of damage.

If the above described DDR signalling is efficiently activated, it ensures DNA repair and consequent rescue of physiological cell functionality. Instead, if the damage is not repaired DDR is chronical stimulated thus leading to apoptosis or cell-cycle arrest, defined as cellular senescence (Campisi and d'Adda di Fagagna 2007).

1.1.3. DNA DSBs response: a matter of phosphorylation and ubiquitination

Most of the times DSBs is related to severe genome instability conditions which require an immediate DDR activation. Particularly, among the lesions that may occur on DNA, DSBs represent the most harmful form of DNA damage. About that, there are two main explanations: first DSBs are often associated with lack of an intact template strand thus reducing repair efficiency and second, DSBs strongly affect DNA structure and consequently impairing genome stability (Mladenov and Iliakis 2011). DSBs generation triggers the local recruitment of the MRN complex, which acts as DNA damage sensor stimulating the localization of the apical kinase ATM to DSB site (Shiloh and Ziv 2013) (Fig. 1). Locally, ATM protein is activated through different Post Traslational Modifications (PTMs) including auto-phosphorylation on target sites at Serine 1981 (Bakkenist and Kastan 2003) and others (Kozlov et al. 2011) and acetylation of Lysine 3016 (Sun et al. 2007). Moreover, NBS1 protein within the MRN complex stimulates ATM re-localization and retention at DSBs (Difilippantonio and Nussenzweig 2007). ATM activation represents the turning point of DSB signalling since it promotes the phosphorylation of the local histone variant H2AX on Serine 139 (referred as γH2AX) which is simultaneously dephosphorylated at the Tyrosine 142 (Lukas, Lukas, and Bartek 2011). Together, these events profoundly affect chromatin structure making available the binding site for the scaffold protein MDC1 (mediator of DNA damage checkpoint protein 1) (Stucki et al. 2005), which is also phosphorylated by ATM (Jungmichel et al. 2012). MDC1 phosphorylation ensures the DSB localization of the E3 ubiquitin ligase RNF8 (RING finger protein 8) via its forkhead-associated (FHA) domain (Mailand et al. 2007): here RNF8 actively stimulates the formation of ubiquitin chains at site of damage that in turn triggers the recruitment of a second E3 ubiquitin ligase RN168 (RING finger protein 168) (Pinato et al. 2009; Doil et al. 2009; Stewart et al. 2009). In particular, RNF168 contains two MIU (Motif Interacting with Ub) domains required for the binding with ubiquitinated proteins (Pinato et al. 2009; Doil et al. 2009; Stewart et al. 2009)(Fig. 1.1.). Upon DNA damage induction by IR, RNF168 selectively mono-ubiquitinates Lysine 13 and 15 on H2AX and H2A histone variants followed by the conjugation to these sites of the Lysine 63-linked ubiquitin chains (Mattiroli et al. 2012; Gatti et al. 2012) (Fig. 1.1.). Importantly, RNF168-DSB localization and its ubiquitination activity is essential for the recruitment of downstream key players including BRCA1 (breast cancer

type 1 susceptibility protein) and 53BP1 (p-53 binding protein 1) thus leading to proper DNA repair pathway activation (Mattiroli et al. 2012; Gatti et al. 2012). In this scenario, the histone H1 has been identified as a target of the signalling RNF8 and the E2 ubiquitin-conjugating enzyme 13 (UBC13) responsible of the chromatin proper ubiquitin signalling which ultimate in RNF168 recruitment at DSB (Thorslund et al. 2015). Recent evidences helped to shed light on this intricate mechanism and add a novel key player which is L3MBTL2 (lethal-3-malignant brain tumour-like protein2) (Fig. 1.1.). In details, once DSBs occur ATM actively phosphorylates L3MBTL2 at Serine 335 promoting its localization at break sites and also its interaction with MDC1 (Nowsheen et al. 2018) (Fig. 1.1.). Additionally, L3MBTL2 promotes RNF168 recruitment ad DSBs thus ensuring DDR signalling continuation as previously described (Nowsheen et al. 2018) (Fig. 1). It is important to note that the resulting chromatin modification by ubiquitination promotes partial transcription repression around (Shanbhag et al. 2010) until the DNA lesion is fully restored (Capozzo et al. 2017).

The continuous recall of proteins at site of lesions causes the increased accumulation of DDR mediators in large segments of lesion-flanking chromatin, that in turn promotes the formation of nuclear foci detectable by light microscopy (Lukas, Lukas, and Bartek 2011). In particular, the apical recruitment of DDR sensor like MRN complex, occur autonomously forming the so-called primary recruitment. Subsequently, DDR foci originate from the ATM-dependent H2AX phosphorylation which ensure the recruitment of downstream DDR factors as well as further MRN-ATM complexes, establishing a positive feedback loop that promotes the spreading of γH2AX for hundreds of kb from the DSB and the so-called secondary recruitment of DDR factors to the damaged genomic locus (Celeste et al. 2003).

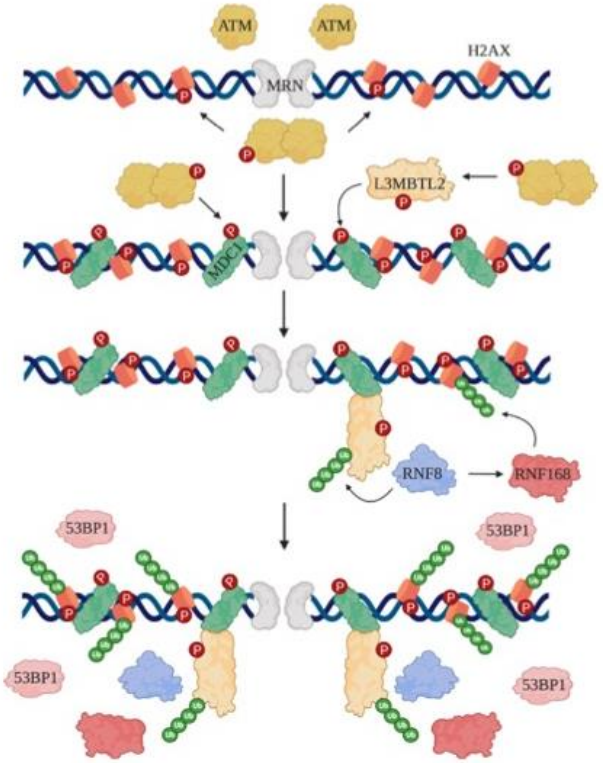


Figure 1.1. Ubiquitin and phospho-dependent assembly at double strand break (DSB) triggered by L3MBTL2-RNF8-RNF168 pathway (this thesis). DNA damage is sensed by the MRN complex which stimulates the activation of ATM trough auto-phosphorylation and its dimerization. Once activated, ATM phosphorylates many downstream proteins including the MDC1 and L3MBTL2 that interact each other in ATM dependent manner. L3MBTL2 mediates RNF8 recruitment which in turn ubiquitinates L3MBTL2. Then, RNF8 triggers RNF168 localization at site of damage which ubiquitinates the histone variant H2AX. Finally, the 53BP1 protein is recruited locally leading to DNA repair activation.

1.1.3.1. DSBs repair mechanisms

To date, DNA damage can activate five major DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) which can be selectively active through different stages of cells cycle. More in detail, BER pathway is activated in case of DNA lesions that are not sensed as significant distortions on DNA helix (e.g. deamination, oxidation and alkylation), NER is mainly activated in order to remove bulky lesions derived from UV or chemotherapeutic agents, while MMR intercedes in case of

base mismatches that may occur during replication (Chatterjee and Walker 2017). Differently, oxidative damage generates SSBs and if not promptly corrected by single-strand break repair (SSBR) may lead to DNA replication collapse. On the other hand, the more genotoxic DSBs trigger both NHEJ and HR which is specific of in S-G2 phase. In addition, NHEJ mechanism ligates close DNA ends thus producing small insertions and deletions while HR provides a more error-free DNA repair since acts on homologous sequences on paired chromosome (Sirbu and Cortez 2013).

Given the high dangerous effects of DSBs on genome integrity and its impact on cell survival in eukaryotes, from yeast to human, along evolution it has developed different DSBs repair pathways that can be grouped in two main categories: NHEJ and HR (Karpenshif and Bernstein 2012) (Fig. 1.2.).

NHEJ repair mechanism is the most error-prone pathway for DNA repair since it involves the direct ligation of DNA ends with reduced or none homology thus increasing the chances of loss or gain of nucleotide at the DNA ends before ligation. Moreover, it is mainly active during G1 phase of the cell cycle. On the contrary, HR pathway is restricted to S and G2 phases of the cell cycle where is ensured the presence of duplicated sister chromatins. This is essential since HR mechanism requires homologous DNA duplex as a template to recover the missing nucleotide sequence, and for this reason, is the most accurate repair mechanism (Kadyk and Hartwell 1992).

The two-step nucleolytic degradation producing 3' single-stranded DNA (ssDNA), known as DSB resection, is the major regulator of the MRN complex and triggers to HR pathway activation. Moreover, DSBs resection requires others key factors including the C-terminal binding protein (CtIP), the helicase Sgs1 and the nucleases Dna2 or EXO1 (Fig. 1.2.). Importantly, DSBs resection is the key event by which cells must chose HR repair mechanism since long ssDNA are mainly substrates for this pathway and not for the NHEJ ones (Symington 2016). At DNA lesion, 3' ssDNA tails are immediately bound by RPA (Replication protein A), in order to protect those extremities from further damage that may occur (Wang and Haber 2004) (Fig. 1.2.). Subsequently, RAD51 recombinase replaces RPA through the involvement of the recombination mediators BRCA2 and RAD52 (Fig. 1.2.). This complex, and in particular the RAD51 nucleoprotein filament mediates the binding with the homologous double stranded DNA and ensures its invasion, thus leading to the displacement of the non-complementary strand and consequently formation of structure termed displacement-loop (known as D-loop) (San Filippo, Sung, and Klein 2008). Once invaded, the end of the D-loop became substrates

of the DNA polymerase which ensure the extension of the D-loop ends thus copying the sequence lost at the break site (Fig. 1.2.). Finally, the D-loop resolution can be solved by two different mechanisms resulting in both crossover or non-crossover products (West et al. 2015) (Fig. 1.2.).

Concerning NHEJ, damaged DNA ends are rapidly recognized by the Ku70-Ku80 heterodimer which has high affinity with for DNA and mediates the recruitment of the catalytic subunit of DNA-PK (DNA-PKs) (Fig. 1.2.). The kinase activity not only targets the auto-phosphorylation but the concomitant activation of NHEJ proteins including the nuclease ARTEMIS that selectively processes the DNA ends by removing useless nucleotide and made them compatible for ligation (Fig. 1.2.). Then, the ligase complex composed by the DNA ligase IV together with XRCC4 and XRCC4-like factor XLF ensure the final end joining of the flaking ends (Schwertman, Bekker-Jensen, and Mailand 2016) (Fig. 1.2.). Furthermore, an Alternative non-Homologous and joining mechanism (alt-NHEJ) may occur at the expanse of the canonical NHEJ. Particularly, short (<18 nucleotide) or longer (>200 bp) within homologous sequences close to the break site can be revealed by end-resection, triggering the microhomology-mediated end-joining (MMEJ) (Sfeir and Symington 2015) or to the single-strand annealing (SSA) (Bhargava, Onyango, and Stark 2016), respectively.

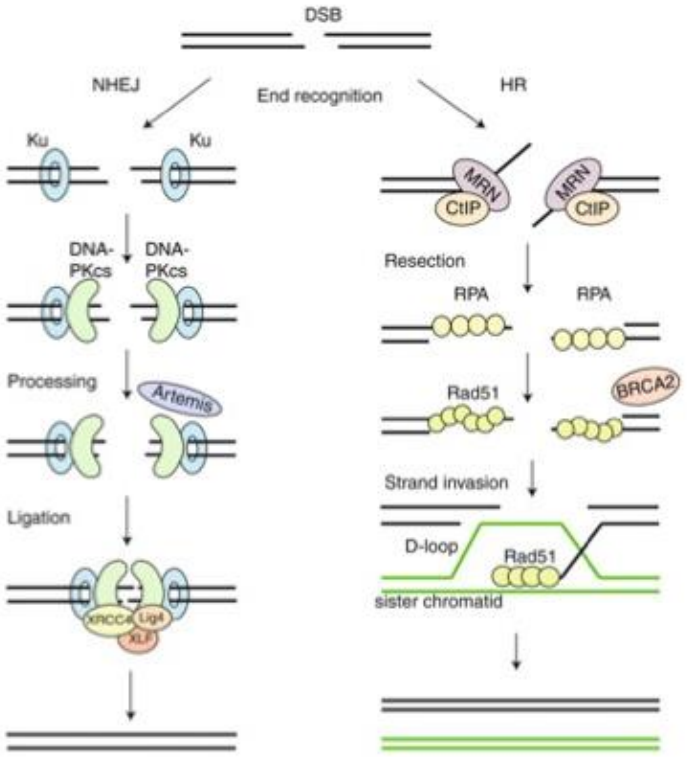


Figure 1.2. Scheme of NHEJ and HR repair pathways (Brandsma & van Gent, 2012). In NHEJ the KU70/80 heterodimer recognizes the DNA ends and recruits DNA-PKcs. Different nucleases can act on incompatible DNA ends, including Artemis. Finally the XRCC4-DNA Ligase IV-XLF ligation complex close the break. In HR the MRN-CtIP complex stimulates DNA ends resection at break level to generate single stranded DNA (ssDNA). At this point the DNA lesion can no longer be repaired by NHEJ. The resulting ssDNA is primarily coated by RPA which is then replaced by RAD51 with BRCA2 mediation. These events ultimate with the strand invasion on the homologous template by the Rad51 nucleoprotein filaments. The formed D-loop and capture of the second end result to DNA repair.

1.1.4. The contribution of Non-coding RNAs to DDR

Nowadays many evidences support the notion that RNA, in the form of non coding RNAs (ncRNAs), plays a key role in genome integrity maintenance, especially in DDR regulation (d'Adda di Fagagna 2014). The largest contribution within ncRNAs population is represented by micro-RNA (miRNAs) described as transcription regulators of many DDR factors including ATM (Hu et al. 2010), DNA-PKcs (Hu et al. 2010), BRCA1 (Moskwa et al. 2011), and RAD51 (Wang et al. 2012). Besides, long ncRNAs also

regulates the expression of downstream genes of DDR (Hung et al. 2011). A striking example is represented by the DNA-damage induced antisense transcript PANDA which is generated from the Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) gene. It has been demonstrated that p53 promotes both CDKN1A and PANDA transcripts thus mediating G1 arrest and cell survival respectively in order to regulates cell cycle checkpoint (Hung et al. 2011). Furthermore, few DDR factors actively bind different kinds of ncRNAs. For example, within 53BP1 structure there is a region responsible for foci targeting which includes a tudor domain, characterized as typical of proteins with a role in RNA metabolism. Importantly, 53BP1 foci are sensitive to RNase A treatment indicating that the RNA plays a key role in the accumulation of this DDR mediator close to DNA lesion (Pryde et al. 2005).

The direct role of ncRNas in DSBs repair regulation was further characterized. Particularly, scatter evidences suggest that RNA can serve as a template for DNA synthesis in different model system such as Saccharomyces cerevisiae (Storici et al. 2007), E. Coli and human cell models (Shen et al. 2011).

Among the ncRNAs, certain species of site-specific small ncRNAs have been suggested to play a role in DDR modulation. Using an inducible DSB site in Arabidopsis thaliana and once DNA break occur, small RNAs of 21-24 nts were detected and were defined as DSB-induced RNAs (diRNAs). Moreover, diRNAs generation and processing involve ATR, the RNA polymerase IV (Pol IV), and several Dicer-like proteins while Ago2 exhibits an effector activity since recruits diRNAs (Wei et al. 2012). The role of such diRNAs was also characterized in both HR and NHEJ repair mechanisms. The complex formed by Ago2 and diRNAs stimulates Rad51 recruitment and retention at DSBs thus modulating HR (Gao et al. 2014). On the other hand, using Arabidopsis thaliana as a model, the role of diRNAs was also characterized in NHEJ (Qi et al. 2016). In addition, a study conducted with a site-specific DSB system in Schizosaccharomyces, RNA polymerase II (RNAPII) has been characterized to localize at break site causing the formation of RNA-DNA hybrids involved in HR repair pathway (Qi et al. 2016).

1.1.4.1. DDRNA and dilincRNA: role in DDR signalling and DNA repair

In the last decade, our group and others, discovered a novel class of ncRNAs that play an active and crucial role in DDR, thus are defined as DNA damage response RNAs (DDRNAs) (Francia et al. 2012). Two double-stranded RNA-specific endoribonucleases type III, DICER and DROSHA, are responsible for DDRNAs processing and depletion of both RNAses strongly impairs IR-induced DDR foci containing pATM and 53BP1 as

observed in both human cells and zebrafish larvae. Besides, in zebrafish model, where DNA-PK kinase is absent, γH2AX containing foci decrease after DICER and DROSHA depletion while they keep unaltered in human model, likely due to redundant kinase activity by DNA-PKcs (Francia et al. 2012). Hence, RNase A treatment upon irradiation and transcription inhibitors arises comparable DDR foci reduction, suggesting that RNAs are crucial for foci assimilation and maintenance. Moreover, incubation with small RNAs (20-35nt) appears to be sufficient to restore DDR foci formation even in absence of messenger RNAs. In order to shed light on DDRNAs biogenesis, it was engineered a cell line containing a target site for an inducible endonuclease specifically localized in between of bacterial repeats. This system reveals that once the cut is stimulated in these cells upon RNase A treatment the DDR focus is restored only after additional incubation with RNA molecules extracted from the same parental cell line thus devoid of integrated construct and used as RNAs source (Francia et al. 2012). These evidences demonstrate that DDRNAs are generated at damage sites and next-generation sequencing of small RNAs from the inducible cell system confirmed the presence of several short RNAs mapped at the cut site, including those with potential ability to form double stranded species (Francia et al. 2012).

More recently, our group made a step forward in the biogenesis and activity characterization of DDRNAs. We demonstrated that DDRNAs arises from longer RNAs newly synthesized at DSBs sites and named damage-induced long non-coding RNAs (dilncRNAs) (Michelini et al. 2017). Starting from DNA ends, dilncRNAs are actively transcribed by RNAPII in both convergent and divergent directions, and more importantly, the MRN complex is responsible for the proper localization at DSBs of the RNAPII (Michelini et al. 2017) (Fig. 1.3.). In this scenario, dilncRNAs are precursor of DDRNAs and are processed by DICER and DROSHA. Moreover, dilncRNAs facilitates DDRNAs association to damage site by complementary base pairing as also observed at centromeres to silence transcription of satellite repeats (Francia 2015).

Intriguingly, both dilincRNAs and DDRNAs physically interact with 53BP1 via its Tudor domain thus contributing to DDR foci formation (Michelini et al. 2017) and more likely, upon RNAPII inhibition, a strong reduction of DDR activation was observed due to loss of DDRNA recruitment at damaged site (Michelini et al. 2017). Finally, incubation with sequence-specific antisense oligonucleotides (ASOs) affect DDR activation since they bind with dilncRNAs consequently reducing their interaction with DDRNAs (Michelini et al. 2017) (Fig. 1.3.).

Another important aspect of this well characterized mechanism is its reproducibility at telomeres level where de-protected telomeres are well known activators of DDR. This aspect is crucial in different pathological contexts associated with telomere dysfunction including aging and progeria syndromes (Rossiello et al. 2017). Like DSBs, telomere dysfunction induces the local transcription of telomeric non-coding RNAs (tncRNAs) including telomeric dilncRNAs (tdilncRNAs) and telomeric DDRNAs (tDDRNAs), which are required for proper DDR activation. Moreover, incubation with ASOs inhibits both tdilncRNAs and tDDRNAs thus inhibiting telomere-specific DDR in different model systems (Rossiello et al. 2017). Intriguingly, a recent study from our group demonstrates that treatment with sequence-specific antisense oligonucleotides (tASOs) which inhibits tncRNAs functionality ameliorates progerin-induced telomere dysfunction resulting in extended healthspan and lifespan in transgenic mouse model for Hutchinson-Gilford Progeria Syndrome (HGPS) (Aguado et al. 2019).

Taken together these evidences strongly support the knowledge that RNA metabolism is crucial in DDR signalling and should be considered as the key and apical event triggering DNA repair activation. Therefore, in this scenario the RNA binding factors may exhibit an important role in modulating DDR activation thus opening a novel and intriguingly window of investigation where RNA-protein interaction can be the major protagonist at sites of DNA damage.

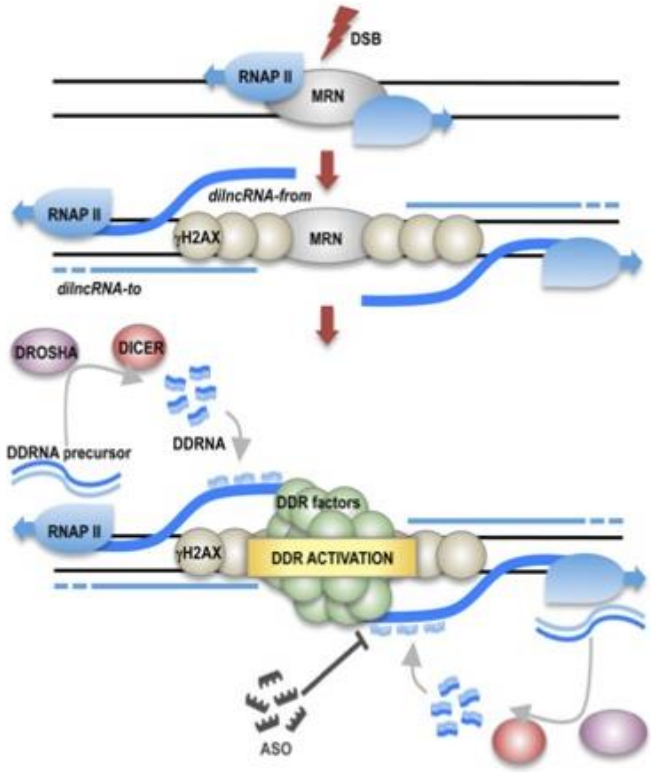


Figure 1.3. Model of dilincRNAs and DDRNAs biogenesis and functionality (adapted from Michelini F. et al 2017). MRN recruits RNAPII to the DSB and induces the bidirectional synthesis of dilincRNA-from (blue) and dilincRNA-to (light blue). DROSHA and DICER process the resulted long double-stranded RNA, generating DDRNAs, which pair with nascend unprocessed single-stranded dilincRNAs; together they bind to 53BP1 and fuel DDR focus formation. ASOs promote site-specific inhibition of DDR, interfering with dilincRNA:DDRNA pairing.

1.2. THE ROLE OF DNA DAMAGE IN NEURODEGENERATION AND AGING

Neurodegeneration is often associated with congenital causes although recent evidences indicate that impaired DNA repair may also arise to age-associated neurodegeneration. Each cell endures with thousands DNA lesions per day thus requiring active and prompt response that ultimate in DNA repair. Accordingly, some cells characterized by short lifespan (e.g. epithelial cells) are rapidly replaced hence reducing the chances to accumulate DNA damage. Some others, especially in neuronal context, are more predisposed to dangerously increase DNA lesions since they are in post-mitotic state.

Moreover, neuronal cells display a high metabolism rate causing the consequent ROS accumulation (Madabhushi, Pan, and Tsai 2014). In brain, the resulting potential mutagenic alterations caused by the ROS-mediated oxidative base modifications are mainly repaired by BER and NER mechanisms, which sense the lesions, proceed with gap-filling DNA synthesis followed by ligation (Madabhushi, Pan, and Tsai 2014). Furthermore, ROS accumulation may also trigger SSBs (Caldecott 2008) that in turn my predispose to DSBs formation (Mladenov and Iliakis 2011).

As mentioned before, mature neuronal cell are fixed in the mitotic phase limiting their capacity to reduce potential DNA lesions accumulation. Particularly, during gestation neuronal progenitor switch from symmetric to asymmetric division mode producing one progenitor cell and one post-mitotic neuron. This pool of neurons moves to their final destinations then constituting a functional network after further differentiation events (Madabhushi, Pan, and Tsai 2014). In these initial stages DNA repair is crucial since unrepaired lesions may lead to irreparable damage to nervous system (McKinnon 2013). Accordingly, HR repair pathway during progenitor proliferation is essential especially because it preserves bases misleading reducing the potential loss of genetic information. Besides, NHEJ is the selected DSB-repair mechanism in adult neuron at the expanse of HR (Madabhushi, Pan, and Tsai 2014).

Progressive accumulation of DNA damage is intimately related to aging since unrepaired DNA lesions accelerates cellular senescence (d'Adda di Fagagna 2008) and accumulates along age progression (Mladenov and Iliakis 2011). Intriguingly, gene expression profile strongly changes with aging as observed by microarray analysis on post-mortem human brain specimen (from age of 40 onwards) which indicates down regulation of genes involved in neuronal function while up regulation of genes responsible for stress response (Lu et al. 2004). These events occur together with the accumulation of oxidative damage in the promoter region of genes that are down regulated the progressive reduction of cognitive faculties typically observed with age (Lu et al. 2004).

An important aspect of DNA damage is its impact on helix structure and chromatin organization. Although some chromatin changes are required for proper DDR signalling (e.g. ubiquitination and phosphorylation), some others profoundly affect chromatin conformation with few chances to restore physiological structural state (Oberdoerffer and Sinclair 2007). Nevertheless, long SIRT1 stalling and its distribution among chromatin induced by chronic genotoxic stress, causes a global down regulation of genes targeted by SIRT1 (Oberdoerffer et al. 2008).

DNA damage can also contribute to aging by stimulating inaccurate DNA repair resulting in mutagenic outcomes among DNA sequence. As a consequence, these mutations can directly compromise DNA repair activity since impairs the proper gene expression of DDR factors (Vijg and Suh 2013). Mutations in gene encoding for DNA repair factors have been detected in different congenital diseases indicating that the protective mechanisms for genome stability are essential for nervous system and its impairment triggers neurological phenotype (Madabhushi, Pan, and Tsai 2014). Accordingly, null mutations in gene encoding for the apical kinase ATM causes the ataxia telangiectasia (A-T) which exhibits marked neurological defects, like ataxia and cerebellar atrophy, assimilated as progressive neurodegenerative phenotype (Biton, Barzilai, and Shiloh 2008). Additionally, mutations in MRE11 trigger the rare A-T like disease (ATLD) with which shares neurological symptoms although in ATLD those features appears later compare to A-T (Taylor, Groom, and Byrd 2004). Another component of MRN complex, NBS1, if mutated, causes a disease defined as Nijmegen breakage syndrome which lead to microcephaly differently to canonical cerebellar degeneration observed in AT and ATLD diseases (Digweed and Sperling 2004). Different phenotypes detected in neurodegenerative diseases all carrying defective ATM activation arises the necessity to investigate the mechanisms by which such divergent events occur in apparently shared pathological contexts (Shull et al. 2009). Intriguingly, it has been observed that ATM stimulates apoptosis of excessively damaged post-mitotic neural cells (Lee, Chong, and McKinnon 2001). In this scenario, ATM loss of function together with mutations in MRE11, responsible for DNA damage accumulation in A-T and ATLD respectively, abolish the apoptosis ATM-mediated in damaged cells (Lee, Chong, and McKinnon 2001; Shull et al. 2009) thus increasing the population of damaged cells which progressively die over time and possibly contributing to neurodegeneration (Madabhushi, Pan, and Tsai 2014).

Defective DNA repair has been observed in age-related neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Adamec, Vonsattel, and Nixon 1999; Bender et al. 2006; Martin 2001; Mullaart et al. 1990). The high metabolism rate in neuronal cells trigger the prevalent detection of oxidative lesions and SSBs and the concomitant reduction of gene expression of BER factors may enhance the disease progression in age related diseases, including AD (Borgesius et al. 2011; Canugovi et al. 2013). Furthermore, the accumulation of DNA strand breaks has been detected in both AD and ALS (Adamec, Vonsattel, and Nixon 1999; Martin 2001; Mullaart et al. 1990). Consistently, several studies carried out in mouse models of neurodegeneration clearly show the increases level

of DSBs (Suberbielle et al. 2013). To this end, studies involving the p25/Cdk5 mouse model are widely recognized as the most interesting ones to investigate the missing link between DSBs and neurodegeneration (Cruz et al. 2003). Nevertheless, why and how DSBs arises in neurodegeneration is still under debate since DSBs generation is rarer than other types of DNA lesions in neuronal context and even in proliferating cells which are exposed to DNA replication stress. One possible explanation could be that the neuronal activity by itself can stimulate DSBs generation as also suggested by the high DSBs levels observed in AD mice model: in this case DSBs can be a consequence of the synaptic dysfunction due to beta amyloids accumulation (Dobbin et al. 2013).

In the last decades the accumulation of RNA:DNA hybrids has been associated to neurological diseases. These structure, commonly defined as R-loops, are generated by the nascent RNAs protruding from the transcribing RNA polymerase, to the DNA template strand (thread-back model) and are composed by the RNA-DNA duplex with the remaining displaced ssDNA (Aguilera and Garcia-Muse 2012). In physiological context, R-loops mediates DNA replication of mitochondrial and plasmidic DNA together with the Ig class-switch recombination (CSR), although R-loop exhibits a role in transcription activation and termination and in the regulation of chromatin structure (Santos-Pereira and Aguilera 2015). Besides, R-loops can also trigger genome instability since once generated they should only persist temporary or can lead to DNA damage. Interestingly, some RNA-binding proteins (RBPs) maybe also involved in R-loops prevention by interacting with the nascent mRNA and abrogate its association with DNA (Santos-Pereira and Aguilera 2015). However, R-loops can stimulate genome instability in different ways thus being one of the major source of DNA damage and replication stress (Santos-Pereira and Aguilera 2015). For instance, R-loops were found to stimulate non canonical and origin-independent DNA replication events that in turn may represent mutagenic sources in both E.coli and yeast cells (Kogoma 1997; Stuckey et al. 2015). Interestingly, it has been observed in different organisms that R-loops can interfere with replication-forks progression thus promoting DNA breaks formation (Brambati et al. 2015; Santos-Pereira and Aguilera 2015).

R-loops biogenesis is also associated with ALS cases carrying the expansion of GGGGCC hexanucleotide repeat in chromosome 9 open-reading frame 72 (*C9orf72*). In this context, the contribution of R-loops in the generation of aborted transcripts harbouring the repeats causes severe impairment of nucleolar function (Haeusler et al. 2014). To this regards, C9orf72 repeat expansions has been also related to R-loops-DSBs induction and impaired ATM activation which both ultimate in downstream alteration of DDR and DNA repair (Walker et al. 2017). All the mentioned effects were consistently

observed in mice nervous systems where, after C9orf72 RNA or dipeptide repeats expression, was observed a neurodegenerative phenotype supporting that genome instability is the primary promoter of neuronal degeneration (Walker et al. 2017). In line with the concept that R-loops pathological accumulation is the key mechanism behind neurodegeneration, the defective activities of enzymes implicated in R-loops resolution, like SETX, have been correlated with neurodegenerative disease including ataxia-ocular apraxia type 2 (AOA2) (Moreira et al. 2004) and ALS type-4 (ALS4) (Chen et al. 2004).

In conclusion, DNA damage has been widely observed and mostly implicated in many congenital degenerative diseases of nervous system, stimulating the necessity to further investigate the mutual connection between DNA damage and neurodegenerative diseases.

1.3. THE INTRICATE ROLES OF RNA BINDING PROTEINS AND DNA DAMAGE IN AMYOTROPHIC LATERAL SCLEROSIS

..1.3.1. Amyotrophic Lateral Sclerosis: clinical, histological, genetic features and role of DNA damage

The Centers for Disease Control and Prevention (USA) estimated in 2016 that between 14.000-15.000 Americans suffers of Amyotrophic Lateral Sclerosis (ALS) with no distinction of races and ethics backgrounds. ALS is characterized by the progressive degeneration of upper and lower motor neurons. Cortico-spinal motor neurons, better known as upper motor neurons (located in layer V of the cerebral cortex) make synapses connections between motor cortex in brainstem and bulbar or spinal motor neurons (lower motor neurons) which finally coordinate skeletal muscles (Taylor, Brown, and Cleveland 2016). Analogous to other neurodegenerative disorders, ALS arises locally and progressively spreads in target tissues (Ravits and La Spada 2009) leading to final muscular paralysis and respiratory failure, which in most of the times ultimate in death within 3-5 years from the diagnosis (Taylor, Brown, and Cleveland 2016) (Fig. 1.4.). ALS clinical onset is generally about 60 year of age and an epidemiological study conducted on European population estimates its incidence is 2-3 individuals per one hundred thousand each year (Al-Chalabi and Hardiman 2013). Nowadays, the aetiology of ALS is well assessed although the wide range of symptoms, especially in the first stage of the disease, made necessary a clinical distinction within the ALS. Particularly, ALS phenotypes are classified based on the initial region of onset and the consequent ratio of lower and upper motor neurons affected thus implying different clinical manifestations (Ravits and La Spada 2009). For instance, progressive muscular atrophy primarily affects lower motor neurons causing limbs weakness and deteriorations while primary lateral sclerosis causes spasticity but no muscle atrophy. Moreover, tongue atrophy and reduced speech ability

are distinctive symptoms of bulbar ALS since it affects motor neurons located in brainstem which innervate tongue (Taylor, Brown, and Cleveland 2016). ALS pathology shares clinical features with other neurodegenerative diseases such as Frontotemporal Dementia (FTD) which belongs to the family of neurodegenerative disorders and it is characterized by the loss of fronto-temporal cortical neurons (Conlon et al. 2018; Seelaar et al. 2011; Taylor, Brown, and Cleveland 2016). It was observed that 20% of ALS cases also develop cognitive and behavioural defects typical of FTD. This evidence suggest shared pathological hallmarks and mechanisms (Ling, Polymenidou, and Cleveland 2013).

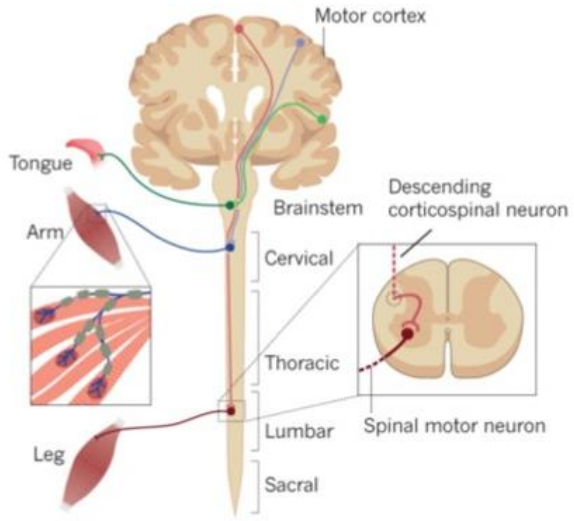


Figure 1.4. Overview of the components within the nervous system, which are affected by ALS (adapted from Taylor, Brown, and Cleveland 2016). ALS mainly affects motor neurons in the motor cortex whose axons prolong into synapses in brainstem, spinal cord and lower motor neurons.

From genetic point of view, ALS is characterized by autosomal dominant, autosomal recessive or X-linked origin in the case of familiar inheritance (familiar ALS-fALS) but 90% of cases are sporadic with no known family history (Chen et al. 2013).

Mutations associated with fALS occur in genes involved in many aspects of motor neurons physiology including RNA metabolism, protein homeostasis, clearance of unfolded proteins and cytoskeletal structure (Taylor, Brown, and Cleveland 2016). The first genetic variants identified in about 20% of fALS and 2% of sALS patients was

described in the SOD-1 gene (Rosen et al. 1993). SOD-1 gene encodes for Cu-Zu superoxide dismutase enzyme involved in O_2 and H_2O production from antioxidant action on reactive superoxide (Rosen et al. 1993). Subsequently about 170 SOD-1 mutations have been identified in ALS diseases causing different levels of impairment of dismutase activity (Bruijn et al. 1998; Taylor, Brown, and Cleveland 2016). A significant fraction of SOD-1 variants causes dismutase misfolding responsible for its toxic cytoplasmic accumulation in motor neurons and astrocytes (Bruijn et al. 1998; Taylor, Brown, and Cleveland 2016).

Another widely identified genetic variant among fALS and sALS is the amplification of the GGGGCC repeats in C9orf72 (DeJesus-Hernandez et al. 2011; Renton et al. 2011). In healthy individuals, repeats lengths range from 2 to 23 copies while they are expanded to hundreds of copies in ALS-FTD subjects. Furthermore, this expansion can even increase from 10% to 50% the probability that FTD patients develop ALS also (Bruijn et al. 1998). The mechanism by which the hexanucleotide expansion C9orf72 can promote toxicity in ALS-FTD (C9 ALS—FTD) is still under debate although three possible main mechanisms have been proposed to explain this phenomena: transcriptional repression of the C9orf72 locus; repeated RNA accumulation and folding in secondary structure; toxic dipeptide production. Particularly, since C9orf72 is less expressed in ALS/FTD cases, its pathogenicity could be due to C9orf72 loss of function as guanine-nucleotide exchange factor (GEF) for different Rab GTPases (Waite et al. 2014; Webster et al. 2016). C9orf72 loss of function is associated with splenomegaly, lymphadenopathy and altered immune responses in macrophages and microglia in mouse model system deleted for C9orf72 suggesting that induction of a specific neuroinflammation phenotype in C9orf72 ALS is an additional pathological mechanism (Burberry et al. 2016; O'Rourke et al. 2016). Nevertheless, strong emerging data support a gain of toxic function caused by this expanded repeats in C9orf72 ALS-FTD cases (Balendra and Isaacs 2018).

Pathological accumulation of RNA molecules transcribed from both directions thus resulting in secondary structure and formation of RNA foci observed in model system of C9orf72-associated ALS is another important aspect to discuss (Gendron et al. 2013; Mori et al. 2013). The accumulated RNA exert cell toxicity by binding and sequestering different RBPs that are therefore no more available for their physiological functions (Conlon et al. 2016; Lee et al. 2013). In addition, C and G reach expanded C9orf72 repeats are prone to form secondary structures like G-quadruplets that cause premature transcription termination and abortion and also boost R-loops formation, thus potential genome damage (Taylor 2014). The third mechanism proposed for C9orf72 toxicity in

ALS-FTD involves dipeptide repeats (DPR) translated from the hexanucleotide repeats in AUG-independent manner (Zu et al. 2011; Zu et al. 2013). Translation of repeat-associated RNAs generates dipeptide composed by glycine-alanine (GA), glycine-arginine (GR), proline-arginine (PR) and proline-alanine (PA). These products accumulate in cytoplasm and nuclear inclusions distinctive of C9 ALS-FTD (Zu et al. 2013). Intriguingly, inclusions containing arginine rich dipeptide appear to be the most harmful since reduce RNA processing in nucleoli thus causing cell death (Kwon et al. 2014). Harbouring low-complexity domains (LCDs), dipeptides can interfere with the biophysical and structural properties of membrane-less organelles like stress granules (SGs) (Lee et al. 2016).

Although genetic mutations of SOD-1 and C9orf72 genes are the most represented among ALS cases, there are several other mutations identified in ALS. This includes those occurring in several RNA binding proteins such as TARDBP, fused in sarcoma (FUS), HNRNPA1, SQSTM1, VCP, OPTN, PFN1, alsin (ALS3) and senataxin (SETX) (Coppede 2011; Taylor, Brown, and Cleveland 2016). Besides, additional mutations in ATXN2 (Elden et al. 2010) and EPHA4 (Van Hoecke et al. 2012)have been proposed to enhance disease predisposition.

The most diffused pathological hallmarks of ALS and FTD in common of all these genetic mutations is the formation of neuronal CI (NCI) positive for phosphorylated and ubiquitinated proteins (Forman, Trojanowski, and Lee 2004; Wightman et al. 1992).

The identification of the components of NCI has been one of the main focus of ALS research in the last decades and unveil that TDP43, FUS and hnRNP A1 are the main component (Taylor, Brown, and Cleveland 2016) together with constitutive components of SG such as TIA-1 and G3BP1. Histopathological analysis performed on post-mortem tissues from ALS patients show that TDP-43 is present in all types of NCI of more the 90% of ALS cases, both of sporadic and familiar origin with the exception of those caused by SOD-1 gene mutations, and SOD-1 positive inclusions (Mackenzie et al. 2007). In addition, ALS cases with FUS mutations, present FUS-positive NCI which are normally devoid of TDP-43 (Baumer et al. 2010). Other scattered evidences show that FUS NCI can occasionally include TDP-43 in both sALS and fALS cases (Deng et al. 2010). In this scenario, the RNA binding protein FUS has been described in a certain subset of ALS cases and particularly the ALS-linked mutation FUS P525L localized in the NLS domain has been associated with a severe juvenile onset (Conte et al. 2012) and immunohistochemistry performed on post mortem tissues carrying this mutation revealed the peculiar cytoplasmic localization of the protein (Fig. 1.5).

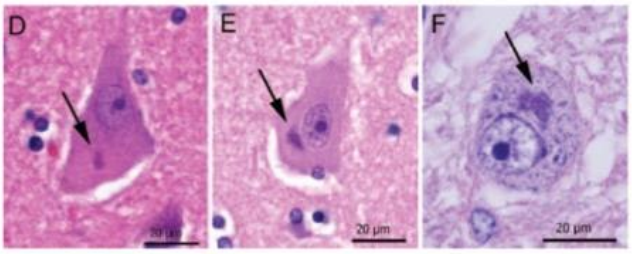


Figure 1.5. Immunostaining of the motor cortex and lower motor neurons in ALS. (adapted from Bäumer D. et al 2010). Basophilic neuronal CIs (indicated by the black arrows) were identified in upper and lower motor neurons of all cases with FUS mutation. (D, E) FUS-P525L (E) novel 4 base pair deletion in exon 15 (c.1554_1557delACAG) predicted to lead to a frame shift affecting the last 8 amino acids of FUS.

Neurons are not the only affected tissues and pathological phenotypes appear to be dependent on defects on other tissues and on non-cell-autonomous mechanisms. Microglia, oligodendrocytes and astrocytes have been associated with defective processes observed in SOD-1 and C9orf72 ALS cases (Taylor, Brown, and Cleveland 2016). For instance, it has been observed a neuronal inflammation due to a counter-intuitively boost by SOD-1 on the activation of the GTPase RAC1 (Harraz et al. 2008). Moreover SOD-1 mutations demonstrated to impair the energy supply provided by oligodendrocytes to motor neurons (Lee et al. 2012).

Astrocytes can limit motor neurons hyperactivation by promptly picking up synaptic glutamate through the excitatory amino acid transporter 2 (EAAT2), whose levels showed to be drastically reduced in fALS and sALS patients, thus resulting in excitotoxic effects (Rothstein et al. 1995).

Axonal transport is essential for motor neuron physiology since it ensure the continuous sourcing of proteins and RNAs in neuritis and synaptic sites that are far away from the cell bodies, where this molecules are mainly synthetized (Taylor, Brown, and Cleveland 2016). Intriguingly, mutation of the RBPs, including FUS, TDP-43 and hnRNPA1 which regulate the delivery of RNA containing granules in axons, have been associated with severe impairment of the mentioned transport thus damaging neuronal cells and their functionality (Taylor, Brown, and Cleveland 2016). Furthermore, axonal transport results impaired since many mutations have been identified in genes encoding for cytoskeletal

motor neurons proteins: this affects both anterograde and retrograde axonal transport and promotes neurodegeneration (Puls et al. 2003; Williamson and Cleveland 1999).

Like most of neurodegenerative diseases, ALS is characterized by the pathological accumulation of misfolded proteins, which undergoes in liquid to solid phase separation thus leading to the formation of fibrillar-like structures. Accordingly, if those structure are not promptly solved trigger severe impairment of many aspect of neuronal cell physiology including DDR. Hence, mutations in genes involved in autophagy and proteasomal pathways have been identified in different ALS cases. These genetic variants occur in gene like ubiquilin-2, sequestrome1 (SQSTM1 or p62), optineurin and the valosin-containing protein (VCP) (Taylor, Brown, and Cleveland 2016), suggesting that defects in clearance pathways and consequent accumulation of misfolded proteins may be one of the key pathological mechanism in ALS disease progression. The role of liquid-liquid phase separation (LLPS) of key ALS-linked factors and the impact on DDR will be discus below. Moreover, since the clearance of these pathological accruals appears to be the key event triggering many downstream effects, a dedicated part to the novel emerging interplay between autophagy and DDR will be found in this thesis.

1.3.2. The DNA/RNA binding protein FUS: structure, functions and role in DDR

Fused in sarcoma (FUS) is a DNA/RNA binding protein belonging to the nuclear ribonucleoprotein (hnRNP) family. The encoding gene was identified as a fused oncogene on chromosome 16 in human liposarcoma, whose translocation and fusion to transcription factors causes the over expression of the protein (Crozat et al. 1993). FUS is mainly an RNA binding protein (RBP) localizes in the nucleus where it forms stable complexes with other hnRNPs (Nakaya et al. 2013).

FUS is 526 amino acids protein and structurally is composed by: an N-terminal domain rich of glutamine-glycine-serine-tyrosine (QGSY) residues, three arginine-glycine-glycine repetitive regions, known as RGG1, RGG2 and RGG3, a RNA recognition motif RMM, a Cys2-Cys2 zinc finger domain (ZnF), a nuclear export signal (NES) and a the C-terminus nuclear localization signal (NLS)(Guerrero et al. 2016) (Fig. 1.6).

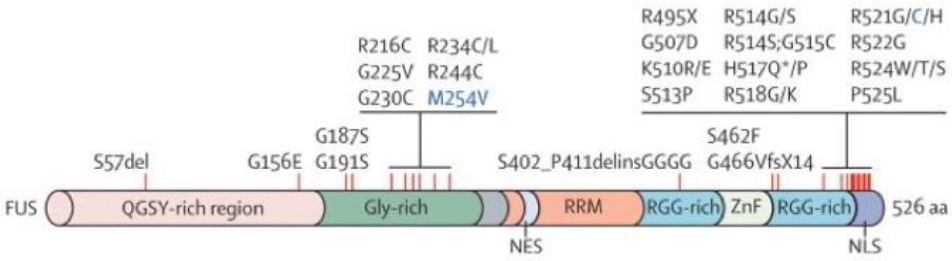


Figure 1.6. Protein structure of FUS and the mutations identified in ALS and FTD patients (Mackenzie IRA et al 2010). Mutations identified so far in FUS which are related to ALS only (in black) and also associated with FTD (in blue). NES=nuclear localization signal. QGSY=Gln-Gly-Ser-Tyr-rich region. RGG=Arg-Gly-Gly-rich motif. RRM=RNA recognition motif. ZnF=Cys2/Cys2-type zinc finger motif

Importantly, the QGSY-rich domain with a small portion of RGG1 domain (1-239) represent a high intrinsically disorder region (IDR) which is prone to aggregate like prion protein thus this domain is also called prion-link domain (PrLD) (Chen et al. 2019).

Moreover, according to bioinformatic approach, FUS has an additional PrLD located at 391-407 amino acid (Sun et al. 2011), although the most characterized ones is located at N-terminus. The high presence of polar amino acids within the PrLD drives FUS aggregation and consequent membrane less organelles formation (Pessina et al. 2020). In tissues FUS is exclusively localized mainly in the nucleus and in its de-localization exerts pathological consequences as observed in ALS (Andersson et al. 2008).

FUS is involved in many cellular processes especially concerning RNA metabolism including transcription. For instance, FUS exhibits a transcription regulatory role where, under specific condition, it may stimulates transcription of certain nuclear hormone receptors by interacting with their DNA-binding domain (Tan and Manley 2009). Moreover, FUS can directly bind the C-terminal domain (CTD) of RNAPII and RNA can modulate this interaction (Schwartz et al. 2012). In this scenario, FUS has been indicated as DROSHA interactor (Gregory et al. 2004) and it is able to modulate the miRNA biogenesis (Morlando et al. 2012). Accordingly, upon FUS depletion the expression of several miRNAs involved in neuronal function, was strongly decreased as observed in two different in vitro systems (Morlando et al. 2012). Moreover, FUS downregulation also impairs DROSHA chromatin recruitment at specific miRNA coding loci suggesting that FUS-DROSHA interaction is required for proper enzyme localization

at transcription sites (Morlando et al. 2012). More recently, it has been demonstrate that FUS play an key role in the modulation of circRNAs in vitro (Errichelli et al. 2017). CircRNAs are a novel class of single strand RNAs which arise from a back-splicing reaction where the downstream 5' splice site interacts with an upstream 3' splice site then the resulted circRNA is locked covalently (Starke et al. 2015). Noteworthy, biogenesis of 19 identified circRNAs appears to be FUS-dependent as validated in both mouse and human in vitro system (Errichelli et al. 2017). Furthermore, in IPSc derived motor neurons carrying one of the most severe FUS ALS-linked mutation P525L, the biogenesis of two distinctive circRNAs is strongly reduced in homozygous condition (Errichelli et al. 2017).

There are several evidences that demonstrate the active role of FUS in DDR signalling. The first evidence has been suggested in a study where the binding of FUS with ssDNA and D-loop was demonstrated (Baechtold et al. 1999). This event strictly promotes the proper annealing of complementary ssDNA and D-loop formation in super helical dsDNA, which in turn stimulate HR DSB repair mechanism (Baechtold et al. 1999). Moreover, FUS is directly recruited at site of damage upon UVA laser and micro irradiation and this event occur in PARP1-dependent manner (Mastrocola et al. 2013; Rulten et al. 2014). Particularly, RGG2 domain within FUS drives protein recruitment at DSB suggesting that PrLD, partially located in this domain, play a key role to targeting FUS at site of lesion (Mastrocola et al. 2013). Upon DNA damage induction, FUS is both phosphorylated by ATM, at Ser42 (Gardiner et al. 2008) and by DNA PK (Deng et al. 2014) indicating FUS as a new component of the PIKK signalling in DDR pathway. FUS is also involved in chromatin structure in DDR as demonstrated by its interaction with the Histone deacetylase 1 (HDCA1) thus stimulating both HR and NHEJ (Wang et al. 2013).

The mechanism, or the mechanisms by which FUS mutations exhibit toxic effects and how those mutations impairs DDR leading to neurodegeneration needs further investigation.

1.3.2.1. Post translational modifications (PMTs) and other factors that modulate LLPS

Protein aggregation is a common hallmark of neurodegenerative diseases although the pathological mechanisms that drive their formation are still under debating (Aguzzi and O'Connor 2010). It has been shown that low-sequence complexity domains (LC domains) are responsible for the formation of membrane-less compartments including P-

granules, stress granules (SGs) and Cajal bodies (Toretsky and Wright 2014). LC domain is peculiar of yeast prion proteins which assemble into fibers rather than a liquid states (Alberti et al. 2009); proteins holding these domains are prone to form amyloid-like structures and are defined as "prion-like". Moreover, prion-like LC domains are widely represented among RNA binding proteins (RBPs) and mutations in prion-like domains cause severe protein misfolding typically leading to solid inclusions formation (Li et al. 2012).

One of the most investigated prion-like protein involved in the cytoplasm compartmentalization is the RNA-binding protein FUS. The polar PrLD allows FUS protein to undergo liquid-liquid phase separation (LLPS) responsible for the formation of membraneless organelles: this process is thinly regulated by protein concentration, DNA and RNA levels and SYGQ-rich domain post-translational modifications (Patel et al. 2015; Shorter 2017). Structurally, FUS aggregation appears to be solved as β-sheet structures (Hughes et al. 2018) although recent evidences suggest that within the liquid phase separated state FUS appears predominantly disordered (Burke et al. 2015). The proper maintenance of FUS LLPS is due to different type of interactions, including electrostatic and hydrophobic interactions among glutamine and tyrosine residues that significantly contribute to the formation of hydrophobic bond essential for condensed phase (Burke et al. 2015). Moreover, multivalent cation-π interactions between multiple arginine residues in C-terminal domain and multiple tyrosines in the LC domain trigger FUS phase-separation in an arginine dose-dependent manner (Qamar et al. 2018). Physiologically, FUS can shuttle between the two phases stimulated by different stress types although aberrant LLPS are thought to be the key event that lead to fibrils formation especially in neuronal context (Guerrero et al. 2016). Mutations in PrLD or in the NLS of FUS raise protein concentration that in turn may enhance the conversion from liquid to solid phase (Guerrero et al. 2016).

Low complexity domains (LCDs) are key players in the LLPS transition and the lack of well ordered secondary structure made them target of post-translational modifications (PTMs). Thus, such events represent crucial regulatory factors of phase separation (Itakura, Futia, and Jarosz 2018). PTMs can alter phase transition through two main events: i) destabilizing or augmenting multivalent interaction between phase-separating macromolecules involved into; ii) recruiting or excluding other proteins and/or nucleic acid into/from the condensate phase (Owen and Shewmaker 2019).

The two main PTMs that may occur are the insertion of methyl-groups on Arginine and the insertion of phospho-groups on Tyrosine and Serine. While arg-methylation does not alter the charge but it is able to affect charge distribution, phosphorylation introduces a negative charge: both the events control the phase separation behavior (Hofweber and Dormann 2019). Recent evidences suggest that arginine methylation decreases LLPS propensity by reducing Arg-aromatic interaction, which in turn are the main responsible of phase separation of RBPs including hnRP-A2 and FUS (Hofweber and Dormann 2019). Moreover, reduced levels of FUS methylation has been detected in insoluble protein inclusions in brains of FTD patiens, while FUS is normally soluble and dimethylated in healthy brains, suggesting that Arg methylation maybe one of the pathogenic events promoting aberrant LLPS (Suarez-Calvet et al. 2016). Arg methylation has been demonstrated to directly regulate SGs survival: hypermethylation of G3BP1, one of the major component of SG, suppress its assembly (Tsai et al. 2016).

On the other hands phosphorylation, by introducing two negative charges, rapidly and reversibly switch protein behavior in order to provide a prompt response to signals and regulate protein function. This mechanism is highly employed in RBP, which are abundant of serine and threonine residues (Hofweber and Dormann 2019). Phosphorylation differs from arg-methylation by its ability to either enhance or suppress LLPS of RBPs in vitro as clearly demonstrated for the two RNA-binding protein FUS and TDP-43. DNA-PK mediated phosphorylation of putative Ser/Thr residues of FUS protein show suppressive effects on phase separation preventing its subsequent liquid to-solid-state transition and formation of fibril-like structures (Monahan et al. 2017). Moreover, phosphomimetic substitution of serine 48 to acid glutamate (S48E) localized in 91 N-terminal domain of TDP43, which is reported to be highly phosphorylated, lead to reduced LLPS compared to WT TDP43 suggesting reduced intermolecular interaction (Wang, Conicella, et al. 2018). In contrast, phosphorylation of the microtubule-associated protein Tau triggers its phase separation. The binding of Tau with RNA stimulates fibrillization in vitro and it is able to associate with SGs through RBPs constitutive of those structures (e.g. TIA-1) (Vanderweyde et al. 2016; Wang et al. 2006). Additional PTMs that weakness LLPS are Arginine-Citrullination and Lysine Acetylation, while Lysine Ribosylation enhances phase separation; both the mechanisms are involved in SG formation in neurological diseases (Owen and Shewmaker 2019).

High ATP concentration and the presence of triphosphate chain may modulate FUS LLPS. Considering that neurons exhibit high ATP consumption, neuronal context might be more prone then others to favor pathological fibrillar conformation of FUS containing membraneless organells. Indeed, ATP has been described to significantly dissolve LLPS,

rather than AMP. This is possibly due to its interaction with Arg/Lys residues in RGG1, which in turn disrupts interaction of those residues with aromatic residues within PrLD consequently abrogating proper LLPS (Kang et al. 2019).

Aberrant LLPS resulting from the impairment of one of the above-mentioned regulatory mechanisms or the combination of them is thought to be the key event that triggers the formation of FUS fibril-like structures detected in ALS and FTD patients. The conversion from liquid to solid state is also observed in FUS WT droplets over time suggesting that aging is an important regulator of this mechanism, which it is further augmented by FUS mutations (Patel et al. 2015). Mutations in both PrLD and in NLS domain may increases protein concentration in restricted space such as in the context of membrane-less organelles. Noteworthy, one of the most accredited explanation is that FUS solid transition is enhanced by raised protein concentration within phase separated compartment that alter molecules dynamic in turn may stimulating further aggregation reaction among close subcellular compartments as well as neighbor cells (Patel et al. 2015).

All together evidences suggest that FUS LLPS is physiologically regulated by intramolecular interactions that should be transient and what interferes with LLPS might lead to liquid-solid phase transition. Further investigations are required in order to shed light on the exactly mechanisms based on the co-operative interaction between the N-terminus and the C-terminus FUS PrLD domains and how this trigger irreversible solid-phase formation in pathological context.

1.3.2.2. Stress granules and ALS

RNP granules are a category of membrane-less organelles formed by the local assemblies of proteins and RNA. In this family are included Cajal bodies, paraspeckles, stress granules (SGs) and P bodies (Protter and Parker 2016). Both SGs and P bodies were detected nearby the cytoplasm and are mainly constituted of untranslated messenger ribonucleoproteins (mRNPs)(Kedersha et al. 2005). Although P bodies are mainly involved in mRNA degradation (Aizer et al. 2014), SGs generate in response to different stress sources and ultimate in the inhibition of initial steps of RNA translation consequently blocking protein synthesis until the stress is solved (Protter and Parker 2016). SGs arise from many different events causing cellular stress, like viral infections, oxidative agents exposure, heat shock, chemical components (e.g. puromycin), increased osmolarity and serum deprivation. These events converge on the phosphorylation of the translation initiation factor eIF2a which in turn prevents the formation of the ternary

complex eIF2-GTP-tRNA (Met) which normally bind the 48S pre-initiation complex in order to start protein translation (Kedersha and Anderson 2002). Subsequently, the 48S complex binds the TBP TIA-1 and the TIA-1 related protein (TIAR), which abrogate translation through polysomes decay (Kedersha and Anderson 2002). The translation stall is though to be essential for progressive reduction of energy consumption while cell is engaged by exogenous stress (Yang et al. 2014) and once the stress is solved, mRNA can be reassembled into polysomes to resume translation or can be shipped to P-bodies for degradation (Dewey et al. 2012). Structurally, two different layers compose SGs: a core structure enriched in RNA and protein surrounded by a less concentrated and more dynamic envelope (Jain et al. 2016). Moreover, SGs are dynamic structures: this is the most distinctive feature of such structures. For instance, SGs can flow within the cytoplasm and can undergo fusion or fission. Importantly, through photobleaching (FRAP) approach it has been identified in details the physical properties of SGs components. Indeed not all the SGs components responds at same way to FRAP thus suggesting that the core layer is mainly composed by "immobile pool" with less dynamic properties (Protter and Parker 2016).

Different factors modulate SGs composition including cell types and stress sources (Markmiller et al. 2018). However, there are some SGs protein marker which are constitutively of such structures independently from the surrounded context in which these structures originate. In detail, few proteins are defined SGs constitutive markers including TIA-1, TIAR, the Ras GTPase-activating protein-binding protein (G3BP), the poly-A binding protein (PABP-1) and the eIF3 and eIF4G factors forming the translation initiation complex (Buchan and Parker 2009; Dewey et al. 2012): all of them seeds SGs formation which is later followed by the additional incorporation of RBPs like hnRNPA1, FUS and TDP-43 (Protter and Parker 2016). Despite TDP-43 is only recruited subsequently, its depletion affects SGs structure and dynamics like G3BP1 does, and when TDP-43 levels are reduced both G3BP1 and TIA-1 are down and up-regulated, respectively (McDonald et al. 2011). Besides, FUS depletion doesn't affect SG assembly since no significant differences in terms of SGs population has been observed upon FUS knockdown (Aulas, Stabile, and Vande Velde 2012).

Under certain condition, like heat shock and sodium arsenite (ARS) treatment, both WT and mutant forms of FUS are recruited into SGs (Aulas and Vande Velde 2015). However, FUS WT is less recruited into TIAR-positive SGs showing weak localization in response to oxidative, temperature or ER stress (Andersson et al. 2008; Bentmann et al. 2012; Zhang et al. 2020). Particularly, endogenous FUS strongly co-localize in SGs only

upon hyperosmolarity conditions and other RNA bodies, like processing bodies (PB) result negative for FUS inclusion (Bosco et al. 2010).

Accordingly, in IPSc derived motor neurons the expressing FUS WT show nuclear protein localization even after sodium arsenite treatment (Lenzi et al. 2015). On the other hand, FUS ALS linked mutation strongly increases the number and affects the SGs dynamics. In heterozygous IPSc-FUS$^{P525L/WT}$ line the number of paraspeckles was increased upon ARS treatment and in IPSc- FUS$^{P525L/P525L}$ the protein signal was mainly detected in those cytoplasmic structure resulted positive for TIAR and PABP SGs markers (Lenzi et al. 2015). Mutant FUS also affect the SGs recovery as shown by the delayed SGs resolution observed in IPSc-FUS$^{P525L/wt}$ compared to IPSc-FUS WT upon ARS removal (Lenzi et al. 2015). FUS recruitment into SGs appears to be independent of its QGSY-rich domain but requires its RNA binding activity (Andersson et al. 2008; Bentmann et al. 2012). Besides, ALS linked mutations frequently occur in FUS NLS domain thus driving the protein cytoplasmic aggregation and recruitment into SG (Vance et al. 2013). More recently, the R521C FUS mutation has been associated to SGs processing alteration in knock-in mouse line demonstrating that mutant FUS is intrinsically related to a marked SGs misprocessing with consequent motor neuron impairment both in vivo and in vitro (Zhang et al. 2020).

As already described previously, LLPS is the main driven mechanism generating SGs. Indeed, LLPS seed the SGs formation, which are then stabilized by more fixed interaction among the molecules composing such structure. The resulting mechanism is a multistep model which involve the specific interaction among mRNPs thus ensuring the nucleation of SGs core and subsequently the constitution of SGs shell by further interactions established in close proximity of LCDs within RBPs (Protter and Parker 2016) (Fig. 1.7.).

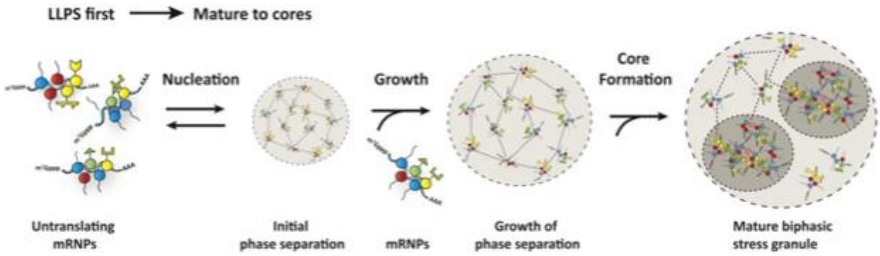


Figure 1.7. Model of SGs assembly (adapted from Protter and Parker 2016). According to the LLPS model, the first step is the nucleation of translationally repressed RNFPs into initial phase-separated droplets and these structures are modulated by weak and dynamic interaction. Then, additional translationally repressed RNPs are recruited within the droplets. Finally the third phase of assembly is the formation of a core within phase-separated granules.

Moreover, after nucleation additional mRNPs are recruited into SGs and forming cores that fuse and are surrounded by envelopes, giving rise to larger and cytologically visible SGs. This mechanism is ascribed to the activity of ATPases as DEAD-box, MCM and TVB helicases and chaperones (Cherkasov et al. 2013; Jain et al. 2016). Upon stress induction, such ATPases ensure the prompt recruitment of SGs component and modulates exchanges between core and shell and with the surrounding environment. This event is possible only because ATPases temporarily abrogate internal interaction within SGs components. Then, as soon as the stress is solved, ATPases are still required in order to modulate SGs disassembly (Protter and Parker 2016). The complete SGs resolution involves the autophagy pathway stimulated by other ATPases including VCP/Cdc48 ubiquitin segregases (Buchan et al. 2013).

1.4. DNA DAMAGE AND AUTOPHAGY: A NOVEL EMERGING INTERPLAY

1.4.1. Mechanisms and regulators of autophagy

The common hallmark of neuropathies, including ALS, is the accumulation of misfolded proteins in the cytoplasm (Soto and Pritzkow 2018; Sweeney et al. 2017). Thus, the proper clearance of misfolded proteins is crucial for cells survival, especially in neuronal context. In cells, protein homeostasis is commonly ensured by two mechanisms: the ubiquitin-proteasome system (UPS) and the autophagy pathway. The UPS facilitates the processing of short-lived proteins while autophagy activation is required to target long-lived proteins towards lysosome dependent clearance (Nijholt et al. 2011).

Autophagy ensures tissue homeostasis and plays a crucial role in different human pathologies including neurodegeneration, cancer, autoimmunity and aging: all of them have been associated with autophagy deregulation (Mizushima et al. 2008). So far, three different types of autophagy have been identified based on the final delivery mechanisms of the cargo proteins to the lysosomes. Micro-autophagy is the mechanism where cargo is directly delivered to lysosome through lysosomal membrane invagination while chaperone-mediated autophagy (CMA) involves the interaction with specialized chaperones which stimulate lysosomal import of proteins. Macro autophagy (referred as autophagy) is the most investigated mechanism of autophagy which requires the initial

capture of the cargo into double membrane vesicles known as autophagosomes (Fig. 1.8.). These structure are able to sequester large portions of the cytoplasm and are modulated by an intricate interconnection between of 15 autophagy-related (ATG) proteins (Rubinsztein, Shpilka, and Elazar 2012). Particularly, autophagosome formation requires three steps: initiation, nucleation and elongation (Fig. 1.8.).

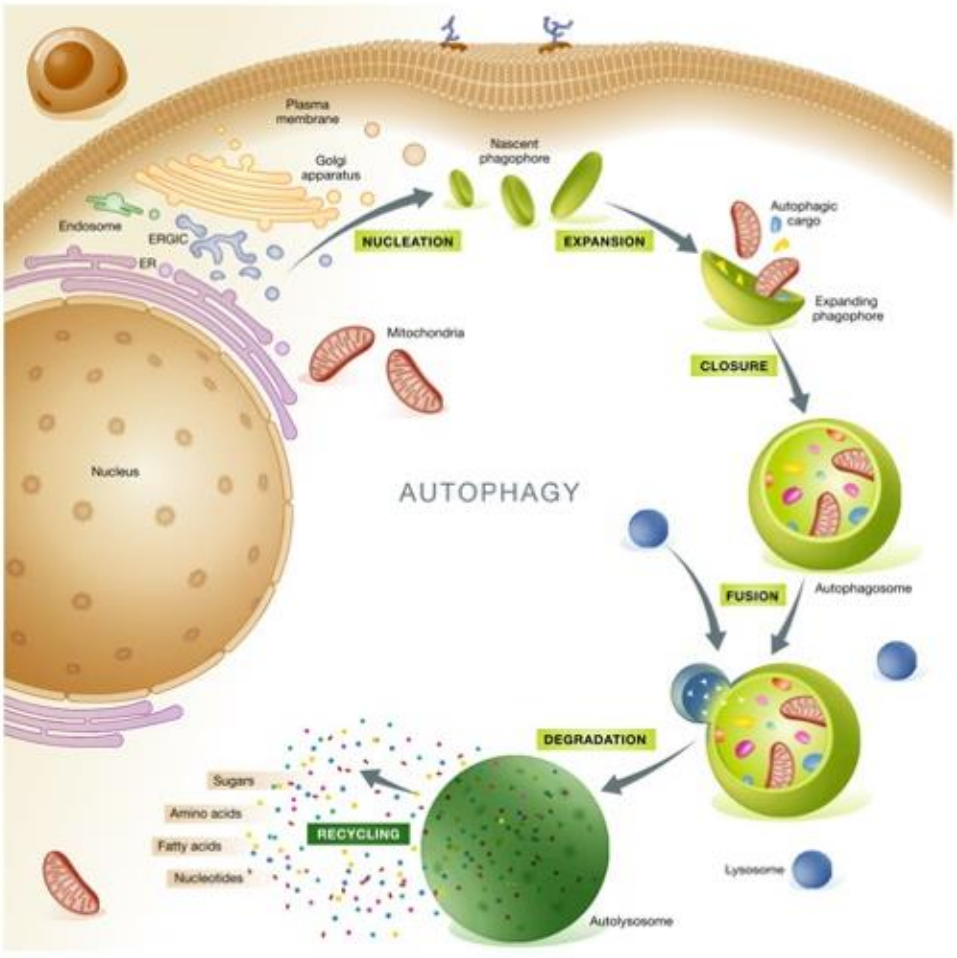


Figure 1.8. The Autophagy pathway (Galluzzi L. et al 2015). Autophagy initiates with the segregation of cytoplasmic material through phagosphores, which nucleate from the endoplasmic reticulum (ER). Many membranous organelles (e.g. Golgi apparatus, ER-Golgi intermediate compartment (ERGIC), plasma membrane, mitochondria and recycling endosomes) contribute to phagosphore elongation. Expanding phagosphore ultimate in autophagosome formation can fuse with lysosome to form autopysosome. This event trigger lysosomal hydrolases activation that

degradate the auphagosomal cargo. The resulted products are then recycled by anabolic or bioenergetics circuitries.

Initiation involves the activation of a large proteins complex in order to assets the formation of an initial double membrane structure defined as phagophore or isolation membranes. In general, phagophore generate close to ER-mitochondria contact sites from a specific structure known as omegasome (Galluzzi et al. 2017). Subsequently, the additional recruitment of proteins stimulates the formation of a macromolecular "nucleation" complex modulated by Beclin-1, ATG14 and Vps15 (Eliopoulos, Havaki, and Gorgoulis 2016). Finally, interactions among several ATG proteins ensure vesicle elongation driven by the conjugation between the phosphatidylethanolamine (PE) to the microtubule-associated protein light chain 3 (LC3 or better known as LC3-II) (Eliopoulos, Havaki, and Gorgoulis 2016). This event is crucial for mature autophagosome formation, which is then targeted to lysosomes. During autophagy flux, autophagosomes rapidly fuse with endosomes forming amphisomes or with lysosome generating the autolysosome (Galluzzi et al. 2017). Noteworthy, autolysosomes are positive for lysosomal markers such as LAMP1 and LAMP2 but if the autophagic flux is high (e.g. lysosomal hydrolases are inibithed genetically or upon pharmacological treatments) they can be negative for autophagosomal marker (Klionsky, Eskelinen, and Deretic 2014). Once there, all the materials is digested in autolysosomes which are assumed to convert into lysosomes still able to fuse with other endosomes or autophagosomes. Another option could be that if the degradation is not fully completed, autolysosome can becomes itself a residual body containing indigested material and lipofuscin pigment (LF) (Eskelinen and Saftig 2009). Intriguingly, LF accumulation has been associated with age-related neurodegeneration suggesting the connection with impaired protein homeostasis and neurophaties (Moreno-Garcia et al. 2018). Besides the canonical macroautophagy other two types of macroautphagy have been recently identified. These includes mitophagy where the autophagy flux is directed to mitochondria, pexophagy where it ultimates in peroxisomes and xenophagy in which the autophagy conclude its flux in intracellular bacteria (Klionsky et al. 2007). Among the key factors which modulate autophagy pathways the two receptors SQSTM1 (also known as p62) and NBR1 act as adaptors since they bind ubiquitinated protein substrates and target them to degradation within autophagy machinery: both result upregulated when autophagy is inhibited (Kirkin et al. 2009; Mathew et al. 2009). Among the key factors which modulate autophagy pathways the two receptors SQSTM1 (also known as p62) and NBR1 act as adaptors since they bind ubiquitinated protein substrates and target them to degradation within autophagy machinery: both result upregulated when autophagy is inhibited (Kirkin et al. 2009; Mathew et al. 2009). Recent evidences also

show that p62 protein represents the link between UPS an autophagy mechanisms. In this regard, the UPS is strongly compromised in case of autophagy deficiency since this stimulates p62 abundance without affecting the proteasomal catalytic activity (Liu et al. 2016). Autophagy mechanism can be primarily regulated by nutrient availability, especially amino acids (Kadowaki et al. 2006), since autophagy is induced by amino acid starvation and amino acids generated during latest steps of autophagy flux acts as feedback inhibitor of autophagosome formation (Eskelinen and Saftig 2009). Moreover, cellular amino acids availability regulates the activity of mammalian target of rapamycin (mTOR) which in turn plays a pivotal role in autophagy regulation (Jung et al. 2010). Accordingly, increased mTOR activity inhibits autophagy while rapamycin treatment (mTOR inhibitor) activates autophagy (Kamada et al. 2000).

1.4.2. DDR and autophagy: possible synergy in human diseases?

The progressively advanced understanding of autophagy suggests that abnormal autophagy could modulate neurodegenerative diseases and cancers. Particularly, p62 dysfunctions have been already described in ALS where immunohistochemistry performed revealed that different inclusions (e.g. skein-like inclusions, Lewy body-like inclusions and basophilic inclusions) result positive for p62 (Mizuno et al. 2006) and more importantly, many mutations on the p62 encoding gene have been described in ALS cases (Fecto et al. 2011; Teyssou et al. 2013).

Moreover, impaired DDR has been associated with both tumour progression and neurodegeneration. In this scenario, many evidences have suggested that autophagy can be activated by DNA damage as observed for ATM kinase, which is one of the apical kinase activated following formation of DSBs. Once activated, ATM may induce autophagy by activating AMPK, which in turn is able to remove the inhibitory effect on mTORC1 and consequently induce autophagy (Alexander, Kim, and Walker 2010). Moreover, the activation of PARP1 upon DSBs formation stimulates the reduction of both NAD^+ and ATP and consequent increased level of AMP thus activate AMPK and induce autophagy (Rodriguez-Vargas et al. 2012). The autophagy regulation DNA-mediated can also occur through several transcriptional and post-transcriptional events. For instance, the two AMPK subunits β1 and β2 are activated by p53 through Sestrin1 and Sestrin2 and, more importantly, p53 modulates AMPK subunits transcription (Feng et al. 2007).

As mentioned above, DDR signalling activation ultimate in DNA repair pathways activation and scatter evidences have shown that autophagy alterations also reflect on

DNA repair outcomes. In this scenario, p62 plays a pivotal role mediating the effect of autophagy on DDR. Several reasons may lead to autophagy inhibition thus leading to p62 accumulation and stimulating its binding with the E3 ubiquitin ligase RNF168. This event abrogates proper chromatin ubiquitination and strongly reduces the recruitment of downstream key players such as BRCA1, RAD51 and RAP80 at DSBs thus affecting DNA repair efficiency (Wang et al. 2016). Moreover, p62 binds RNF168 through its LIM-binding (LB) domain (aa 170–220) and overexpression of p62-WT inhibits 53BP1 foci formation while p62-ΔLB overexpression show not differences with control condition (Wang et al. 2016). Finally, C9orf72 repeat expansion has been recently associated with ATM-mediated DDR impairment in ALS (Walker et al. 2017). Particularly, C9orf72 repeat expansions lead to R-loops formation, which in turn increased DSBs trough the impairment of ATM signalling (Walker et al. 2017). Both p62 depletion and the R-loops resolution helicase (SETX) expression restore proper DDR signalling in cells with experiencing C9orf72 DPR (Walker et al. 2017). Moreover, decreased autophagy efficiency results in loss of DNA repair by reduction of checkpoint kinase 1 (Chk1) (Liu et al. 2015). Accordingly, *Atg7* $^{-/-}$ and *Atg7*$^{flox/flox}$ MEFs cells show decreased levels of Chk1 upon irradiation causing impairment of HR efficiency (Liu et al. 2015).

To date, the aberrant interconnection between autophagy and DDR in human diseases is not fully characterized yet. Both processes are essential for cellular homeostasis and their proper functionality results to be crucial for cell survival. For this reason, is not surprising that a link between autophagy and genome integrity has been found in different human diseases including cancer and neurodegeneration. Intriguingly, autophagy can act both as tumour-suppressive by promoting oncogenic and damaged organelles degradation or as tumour promoter since it may support cancer cell survival (White and DiPaola 2009).

Recent evidences demonstrate that the ALS-linked mutations in FUS protein are associated with impairment in autophagy pathway. The exogenous overexpression of the two mutations P525L and R522G impair the early stages of autophagy since the number of omegasomes present per cell was notably reduced in those cells compared to cells expressing the wild type form (Soo et al. 2015). Besides, the levels of p62 protein are strongly increased in cells expressing the mutant forms with the consequent accumulation of ubiquitinated proteins indicating that the clearance of ubiquitinated proteins is less efficient in those cells compared with control (Soo et al. 2015). Moreover, the FUS ALS-linked mutations alter the co-localization between the protein ATG9 and LC3II thus reducing both the formation and translocation of autophagosomes in such cells (Soo et al. 2015). The concomitant overexpression of Rab1 protein with FUS mutations restores

proper autophagy flux. Rab1 protein mediates the ER-trafficking and autophagosome formation (Zoppino et al. 2010) and its overexpression rescues both LC3II vescicles and omegasome formation in cells expressing FUS mutations (Soo et al. 2015). It has been demonstrated that inhibition of mTOR through rapamycin treatment ameliorates FUS P525L induced SGs dynamic in IPSc lines suggesting that rapamycin may stimulates FUS proteins degradation thus reducing its aberrant incorporation into SGs (Marrone et al. 2018). Noteworthy, autophagy induction by the treatment with a more specific mTOR inhibitor (torkinib) promotes protein homeostasis and consequently increased cell survival in P525L iPSC-derived neurons (Marrone et al. 2019).

Collectively, these evidences support the intricate relationship between autophagy and DDR although how they can interact in the human diseases context needs further investigation.

2. AIM OF THE PROJECT

It has been widely reported that FUS ALS-linked mutations lead to protein nuclear de-localization and its aberrant incorporation in toxic CI (Mackenzie et al. 2011). To date, how such protein inclusions trigger neurotoxicity is still under debate.

Besides the presence of cytoplasmic protein inclusions, another important feature of ALS is the detection of DNA damage (Naumann et al. 2018), including SSBs and DSBs (Farg et al. 2017; Martin et al. 2007). In this scenario, FUS-P525L is one of the most severe ALS mutations, and it is associated with the appearance DNA damage accumulation in post mortem ALS tissues and autophagy defects (Leblond et al. 2016; Marrone et al. 2019; Soo et al. 2015).

In cancer context it has been described a crosstalk between DDR activation and autophagy defects (Wang et al. 2016). To our knowledge, instead no previous studies reported a possible mechanistic link between FUS ALS-mutation, DDR and autophagy. In this thesis we address the impact of mutant FUS CI formation on DDR signalling impacting on DNA repair and autophagy defect.

In this perspective, we characterized the impact of FUS-P525L CI on DDR in cultured cells where we investigated the interconnection between DDR and autophagy flux by analysing key players in both basal condition and after induced DSBs generation.

3. MATERIALS AND METHODS

3.1. CELL CULTURE AND PLASMID TRANSFECTION

HeLa cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine and 1% penicillin/streptomycin. Cells were grown at 37°C under a humidified atmosphere with 5% CO2. I-HeLa11 cells were cultured as previous described (Lemaitre et al. 2014). I-SceI expression in I-HeLa111 was induced by administrating 1 μg ml−1 doxycycline for 24 hours. μlCells were plated into 6 multi-well plates so that they were 70-80% confluent at the day of transfection. Cells used for subsequent imaging analysis were grown on coverslips. For each transfected well, 250 μl of serum-free medium (Opti-MEM) were mixed with 1 μg plasmid DNA, and 250 μl of Opti-MEM were mixed with 6 μl of Lipofectamine 2000 Reagent (Life Technologies). The two solutions were then mixed together and incubated for 10 minutes at room temperature (RT) to allow the formation of lipid complexes. The complete medium was replaced with 1.5 ml of fresh Opti-MEM before transfection. The transfection mix was added and then removed from cells 6h later to be replaced with fresh complete growth medium. After 24h of transfection, cells were collected for subsequent analysis. Plasmid used as control in this study is pcDNA3.1+ (Addgene). The plasmids expressing FUS-WT and FUS-P525L were a kind gift of the Dr. Gianluca Cestra (Institute of Biology and Molecular Pathology (IBPM) CNR Rome; Department of Biology and Biotechnology Charles Darwin, University of Rome "Sapienza"), DROSHA WT (FLAG tagged) was kindly donated by Dr. Narry Kim (Seoul National University), RNF168 WT (FLAG tagged) was kindly gifted by Dr. Lorenza Penengo (University of Zurich) and RNF8 WT (GFP tagged) was kindly borrowed from Dr. Simone Sabbioneda (IGM-CNR of Pavia). The plasmid HA-p62 was purchased from Addgene (catalog number #28027).

3.2. CHEMICAL TREATMENTS AND IR INDUCTION

When indicated, DNA damage was induced by treating cells with Neocarzinostatin (NCS) at a final concentration of 50ng/ml for 20 minutes at 37°C. Treatment with DNA-PK, ATM and ATR inhibitor were performed on cells transfected with FUS-P525L by Lipo2000. These cells were treated at 20h post transfection with the DNA-PK inhibitor KU-60019 (Sigma Aldrich) or the ATM inhibitor NU7441 (Tocris Bioscience) at the final

concentration of 5 μM and ATR inhibitor VE-821 (Tinib-Tools) at final concentration of 10 μM overnight. Untreated cells were incubated with the same volume of DMSO. For cell proliferation assay, 6 hours after plasmid transfection cells were incubated over night with 10 μM BrdU (Sigma-Aldrich). 20 minutes after NCS treatment, cells were fixed in 4% PFA and stained with anti-BrdU (Sigma-Aldrich) antibody according to manufacturer's protocol. Cells were exposed to 2 Gy of ionizing radiation with a high-voltage X-ray generator tube (Faxitron X-Ray Corporation).

3.3. RNA INTERFERENCE

Short interfering RNAs (siRNAs) are commonly used to knock-down the specific gene of interest. For siRNAs transfection, cells were plated in 6 multi-well plates in order to reach 40-50% of confluence at the day of transfection. For each transfected well 250 μl of Opti-MEM were mixed with siRNA oligo and 250 μl of Opti-MEM were mixed with 4 μl Lipofectamine RNAi-MAX transfection reagent (Life Technologies). The two solutions were mixed and incubated for 10 minutes at RT to allow the formation of lipid complexes. The growth complete medium was removed from the cells and replaced with 1.5 ml of freshe culture medium. The resulted mix was then added to the cells that were left in the incubator until the analysis. siRNA treatment is transient and usually biological effects are studied within 72 hours post transfection. The sequences of ON-TARGET plus SMARTpool siRNA oligonucleotides (Dharmacon) are reported in the table below.

Table 1: siRNAs used in this thesis

Target mRNA	Sequences
Non-targeting CONTROL	UGGUUUACAUGUCGACUAA
	UGGUUUACAUGUUGUGUGA
	UGGUUUACAUGUUUUCUGA
	UGGUUUACAUGUUUUCCUA
p62	GAACAGAUGGAGUCGGAUA
	GCAUUGAAGUUGAUAUCGA
	CCACAGGGCUGAAGGAAGC
	GGACCCAUCUGUCUUCAAA

TDP43	GCUCAAGCAUGGAUUCUAA
	CAAUCAAGGUAGUAAUAUG
	GGGCUUCGCUACAGGAAUC
	CAGGGUGGAUUUGGUAAUA

3.4. RNA EXTRACTION AND ANALYSIS

Total RNA was isolated using TRIzol Reagent (Thermo-Fisher) and subsequent mRNA analysis was performed by quantitative RT-PCR followed (qRT-PCR) by DNaseI treatment (Thermo-Fisher). Accordingly, small RNAs (<30nt) were gel-purified after fractionation of total RNA along with 10 pg of synthetic *C. Elegans* cel-miR-67* as a spike-in onto 10% Urea-PAGE and analysed using miScript II System by qRT-PCR.

Oligonucleotides	Sequences
primer for cel-miR-67*	CGCTCATTCTGCCGGTTGTTATG
primer for DDRNA FW	TCCACATGTGGCCACAAATTG
primer for DDRNA RV	CAATTTGTGGCCACATGTGGA

Table 2: Primer sequences used for qRT-PCR

3.5. COMET ASSAY

Neutral comet assay was performed following manufacturer's protocol (Trevigen) as previously reported (Gioia et al. 2019). Briefly, 24h after transfection HeLa cells were trypsinized, washed once with ice-cold PBS 1X and then re-suspended in cold PBS at the final concentration of 10^5 cells ml-1. Subsequently, cell suspension was combined with pre-warmed low-melting agarose at ratio 1:10 (v/v) and finally poured onto the slides. The cell lysis was performed over-night at 4°C. The electrophoresis was performed in 1X Neutral Electrophoresis Buffer for 45min at 21V. After DNA precipitation and wash in 70% ethanol, slides were dried up and DNA stained with SYBR Gold (Thermo-Fisher)

before epifluorescence microscopy analysis (Olympus Biosystems). Comet tail moment was calculated taking advantage of OpenComet software (Gyori et al. 2014).

3.6. INDIRECT IMMUNOFLUORESCENCE (IF)

Cells were grown on glass coverslips. Washed twice with ice-cold PBS 1X and for most of the antibodies used it was necessary 4% PFA fixation with for 10 minutes at RT and permeabilized with 0.2% Triton X-100 for 10 minutes at RT. Some antibodies (e.g. pATM and pS/TQ) required different fixation method to work effectively and in such cases cells were fixed with cold methanol (4°C) at for 1.30 minute at RT. In any cases, cells were then washed twice in PBS 1X, incubated overnight at 4°C with a blocking solution PBG (0.5% BSA, 0.2% gelatine from cold water fish skin in PBS 1X) and then stained with primary antibodies diluted in PBG for 1 hour at RT in a humidified chamber. Cells were washed 3 times for 5 minutes with PBG and incubated with secondary antibodies diluted in PBG for 1 hour at RT in a dark humidified chamber. Finally, cells were washed twice for 5 minutes with PBG, twice for 5 minutes with PBS 1X and incubated with 4'-6-Diamidimo-2-phenylindole (DAPI, 0.2 μg/ml, Sigma-Aldrich) for 2 minutes at RT. Cells were briefly washed with PBS 1X and water and coverslips were then mounted with Aqua Poly/Mount mounting medium (Tebu-bio) and let dry overnight at room temperature. Coverslips were air dried before microscope analysis.

3.7. IMAGE CAPTURE AND ANALYSIS

Immunofluorescence images were acquired using a Confocal Laser Scanning Microscope (Zeiss LSM800) equipped with 4 lasers: Diode laser 405 nm (5mW); Diode laser 488 nm (10mW); Diode laser 561 nm (10mW); Diodo laser 640 nm (5mW), two Master gain with high sensitivity and a 63x 1.4Na objective. The system is driven by software Zeiss ZEN Blue 2.6. Moreover, certain acquisitions were carried out with a widefield epifluorescence microscope (Olympus IX71) equipped with PlanApo 60Å~/1.40NA oil immersion objective, a Cool SNAP ES camera (Photometrics) and driven by MetaMorph software (Universal Imaging Corporation).

For co-localization acquisition, images were collected with confocal microscope and cells that showed FUS positive inclusions (stained with Alexa Fluor (AF) 488) were randomly chosen. For each acquisition, we collected 15-30 z-sections (190nm) with Zen Blue 2.6, setting pinhole at 0.6 Air Unit (AU).

Comparative immunofluorescence analyses were performed in parallel with identical acquisition parameters and exposure times using CellProfiler Cell image analysis software (Version 2.1.1) (Carpenter et al. 2006). Numbers of DDR foci per nucleus were quantified by the automated software CellProfiler, applying an ad-hoc-designed pipeline,

that based on size and fluorescence intensity of DDR foci relative to the background signal, recognizes and counts their number in each DAPI-positive cell nucleus. Identical parameters were applied in the analyses of all conditions compared in each experiment.

For all the analysis in which it was required, the distinction between cells with and without FUS positive cytoplasmic inclusions (CI) (upon FUS-P525L transfection) was made through a comparison of the original images and the images in output from CellProfiler where nuclei are numbered in automated manner. The same approach has been used for nuclei expressing high nuclear level of FUS upon FUS-WT transfection.

For co-localization analyses the images were processed using FIJI (Schindelin et al. 2012) making a threshold of cell nuclei using Li equation, creating a mask that were subtracted from all other channels in order to obtain only cytoplasmic areas. Co-localization between RNF168 and p62 were estimated by Manders" coefficients obtained through the application of JACoP plugin (Bolte and Cordelieres 2006) applied on FIJI. We calculated Manders" overlap coefficient M1 and M2, where M1 was the fraction of RNF168 (stained with AF 647) overlapping p62 (stained with AF), and M2 was define conversely for p62. The best-fit lower threshold was determined using threshold tool and visually inspected. We considered the M2 coefficient of three replicates and we performed about 40 acquisition per replicates. The same approaches has been applied to determine the co-localization between RNF168 signal (M1) and FUS signal (M2) reporting M1 values. In order to exclude possible cross-talk artefacts between AF555 and AF647, we performed single immunostaining of AF555 and AF647. We acquired sample marked only with AF555 exciting the track of AF647 with both AF555 and AF647 lasers simultaneously, without reveal any signals. The same procedure was used also for sample marked only with AF647, getting the same results. Finally the same immunostaining, acquisition and analyses approaches have been applied for the estimation of FUS-TIA1 and FUS-G3BP co-localization results.

For p62 quantification images were processed with FIJI and cells with FUS positive inclusions were counted. The cytoplasmic p62 accumulation was determined removing the outliers with a custom pipeline, setting the same limits for all fields in each biological replicate. Particles were detected applying the ComDet 5.2. plugin (https://github.com/ekatrukha/ComDet/wiki), setting the best fit for both approximate particles size and intensity threshold for each replicates. Data obtained were used to calculate the percentage of cells harbouring p62 cytoplasmic accumulation. All data for imaging analyses were plotted with the GraphPad Prism software version 6.04 (La Jolla California, USA).

3.8. PROTEIN EXTRACTION AND IMMUNOBLOTTING

Cells were lysed in Laemmli sample buffer (2% sodium dodecyl sulphate (SDS), 5% glycerol, 1.5% Dithiothreitol (DTT), 0.01% bromophenol blue, 60 mM Tris HCl pH 6.8). Collected cells were sonicated (Diagenode) with 3 bursts of 15 sec and heated for 4 min at 95°C. The chosen volume of lysates was loaded on a 6%/ 8% SDS-polyacrylamide gel or on a Mini protean pre-cast gel (BIORAD) with a width of 1 mm along with 7 µl of molecular weight markers (Biorad). Gels were run in Tris-Glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) until the dye reached the bottom of the gel. For Western blotting analysis proteins were transferred to a 0.2 µm nitrocellulose membrane (Biorad Trans-Blot® TurboTM transfer pack) using the Trans-Blot® TurboTM Transfer System apparatus (Biorad). The transfer was performed at 25V for 3, 7 or 10 min (according to the molecular weight of the proteins under investigation). Membranes were incubated with 5% skim milk in TBS-T buffer (Tween20 0.1%) for 1 h, followed by over-night incubation at 4°C with primary antibody and 3X washed with TBS-T before 1h incubation at room temperature with the specific HRP-conjugated secondary antibody. After additional 3X washes with TBS-T, chemiluminescence detection was performed by incubation with LuminataTM Classico or Crescendo (Millipore). Proteins were visualized by autoradiography on ECL films (Amersham), using various exposure times and manually developed.

Table 3: Primary antibodies used in this thesis.

Antibody	Company	Code	Host	Application	
				IF	WB
FUS	Bethyl	A300-293A	Rabbit	1:1000	
FUS	Bethyl	A300-839A	Goat	1:1000	
FUS	Santa Cruz	Sc-47711	Mouse		1:1000
FUS	Proteintech	60160-1-Ig	Mouse	1:200	
γH2AX	Millipore	05-636	Mouse	1:1000	
γH2AX	Cell Signaling	9718	Rabbit	1:1000	

53BP1	Bethyl	A303-906A	Goat	1:1000	
53BP1	Bethyl	A300-272A	Rabbit	1:1000	
p53BP1	Cell Signaling	2675	Rabbit	1:400	
pATM	Rockland	200-301-400	Mouse	1:600	
pATM	Sigma-Aldrich	05-740	Mouse		1:2000
pDNA-PK	Abcam	Ab32566	Rabbit		1:500
pATR	Abcam	Ab227851	Rabbit		1:500
SQSTM1/p62	Enzo LifeScience	BML-PW9860	Rabbit	1:1000	
SQSTM1/p62	GeneTex	GTX100685	Rabbit		1:1000
RNF168	R&D System	AF7217	Sheep	1:100	
RNF168	Millipore	ABE367	Rabbit		1:1000
DICER	Sigma Aldrich	SAB4200087	Mouse	1:100	
FK2	Enzo LifeScience	BML-PW8810	Mouse	1:1000	
NBS1	Novus Biologicals	NB100-92502	Rabbit	1:100	
Phospho-(Ser/Thr) ATM/ATR Substrate (pS/TQ)	Cell Signaling	2851S	Rabbit	1:400	
pCHK2	Novus Biologicals	NB100-92502	Rabbit	1:100	

G3BP	BD Biosciences	611126	Mouse	1:100	
TIA1	Abcam	ab40693	Rabbit	1:200	
TDP43	Proteintech	10782-2-AP	Rabbit	1:500	
Cyclin A	Santa Cruz	Sc-751	Rabbit	1:1000	
Cyclin A	Santa Cruz	Sc-27-1682	Mouse	1:200	
Cleaved Caspase 3	Cell Signaling	9661	Rabbit	1:200	
DROSHA (mAb)	Abcam	Ab183732	Rabbit	1:200	
DROSHA (pAb)	Abcam	Ab18192	Rabbit	1:250	
MDC1	Sigma Aldrich	M2444	Mouse	1:500	
HA	Roche	11867423001	Rat	1:300	
BrdU	BD Biosciences	347580	Mouse	1:20	
Vinculin	Millipore	MAB3574	Mouse		1:1000
Tubulin	Sigma Aldrich	T8328	Rabbit		1:2000

Table 4: Secondary Antibody used in this thesis.

Antibody	Company	Code	Against	Applications	
				IF	WB
Rabbit	Abcam	ab97064			1:10000
Mouse	Abcam	ab97030			1:10000
Alexa Fluor 488	Abcam	ab150153	Rat	1:400	
		ab150073	Rabbit	1:400	
		ab150129	Goat	1:400	
		ab150105	Mouse	1:400	
Alexa Fluor 647	Abcam	ab150075	Rabbit	1:400	
		ab150131	Goat	1:400	
		ab150107	Mouse	1:400	
NorthernLight Anti-sheep IgG-NL557	R&D System	NL010	Sheep	1:200	
Alexa Fluor 555	Invitrogen	A31572	Rabbit	1:400	
		A31570	Mouse	1:400	
		A21432	Goat	1:400	

3.9. STATISTICAL ANALYSIS

Fluorescence intensity results are shown as means±standard error of the mean (s.e.m.). Graphs were created and statistical analyses performed using Prism software (GraphPad). For comparative analyses where number of DDR foci or mean intensity was investigated, a nonparametric one-way ANOVA test (because data distribution was negative using Shapiro–Wilk normality test) was applied. * indicates p-value<0.05, ** indicates p-value<0.01, *** indicates p-value<0.001, **** indicates p-value<0.0001, according to GraphPad Prism's statistics.

4. RESULTS

4.1. EXPRESSION OF THE ALS-LINKED FUS-P525L MUTANT PROTEIN INDUCES THE FORMATION OF CI WHICH CO-LOCALIZE WITH MARKER OF STRESS GRANULES

4.1.1. Overexpression of the FUS-p525L, and not overexpression of FUS-WT, causes accumulation of FUS CI

One of the most prominent hallmark of neurodegenerative diseases is the presence of CI containing insoluble proteins (Taylor, Hardy, and Fischbeck 2002). In the ALS/FTLD pathology, mutations in FUS gene in the region encoding for the nuclear localization signal (NLS), result in cytoplasmic de-localization of both the mutated and the wild-type (WT) protein, leading to nuclear loss of function and gain of toxic cytoplasmic function (Lopez-Erauskin et al. 2018). Nevertheless, the molecular mechanisms at the base of cell toxicity induced by the formation of CI have not been fully elucidated yet.

ALS is an aged associated pathology with onset of symptoms at 40-50 years of individuals and formation of CI inclusions has been shown to occur at a presintomatic phase, suggesting that the formation of such structures might be the cause of the disease more than a consequence (Grad et al. 2017). Indeed, none of FUS mutations described in ALS leads to complete impairment of protein function allowing affected individuals to reach adulthood without symptoms (Grad et al. 2017). It has been proposed that increased cytoplasmic concentration of mutant FUS, but not its wild-type (WT) version, is *per se* sufficient to induce cytoplasmic inclusion (Patel et al. 2015). For this reason, transient transfection and overexpression of ALS-linked FUS mutant proteins, in parallel to WT FUS as control, is a widely used experimental approach to acutely induce CI in live cells with the aim of studying their impact on different cellular functions (Farg et al. 2012; Ito et al. 2011; Shelkovnikova et al. 2013; Soo et al. 2015; Vance et al. 2013; Wang, Guo, et al. 2018). Thus, we decided to use the same approach of transient transfection of plasmids expressing WT FUS or mutant FUS in human cell line in parallel. We tested different cell lines suitable for immunofluorescence and prone to intake plasmids by transient transfection such as human U2OS, HeLa and SH-SY5Y and NIH3T3 and we observed that HeLa cells were the one more prone to form CI of mutant FUS. Formation of FUS-positive CI is strongly stimulated by the mutations of Prolin 525 into Leucin falling in the Nuclear Localisation Signal (NLS), a mutation associated with the most severe form of ALS with a juvenile onset (Conte et al. 2012). Thus, to study the impact of CI on genome damage and DDR signalling we focused our research interest on this FUS mutant isoform (P525L). Overexpression of FUS-P525L variant along with FUS WT and empty

vector (EV), as control, induce a more frequent formation of CI (Fig. 4.1.A). By immunofluorescence (IF) analysis, we could visualize FUS CI at single cell level and quantify the percentage of cells that present these structures. Moreover, since we are interested in studying the cellular response to DNA damage in cells with CI we incubate transfected cells with the radiomimetic drug Neocarzinostatin (NCS) which randomly induce DSB (Kuo, Meyn, and Haidle 1984; Galbiati, Beausejour, and d'Adda di Fagagna 2017).

By IF analyses with an antibody against FUS we observed that the overexpression of FUS-P525L variant and not the WT form, gives rises to CI formation in ~ 30% of cells (Fig. 4.1.A). The fraction of cells showing FUS positive CI cells overexpressing FUS WT or transfected with the EV, is instead lower than 5% of cells (Fig. 4.1.B) and could be due to spontaneous events in response to chemical transfection stress. Importantly, FUS-P525L cytoplasmic delocalization occurs independently from DNA damage induction and NCS treatment doesn't significantly affect the amount of cells with FUS inclusions (Fig. 4.1-B). By western blotting analysis we observed that the level of expression of the WT and mutant FUS isoform is very much comparable suggesting that the increased frequency of CI formation observed upon FUS-P525L transfection is not due to different expression level compared to FUS WT expressing cells (Fig. 4.1.C). Of note, by confocal analyses we tested if in a defined plain FUS accumulation in CI might result in its nuclear depletion and we noticed that this is normally not the case (Fig. 4.1.A). This may suggest that the phenotypes observed upon FUS P525L overexpression are not dependent on the loss of his nuclear function. These data are in line with the reported literature (Marrone et al. 2018; Naumann et al. 2018) supporting the notion that the presence of FUS P525L mutant in human cells induces the formation of pathological CI.

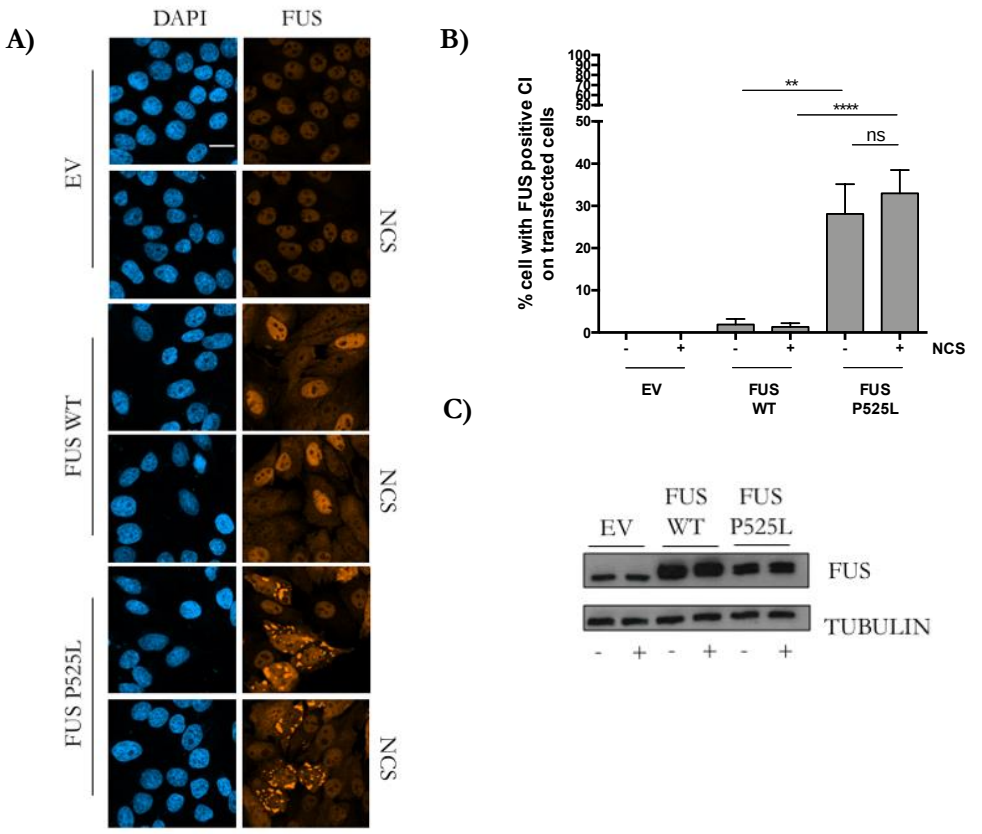


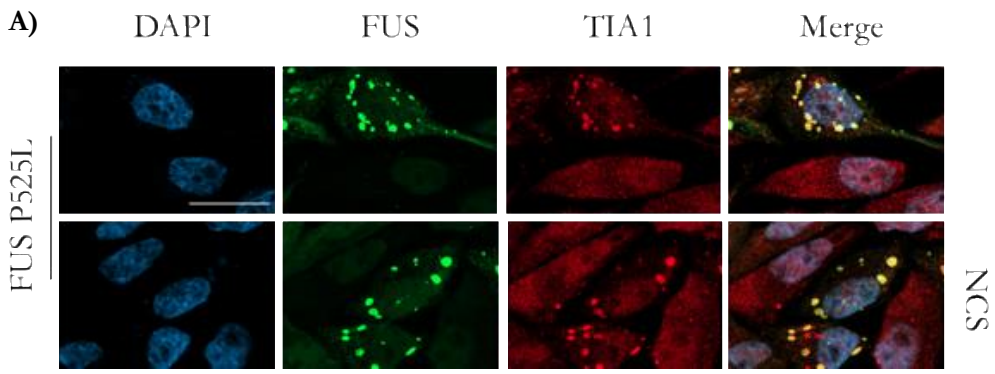
Figure 4.1. Overexpression of FUS P525L variant induces the formation of FUS positive inclusions in HeLa cells. A. Imaging of HeLa cells overexpressing FUS P525L, FUS WT or EV as control. Cells were stained for FUS. Scale bar 20 μm. **B.** Quantification of cells forming FUS positive CI in each indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001. **C.** Western blotting images showing the expression of FUS protein at the indicated condition.

4.1.2. FUS P525L CI co-localize with G3BP and TIA1

One of the better characterized type of cytoplasmic granules that form in response to stress stimuli are stress granules (SGs), which are cytosolic particles belongs to RNA granule family and mainly composed by RNA and protein (Wolozin and Ivanov 2019). They arises upon stress insults including heat shock or sodium arsenite treatment with the main role to protect and prevent mRNAs translation till the stress resolution (Wolozin and Ivanov 2019). Chronic SGs accumulation if cells of ALS patients are considered

hallmarks of the pathology (Dormann et al. 2010). SGs are composed of both constitutive and facultative protein components, and among others, also FUS protein is one of the facultative ones (Aulas and Vande Velde 2015). Different studies attested how the overexpression of ALS-linked mutant proteins is sufficient to induce SGs formation, part of which also incorporate FUS protein (Aulas and Vande Velde 2015). While FUS-WT variant is recruited into less than 10% of TIAR-positive SGs (Bentmann et al. 2012), FUS mutations strongly stimulate protein SGs incorporation (Baron et al. 2013; Dormann et al. 2010). Moreover, immunohistochemistry performed on post-mortem brain and spinal cord tissues from a fALS patient (FUS-R521C) and FLTD reveal that neuronal CI (NCIs) positive for FUS are also positive for stress granule markers (Dormann et al. 2010).

In line of this literature we assessed if FUS positive CIs observed upon FUS-P525L overexpression in our cellular system are also positive to constitutive SGs markers, thus recapitulate some of the feature of inclusions observed in post-mortem patient samples. To this end, cells overexpressing FUS mutant variant were immunostained for two well-known constitutive SG markers like G3BP and TIA1 (Mahboubi and Stochaj 2017). As expected, we confirmed that CI induced by FUS-P525L overexpression, strongly co-localize with both TIA1 and G3BP (Fig. 4.2.A-B). By Manders' coefficient, a tool largely used for this kind of quantification (please see Material and Methods section for more details) we estimated that the co-localization rate between FUS-G3BP and FUS-TIA1 is close to Manders' coefficient of 1 and more than 0.5 respectively, indicating that nearly always FUS CI are also positive to two canonical stress granules markers (Fig. 4.2.C-D). Moreover, the NCS treatment does not impact on the co-localization between FUS and stress granule markers (Fig 4.2.A-B). These evidences indicate that our *in vitro* cellular model system reproduce some of the features of the pathological behaviour of FUS-P525L mutant and mimic what previously observed (Dormann et al. 2010; Kuang et al. 2017).



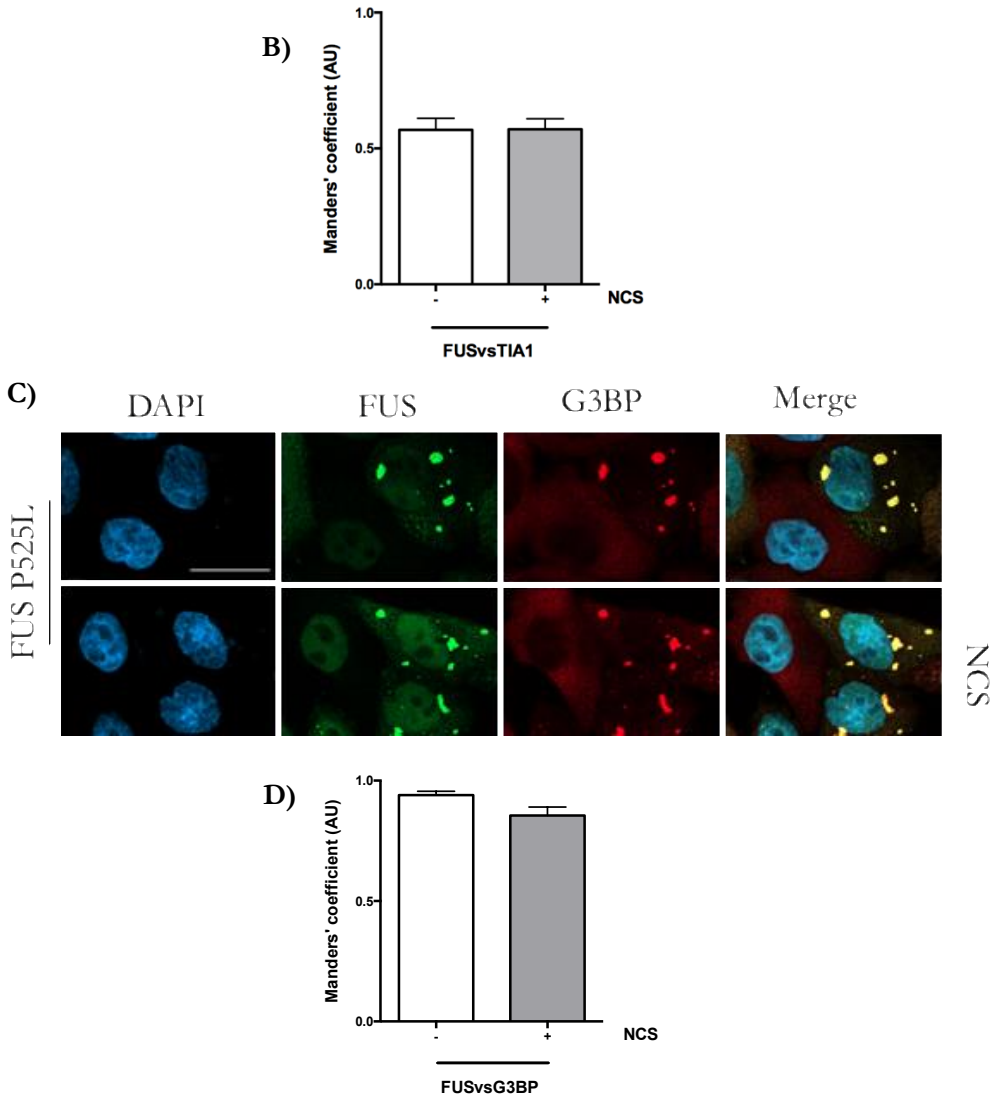


Figure 4.2. FUS P525L-induced CI result positive for SG markers TIA1 and G3BP. A-C. Imaging of HeLa cells overexpressing FUS-P525L were stained for FUS and TIA (A) or for FUS and G3BP (C) antibodies along with co-staining with DAPI. Scale bar 20 μm. **B-D.** Quantification of co-localization between FUS and TIA1 (B) or between FUS and G3BP (D).

4.2. CELLS WITH FUS P525L CI SHOW DNA DAMAGE ACCUMULATION

4.2.1. Cells with FUS P525L CI specifically show γH2AX accumulation, which is dependent on ATM and DNA-PK kinase activity

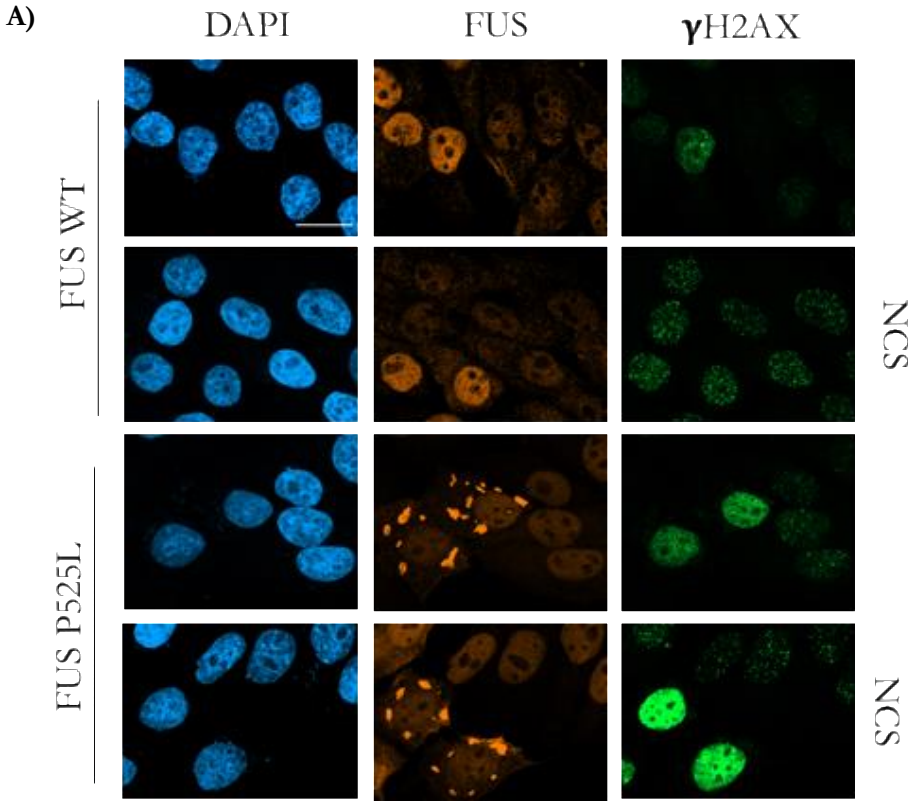
As discussed in the introduction, different studies in literature highlight the detection of DNA damage accumulation in the nucleus of neurons of post-mortem tissues of ALS patients (Kim et al. 2020; Naumann et al. 2018; Wang, Guo, et al. 2018). Importantly, however, it has not been demonstrated yet if DNA damage is an early event causing cell death in neurodegeneration since the molecular mechanism by which DNA damage accumulate in ALS is still unknown. Thus we investigated if we could recapitulate the accumulation of DNA damage associated with the expression of mutant FUS P525L in our *in vitro* system, to characterize how damage is induced.

One of the more upstream event in DDR signalling cascade is the phosphorylation at Ser 139 of the histone variant H2AX (referred as γH2AX) which marks loci of DNA damage and triggers the secondary recruitment of DDR mediator 53BP1 (Celeste et al. 2003). Thus, by staining cells overexpressing FUS-P525L, or FUS-WT as a control, with a specific antibody against the DNA damage marker γH2AX we initially tested if cells expressing FUS-P525L and forming CI, present DNA damage in the form of DSB and/or SSB. Interestingly, the fraction of cells positive for mutant FUS CI presented a very peculiar and strong γH2AX signal (assimilated as pan-nuclear staining) compared to cells expressing WT-FUS or cells expressing mutant FUS but negative for CI (Fig. 4.3.A). The massive accumulation of γH2AX occurs even in the absence of exogenously inflicted DNA damage by treatment with radiomimetic compounds or X-ray, a fact that suggests that the formation of mutant FUS CI induce *per se* a strong genotoxic stress (Fig. 4.3.A-B). Importantly, FUS-P525L overexpression in the absence of CI, do not induce genotoxicity since cells that do not form mutant FUS CI, are negative for γH2AX pan nuclear signal (Fig. 4.3.A-B).

We then moved to characterize the response to exogenously provided DNA damage in cells experiencing FUS CI. To this end the day after plasmid transfection, cells where treated with the radiomimetic drug Neocarzinostatin (NCS), which has been largely used in our laboratory and others to induce DNA damage and study the activation of DNA damage response by immunofluorescence (Francia et al. 2016; Galbiati, Beausejour, and d'Adda di Fagagna 2017; Kang et al. 2012; Kato et al. 2014; Kawale et al. 2018). As expected, upon NCS treatment cells devoid of mutant FUS CI, properly mounted canonical γH2AX-positive DDR foci (Fig. 4.3.A-B). Instead, the strong accumulation of

γH2AX pan-nuclear signal of cells with mutant FUS positive CI as previously described, prevented us from distinguishing discrete γH2AX-positive foci (Fig. 4.3.B). Besides, cells expressing FUS-WT variant show a physiological γH2AX localization at DDR foci (Fig. 4.3.A-B).

These results indicate that expression of FUS-P525L mutant protein leads to its incorporation into CI in a considerable fraction of cells and leads to gain a toxic function thus threat genome integrity.



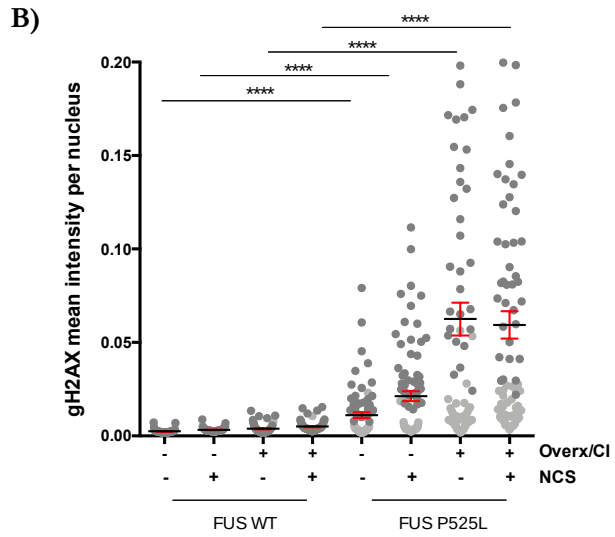
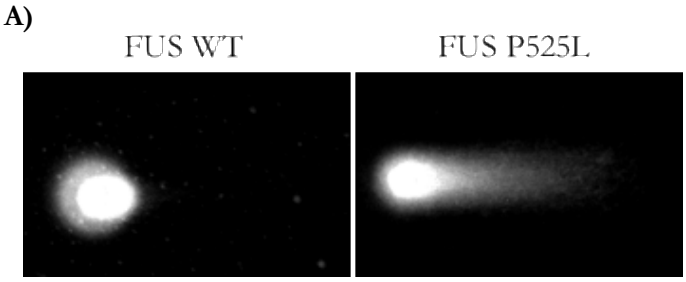


Figure 4.3. FUS P525L-induced CI show accumulation of γH2AX nuclear signal. A,B. Imaging of HeLa cells overexpressing FUS-P525L were stained for FUS and γH2AX antibodies along with co-staining with DAPI. Scale bar 20 μm. **B.** Quantification of nuclear γH2AX intensity measured in cells with and without FUS inclusions in each indicated conditions. Error bars represent SEM calculated among the population. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Different cellular events could lead to γH2AX formation thus we wonder to assess if the strong γH2AX accumulation that we observe in cells with FUS positive CI is due to the accumulation of physical DNA damage. To this end we performed the comet assay under neutral condition in order to detect DSBs. Interestingly we found that the expression of FUS mutant increases the tail moment compared to WT form (Fig. 4.4.A-B) in a certain fraction that reflects the cells with FUS positive CI. This result suggests that the high γH2AX signal that we observe in the nucleus of cells with FUS positive CI is due to the accumulation of a high amount of physical DSBs.



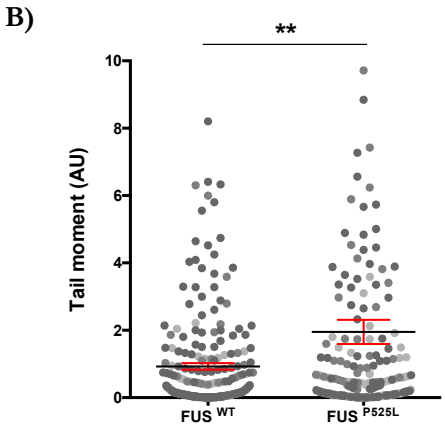


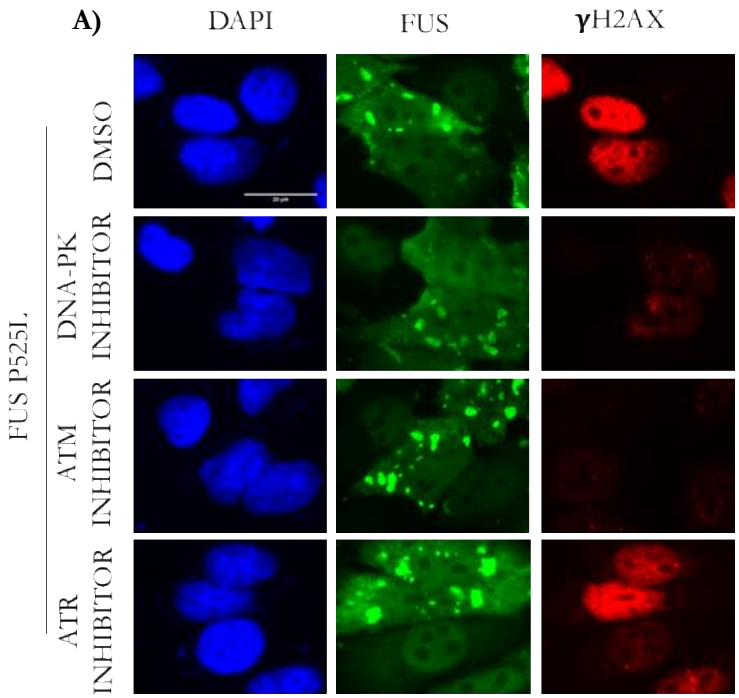
Figure 4.4. FUS P525L mutation affects DNA repair efficiency. A. Representative images of neutral comet assay performed in HeLa cells transfected with FUS WT and P525L. **B** Quantification of DNA damage by tail moment analysis. Red bars indicate the average values ± 95%CI from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

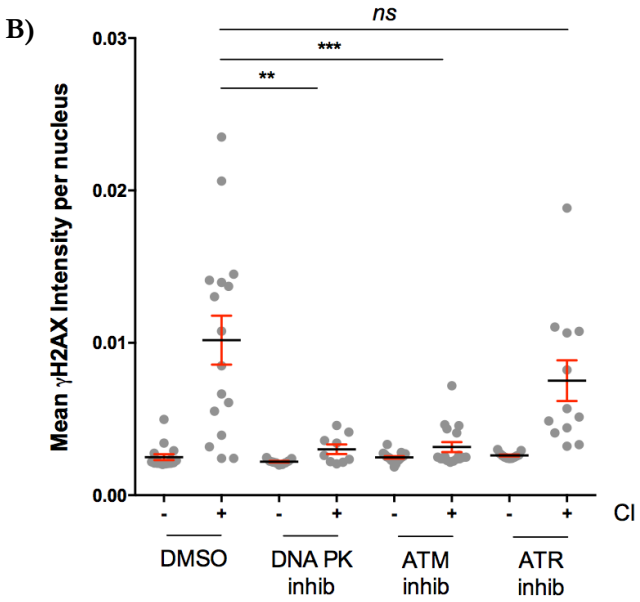
DNA-PK and ATM are the two main kinases recruited at DNA DSB, and a recent study showed that pan-nuclear γH2AX accumulates in response to their activation in different context of chromatin structure alteration (Meyer et al. 2013). Instead, ATR kinase activation has been mainly associated with γH2AX pan nuclear signal in the context of replication stress (Moeglin et al. 2019; Ruiz et al. 2015). Thus, we wondered which of these kinases are responsible for the γH2AX accumulation observed in cells with FUS positive CI. To this end, cells overexpressing FUS-P525L were treated with DNA-PKcs or ATM or ATR inhibitors (KU60019, NU7441 and VE-821 respectively) separately for the last 3h of 24h FUS P525L transfection and the accumulation of γH2AX was then evaluated by IF (Fig. 4.5.A). Intriguingly, cells with FUS positive CI show a γH2AX signal strongly reduced upon treatment with ATM and DNA-PK inhibitor, if compared with cells treated with the solvent DMSO, used as control condition (Fig. 4.5.A-B). Instead, cells treated with ATR inhibitor still show high levels of γH2AX in the presence of FUS CI (Fig. 4.5.A-B). We reasoned that ATR could have phosphorylated H2AX during the previous replication cycle thus before the addition of ATR inhibitor. For this reason, we attempted the same experiment extending for 20h the treatment with the DDR kinases inhibitors together with FUS P525L transfection (Fig. 4.5.C-D). The results obtained were consistent with the previous ones and confirm that ATM and DNA-PK but not ATR, are responsible for H2AX phosphorylation in cells experiencing mutant FUS CI.

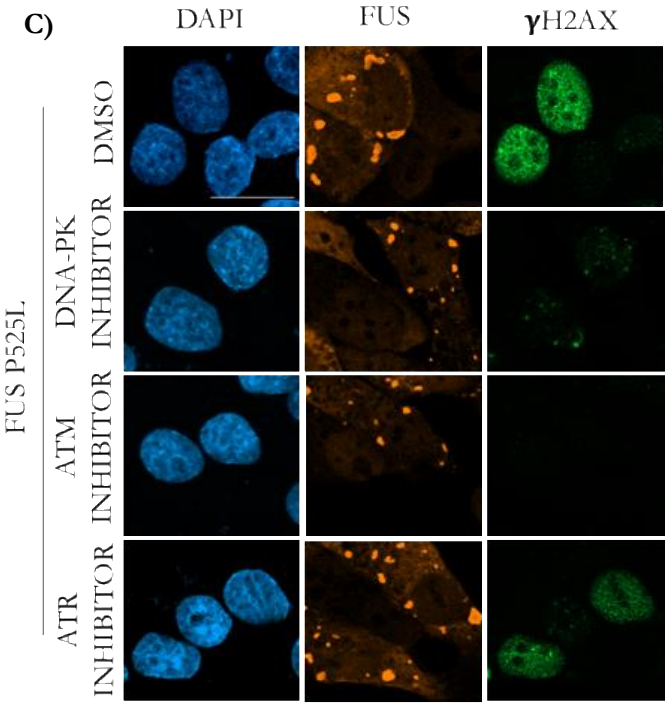
Moreover by western blot we verified the inhibition of the kinases was correctly performed by evaluating the levels of phosphorylated forms of each kinase in basal condition and upon DNA damage induction by IR (2Gy) (Fig. 4.5.E). These data importantly indicate that the γH2AX pan nuclear signal typical of cells with FUS positive CI is due to the activation of both DNA-PK and ATM kinases, rather than ATR. We also observed that the inhibition of ATR phosphorylation also occurs upon DNA PK and ATM inhibition (FIG 4.5.E). In this regards it has been reported that upon DSB formation ATR activation is ATM dependent (Cuadrado, Martinez-Pastor, and Fernandez-Capetillo 2006). Besides we should take in account that the treatment with inhibitors only acts by blocking proteins activity while the proteins still be present locally thus it could be possible that nearby kinases can still work instead of the inactive ones.

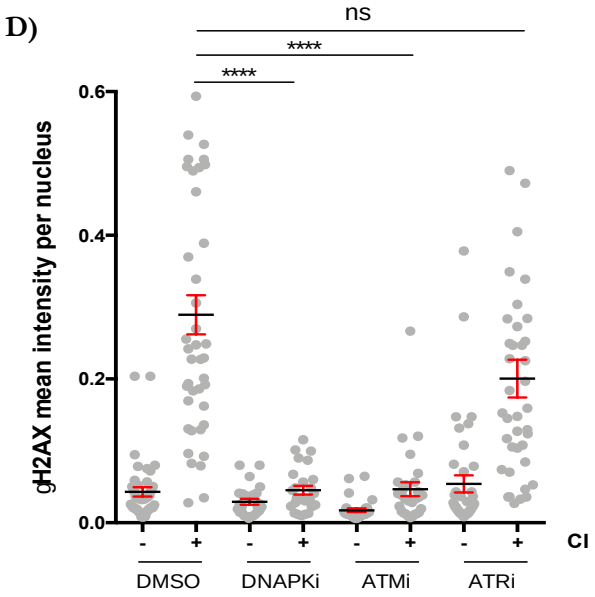
This data may suggest that high DNA damage signal in these cells is due to the presence of DSBs in the genome of these cells and not to replication stress events or single strand DNA accumulation.

Taking in account that both DNA-PK and ATM are able to phosphorylate FUS protein and the reported scattered evidences in the literature suggesting that FUS phosphorylation by DDR kinases might modulate its aggregation propensity (Gardiner et al. 2008; Monahan et al. 2017) we assessed if the treatment with inhibitor of ATM and DNA-PK kinase activity could affect the frequency of FUS CI in the cell population, thus explaining the reduction in γH2AX signal intensity. To this aim, we counted the percentage of cells with mutant FUS CI, in cells treated with ATM or DNAPK or ATR inhibitor, or DMSO as control condition, in presence or not of exogenously provided DNA damage (induced by treatment with NCS). The resulting quantification, shown in figure 4C, indicate that nor DNA-PK or ATM as well as ATR inhibition interfere with the frequency of cells positive for mutant FUS CI, since cells expressing FUS-P525L show the same frequency of CI upon all treatments (Fig. 4.5.F)

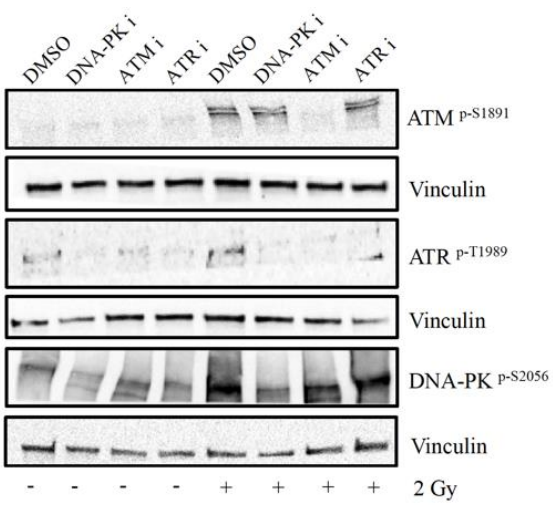

A)
DAPI
FUS
γH2AX
FUS P525L
DMSO
DNA-PK INHIBITOR
ATM INHIBITOR
ATR INHIBITOR


B)
ns
***
**
Mean γH2AX Intensity per nucleus
0.03
0.02
0.01
0.00
- + - + - + - +
CI
DMSO
DNA PK inhib
ATM inhib
ATR inhib


C)
DAPI
FUS
γH2AX
FUS P525L
DMSO
DNA-PK INHIBITOR
ATM INHIBITOR
ATR INHIBITOR


D)
ns
****
****
γH2AX mean intensity per nucleus
0.6
0.4
0.2
0.0
- + - + - + - +
CI
DMSO
DNAPKi
ATMi
ATRi

E)


DMSO
DNA-PK i
ATM i
ATR i
DMSO
DNA-PK i
ATM i
ATR i
ATM p-S1891
Vinculin
ATR p-T1989
Vinculin
DNA-PK p-S2056
Vinculin
- - - - + + + + 2 Gy

F)

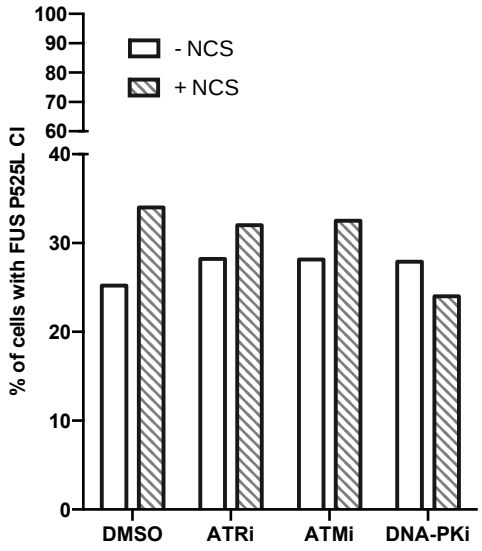
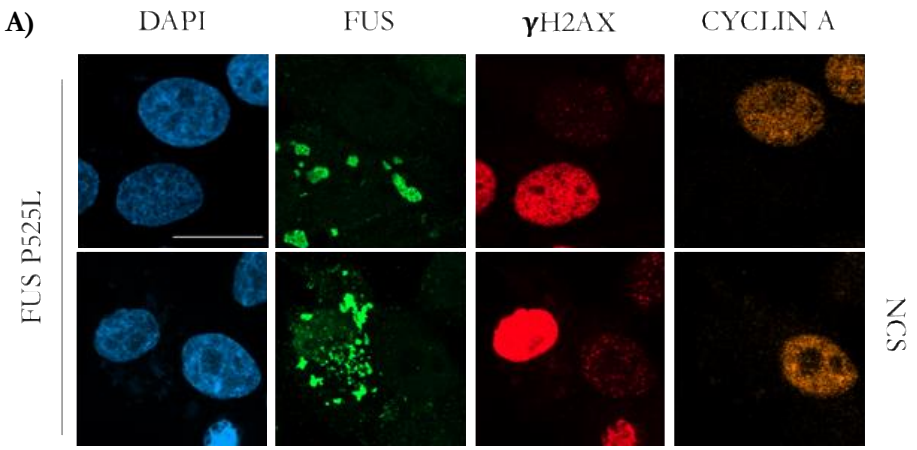
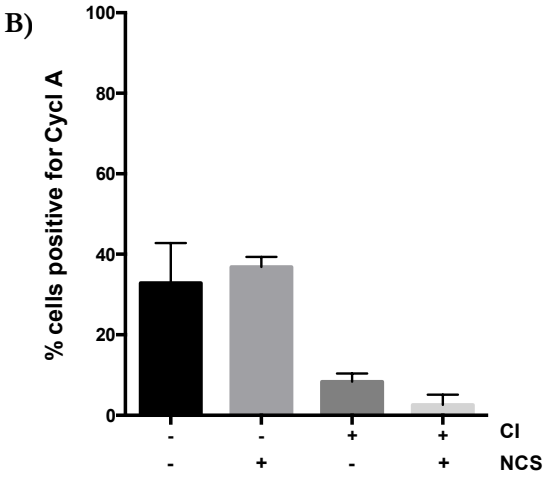

100
90
80
70
60
40
30
20
10
0
- NCS
+ NCS
% of cells with FUS P525L CI
DMSO
ATRi
ATMi
DNA-PKi

Figure 4.5. Pan-nuclear γH2AX in cells with FUS positive inclusions is dependent on DNA-PK and ATM kinases activation. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained to detect FUS and γH2AX after treatment with DNA-PK, ATM and ATR inhibitors or with DMSO as control (3h treatment). Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of nuclear γH2AX intensity measured in cells with and without FUS inclusions in each indicated conditions. Error bars represent SEM calculated among the population. P-value ≤ 0.05. **C.** Imaging of HeLa cells overexpressing FUS-P525L and immunostained to detect FUS and γH2AX after treatment with DNA-PK, ATM and ATR inhibitors or with DMSO as control (20h treatment). Nuclei were counterstained with DAPI Scale bar 20μm. **D.** Quantification of nuclear γH2AX intensity measured in cells with and without FUS inclusions in each indicated conditions. Error bars represent SEM calculated among the population. P-value ≤ 0.05 **E.** Western blot showing the phosphorylated forms of the indicated kinases upon inhibition in both undamaged and damaged condition. **F.** Quantification of the FUS CI (calculated on the whole population) at indicated conditions. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.2.2. Pan-nuclear γH2AX in cells with mutant FUS CI is nor not associated with possible DNA replication neither apoptosis

Previous evidences indicated that pan-nuclear γH2AX marks a high fraction of cells in S-phase upon UV-irradiation (de Feraudy et al. 2010) and other studies suggest it is associated with problems during DNA replication (Moeglin et al. 2019). Thus, even though we observed that ATR activation, normally occurring during replication stress, is not sufficient to explain γH2AX accumulation, we investigated if the γH2AX enrichment observed in cells with mutant FUS CI may be associated with cells in S-phase. To this end, we stained cells expressing mutant FUS for Cyclin A, which is the cyclin expressed in S and G2 phase of the cell cycle (Henglein et al. 1994; Pagano et al. 1992). Contrary to the expectation, upon FUS-P525L overexpression all the cells harbouring FUS positive CI, have very low or null levels of Cyclin A (Fig. 4.6-A) suggesting that such cells are preferentially in G1 phase and are not replicating. Differently, cells devoid of pan-nuclear γH2AX showed the expected Cyclin A positivity with various intensity levels (Fig. 4.6A-B) thus reflecting the different levels of its expression in the various phases of the cell cycle. Of note, mutually exclusion between γH2AX and Cyclin A signals occurs with identical frequency both in damaged and undamaged conditions, indicating that the cell cycle arrest in cells with FUS positive CI occurs before exposure to DNA damage (Fig. 4.6A-B). Thus, the formation of FUS positive CI causes a G1/S cell cycle arrest and this is in line with the notion that pan-nuclear γH2AX could reflect the accumulation of physical DNA damages like DSBs and SSB that in turn may induces cell cycle arrest due to DNA damage checkpoints activation (Branzei and Foiani 2008; Jackson and Bartek 2009).


A)
DAPI
FUS
γH2AX
CYCLIN A
FUS P525L
SCN


B)
% cells positive for Cycl A
100
80
60
40
20
0
- - + +
CI
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NCS

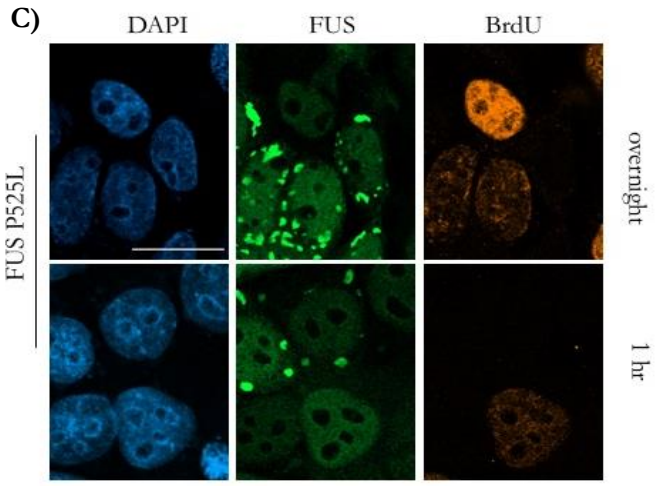
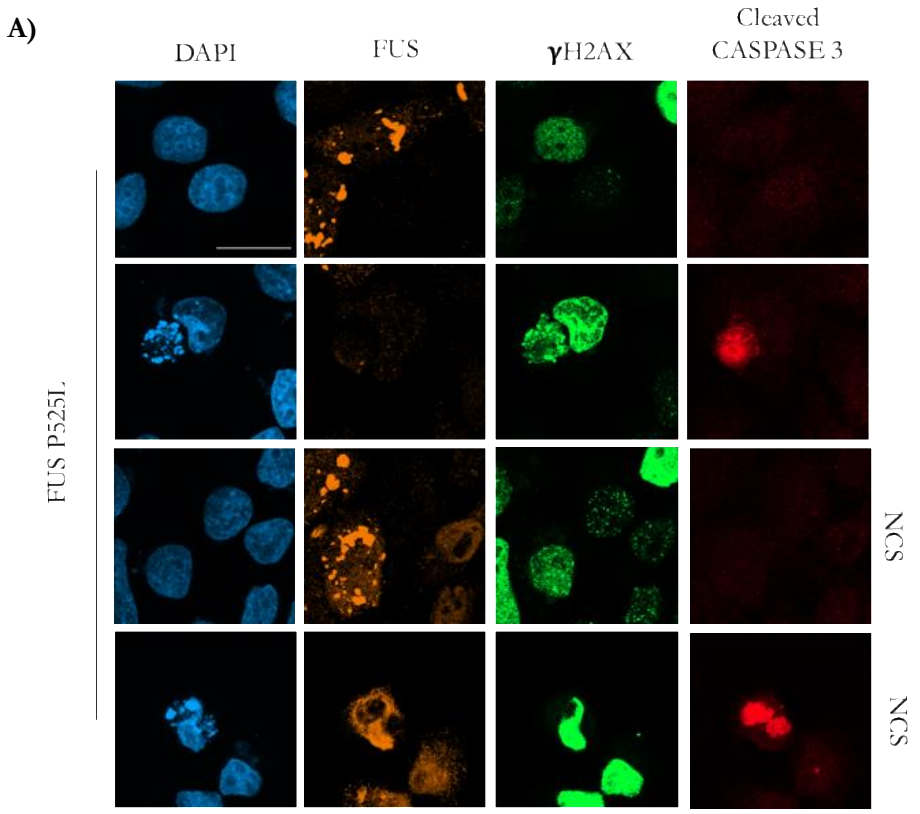


Figure 4.6. Pan-nuclear γH2AX in cells with FUS positive inclusions is not associated with replication stress events. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained to detect FUS and Cyclin A at indicated conditions. Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of cells showing Cyclin A positivity measured in cells with and without FUS inclusions in each indicated conditions. Error bars represent SEM calculated from two independent experiments. **C.** Imaging of HeLa cells overexpressing FUS-P525L and immunostained to detect FUS and BrdU at indicated conditions. Nuclei were counterstained with DAPI. Scale bar 20μm.

To further strengthen this conclusions, we incubated cells expressing FUS-P525L mutant with BrdU for 1 hour or overnight (o/n) and, again we observed that cells with FUS positive CI have reduced capacity to incorporate BrdU compared to cells devoid of CI (Fig. 4.6.C) thus suggesting that those cells replicate significantly less.

Another proposed mechanism for motor neurons degeneration in ALS is the activation of the apoptosis program (Martin 1999; Sathasivam, Ince, and Shaw 2001). Noteworthy, phosphorylation on Ser 139 of γH2AX with a pan nuclear distribution is reminiscent of the induction of chromosomes fragmentation during the apoptotic process (Rogakou et al. 2000) and mark nuclei of cells undergoing intermediate steps of apoptosis (Solier and Pommier 2014). Thus, we stained cells with FUS positive CI for a well-known apoptotic marker like cleaved caspase-3 (Wolf et al. 1999). Unexpectedly, instead upon FUS-P525L overexpression cells with CI and pan-nuclear γH2AX signal result negative for cleaved caspase-3 apoptotic marker (Fig. 4.7.A), while apoptotic bodies randomly present in the cell population even in control conditions, were positive for both cleaved caspase-3 and γH2AX (Fig. 4.7.A-B). We calculated the percentage of cells showing cleaved caspase 3

signal in our images as shown in figure (Fig. 4.7.B). Since to collect these images we actively search for the nuclei with apoptotic-like conformation to be able to confirm the specificity of the signal, we believed that our calculation strongly overestimate the percentage of spontaneous apoptotic events. Nevertheless, we can clearly appreciate that cells with FUS CI do not have the same apoptotic-like shape and indeed are negative for cleaved caspase 3 marker (Fig. 4.7.B). Overall, these results suggest that cells enriched in nuclear γH2AX, associated with mutant FUS CI, are not under replication stress and are not undergoing apoptotic mediated cell death. However it should be mentioned that HeLa cells might be more resistant to apoptosis respect to other primary cell types.



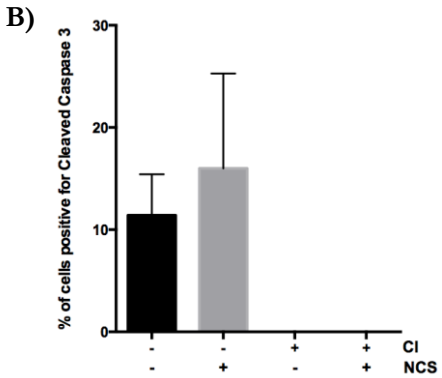
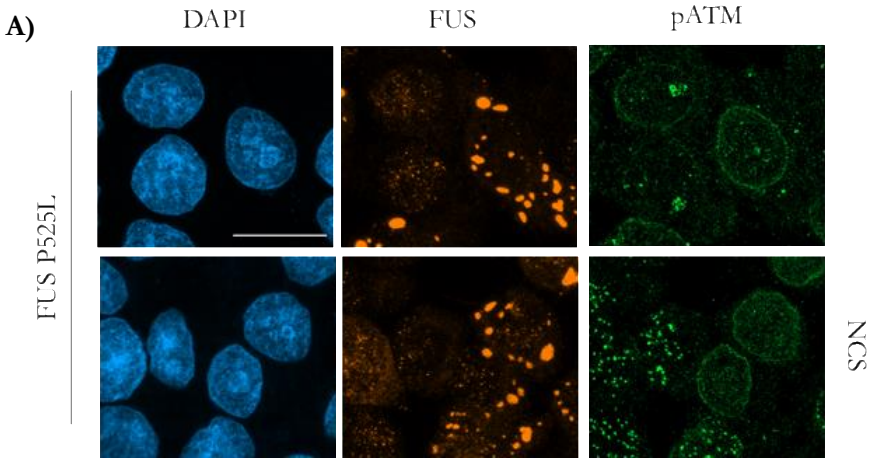


Figure 4.7. Pan-nuclear γH2AX in cells with FUS positive inclusions is not associated with apoptotic events. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained to detect FUS, γH2AX and Cleaved Caspase 3. Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of Cleaved Caspase 3 signal distinguishing between cells with and without CI at indicated conditions. Error bars represent SEM calculated among the population.

4.3. CELLS WITH MUTANT FUS CI SHOW INCREASED DIFFUSE PATM SIGNAL BUT LOSS OF PATM FOCI AND AS A CONSEQUENCE, PHOSPHORYLATION OF ITS DOWNSTREAM TARGETS

As described above, we established that ATM and DNA-PK kinase activity are responsible for H2AX phosphorylation in cells with mutant FUS CI, thus we tested if also ATM was strongly activated in the same cells in the absence of exogenously inflicted DNA damage by radiomimetic treatments. Thus we investigated the activation of ATM in those cells. To this end, we stained cells expressing FUS-P525L mutant with an antibody that recognizes ATM auto-phosphorylation at Ser 19181 (pATM), a widely used approach to study ATM activation. Interestingly, pATM diffused signal is increased in cells with mutant FUS CI if compared to the surrounding cells that lack CI even already at basal condition (Fig. 4.8.A-B). This result indicates that the presence of strong γH2AX signal in cells with mutant FUS CI indeed correlate with a basal induction of ATM activation (Fig. 4.8.A-B). Nevertheless, a diffused activation for ATM is reminiscent with what we detected in the past upon dilncRNA transcriptional inhibition (Michelini et al. 2017), which correspond to a dysfunctional ATM activation unable to localize to site of damage and efficiently drive local DNA repair. Thus we tested the ability of these cells to respond to acute DSB formation by treatment with NCS. Interestingly, upon NCS treatment, cells with mutant FUS CI lack discrete pATM foci, still exhibiting a homogeneous and diffused nuclear ATM activation (Fig. 4.8.A-C). On the contrary, adjacent cells without mutant FUS CI display distinct and bright pATM foci as expected

for cells exposed to DSB (Fig. 4.8.A-C). Overall these results indicate that the ATM kinase is chronically active in cells experiencing mutant FUS CI, nevertheless, this activation is dysfunctional since all cells are defective in mounting proper pATM foci. This might suggest that, cells with mutant FUS CI lose the ability to properly respond to DNA damage induction, thus causing induce DNA damage accumulation. We noticed that pATM show a peculiar peri-nuclear distribution accumulated at nuclear membrane. We reasoned that ATM protein is a PI3-like kinase thus structurally very similar to a transmembrane kinase. Moreover, the closely related ATR protein (which also belongs to PI3 family kinases) has been demonstrated to be a nuclear transmembrane protein able to activate upon mechanical stress (Kumar et al. 2014). In this regard, we can speculate that FUS CI could indeed cause mechanical stress thus leading to ATM activation and accumulation at the level of nuclear membrane..



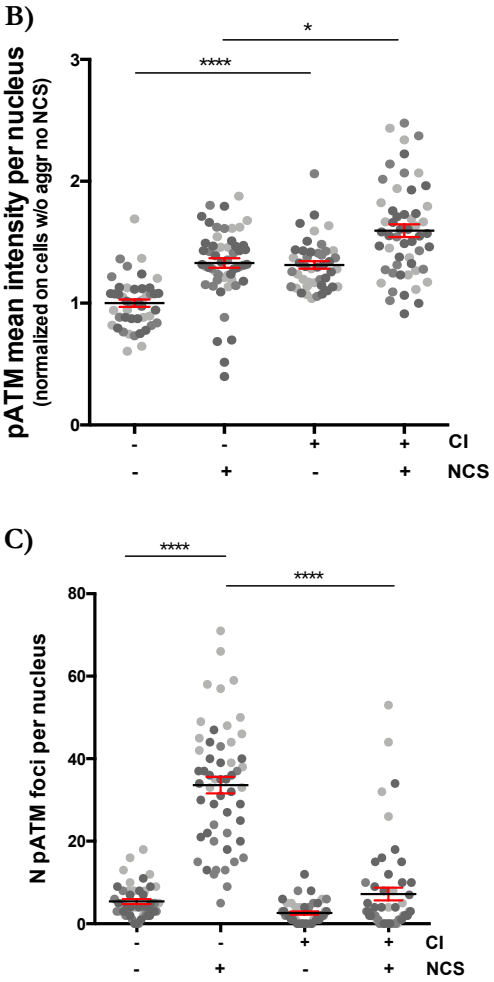


Figure 4.8. FUS positive CI leads to wide-nuclear pATM activation and impairs pATM foci formation upon DNA damage induction. A. Imaging of HeLa cells overexpressing FUS-P525L in basal condition or upon DNA damage induction by NCS. Cells were stained with FUS and pATM antibody. Nuclei were counterstained with DAPI Scale bar 20µm. **B-C.** Quantification of pATM mean intensity (B), and count of pATM foci per nucleus (C) measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

ATM activation through its auto-phosphorylation is essential for the phosphorylation of downstream factors other than H2AX, such as for example the downstream kinases CHK2, in order to transduce the DDR signalling cascade to effector proteins (Jackson

and Bartek 2009). To better characterize if ATM basal activation observed in cells with mutant FUS CI was indeed dysfunctional, and unable to transduce the signal downstream, we tested the phosphorylation of its targets upon exposed to NCS. Upon DNA damage the downstream kinase CHK2 is actively phosphorylated on Threonine 68 by ATM (Zannini, Delia, and Buscemi 2014) thus we used an antibody detecting this phosphorylation in our cellular system. Importantly, upon NCS treatment cells with mutant FUS CI show reduced pCHK2 nuclear signal compared to the surrounded cells suggesting that diffused ATM activation is defective in transducing the signal downstream (Fig. 4.9.A-B).

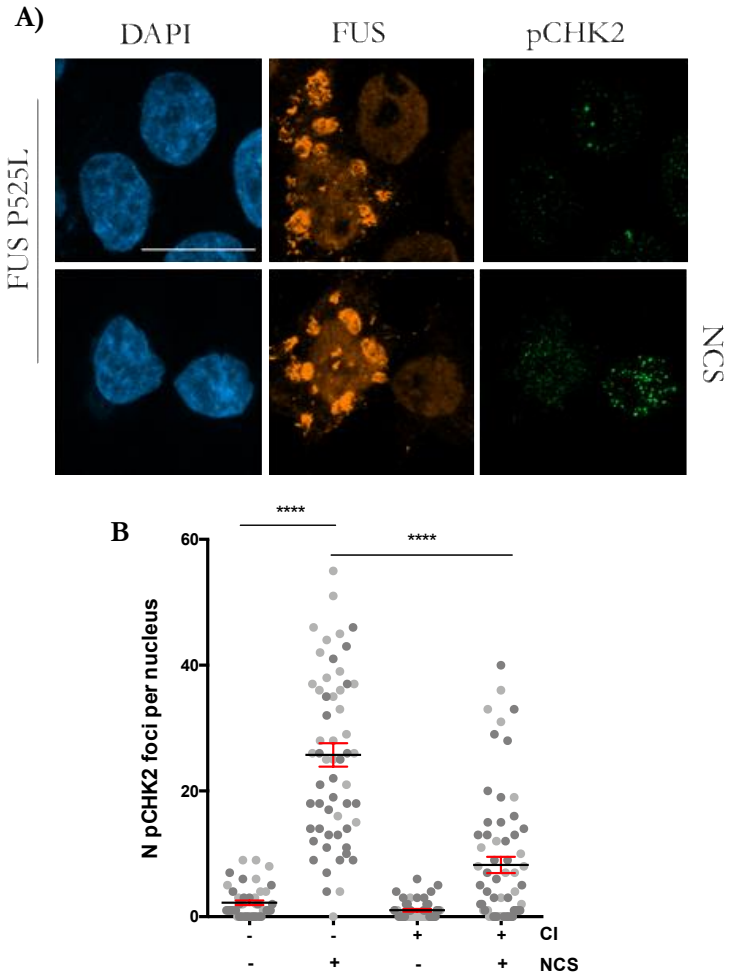
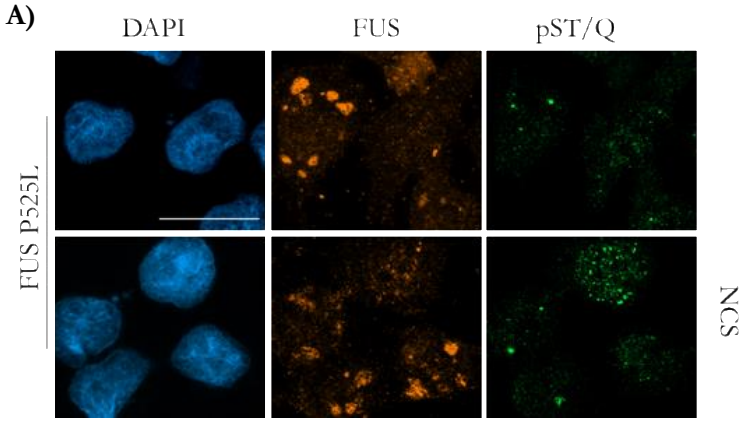


Figure 4.9. The phosphorylation of the ATM downstream target CHK2 is affected in cells with FUS positive CI. A. Imaging of HeLa cells overexpressing FUS-P525L in basal condition or upon DNA damage induction by NCS. Cells were stained with FUS and pCHK2 antibody. Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of pCHK2 foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

ATM has a consensus site for its phosphorylation which is Ser/Thr preceded by Leu or similar hydrophobic aminoacid at the -1 position and followed by Gln at the +1 position (SQ or TQ). A useful antibody has been developed to detect the ATM dependent phosphorylation of these consensus site on different ATM target. Therefore, we also tested cells experiencing mutant FUS CI with this antibody which gives us the chance to visualize several different ATM targets by IF. With this analyses could clearly appreciate that cells with mutant FUS CI exhibit a strong reduction in the number of pST/Q foci per nucleus upon DNA damage (Fig. 4.10.A-B). These data suggest that the primary ATM activation at site of endogenously generated DNA damage in cells experiencing mutant FUS CI is not followed by proper ATM signal transduction thus likely negatively affecting the subsequent DDR signalling cascade and DNA repair.



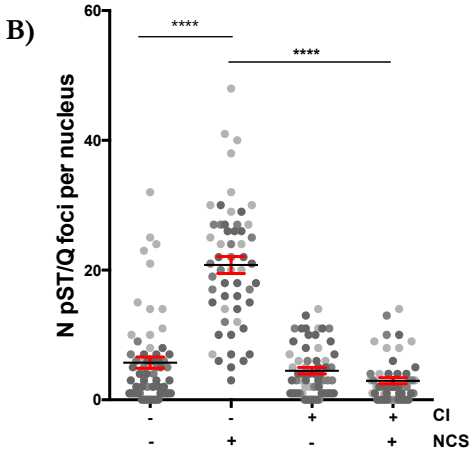
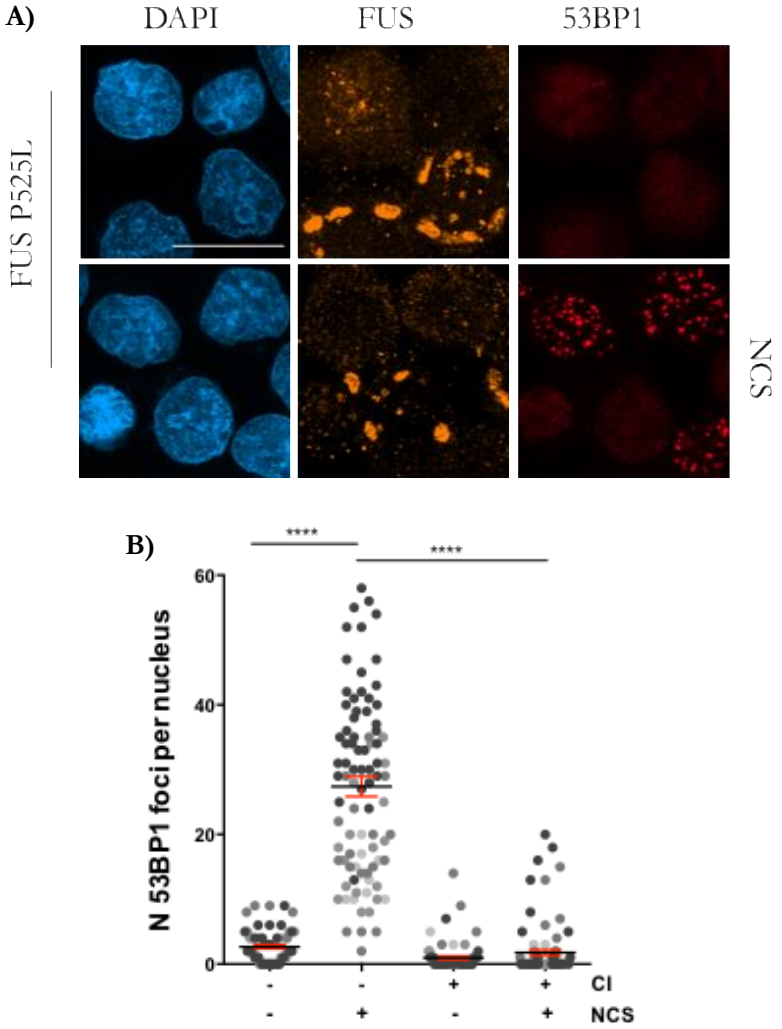


Figure 4.10. The ATM signaling is de-regulated in cells with FUS inclusions A. Imaging of HeLa cells overexpressing FUS-P525L in basal condition or upon DNA damage induction by NCS. Cells were stained with FUS and pS/TQ antibody. Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of pCHK2 foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.4. MUTANT FUS CI NEGATIVELY IMPACTS ON 53BP1 RECRUITMENT AND PHOSPHORYLATION AT DSB BUT NOT ON MDC1

One of the main mediators of the DDR cascade, involved in the NHEJ pathway of DNA repair is 53BP1, a factor that by sustaining protein-protein interactions amplifies the DDR signalling and is widely study for the good tools that by immunofluorescence allow the detection of big a defined DDR foci positive for this marker. Moreover, several S/T-Q motifs have been identified in N-terminal region of 53BP1 and some of these residues have been described to be ATM target (Jowsey et al. 2007). Particularly, the Ser1778 within the BRCT domain of 53BP1 is actively phosphorylated upon NCS treatment and plays a crucial role in DDR repair pathway (Lee et al. 2009). Thus we investigate if the recruitment of 53BP1 is defective in cells with mutant FUS CI upon NCS treatment. While cells without CI clearly and efficiently mount 53BP1 foci, cells with mutant FUS CI are totally devoid of 53BP1 foci (Fig. 4.11.A-B) strengthening our model that DDR signalling is strongly defective in these cells. In addition, when we stained NCS treated cells with mutant FUS CI with an antibody able to recognize phosphorylated form of

53BP1 at Ser1778 (p53BP1) we observed that also this tool confirms that 53BP1 phosphorylation at DDR foci is strongly reduced or totally absent (Fig. 4.11.C-D).



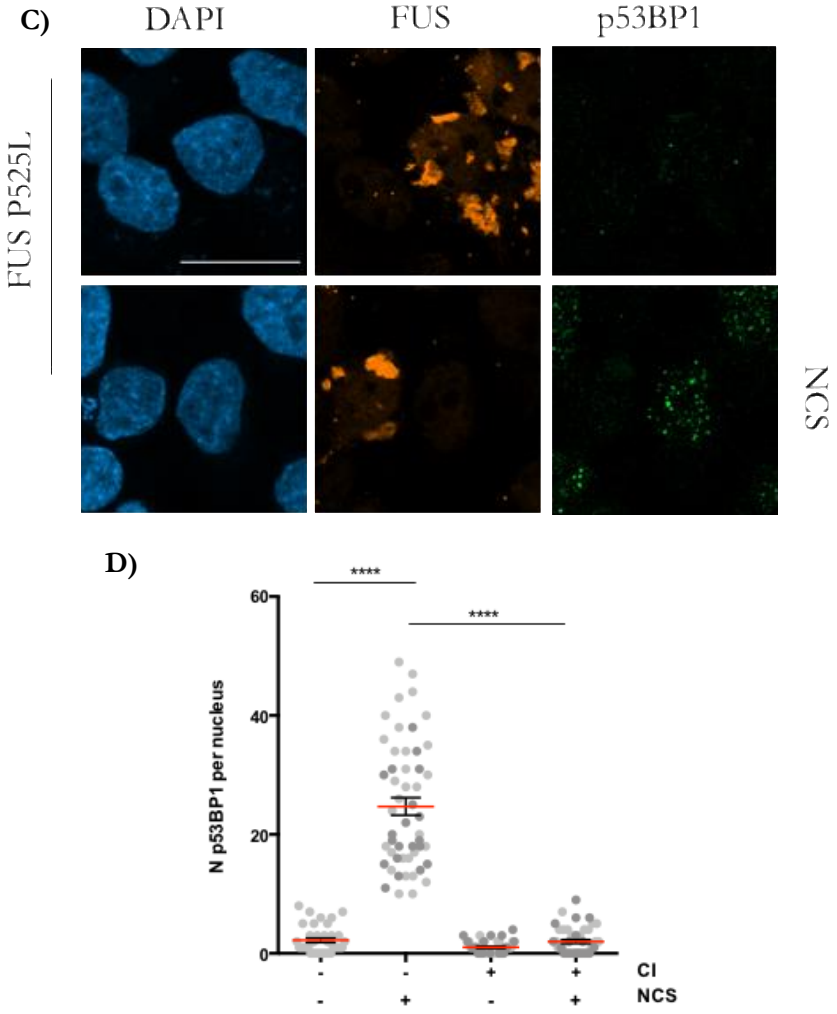


Figure 4.11. Cells harbouring FUS positive CI exhibit loss of both 53BP1 and p53BP1 foci upon DNA damage induction. A-C. Imaging of HeLa cells overexpressing FUS-P525L and immunostained for FUS and 53BP1 (A) or for FUS and p53BP1 (C) in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20μm. **B-D.** Quantification of 53BP1 (B)and p53BP1 (D) foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

All together, these evidences suggest that mutant FUS CI caused genotoxic stress condition and also interfere with the nuclear response to DNA damage impeding the 53BP1-DDR foci formation.

As described in the introductive section, the other mediator and scaffold protein in the response to DSB is MDC1. Importantly, MDC1 recruitment to site of damage is γH2AX dependent but upstream to pATM foci formation (Lou et al. 2006) and differently from 53BP1, primarily functions in homologous recombination, a repair pathway typical of replicating cells (Xie et al. 2007). Therefore, we stained cells expressing FUS-P525L mutant and exposed to NCS with an antibody against MDC1 and, differently from what we detected for 53BP1 we observed that MDC1 recruitment is not affected in cells with mutant FUS CI (Fig. 4.12.A-B).

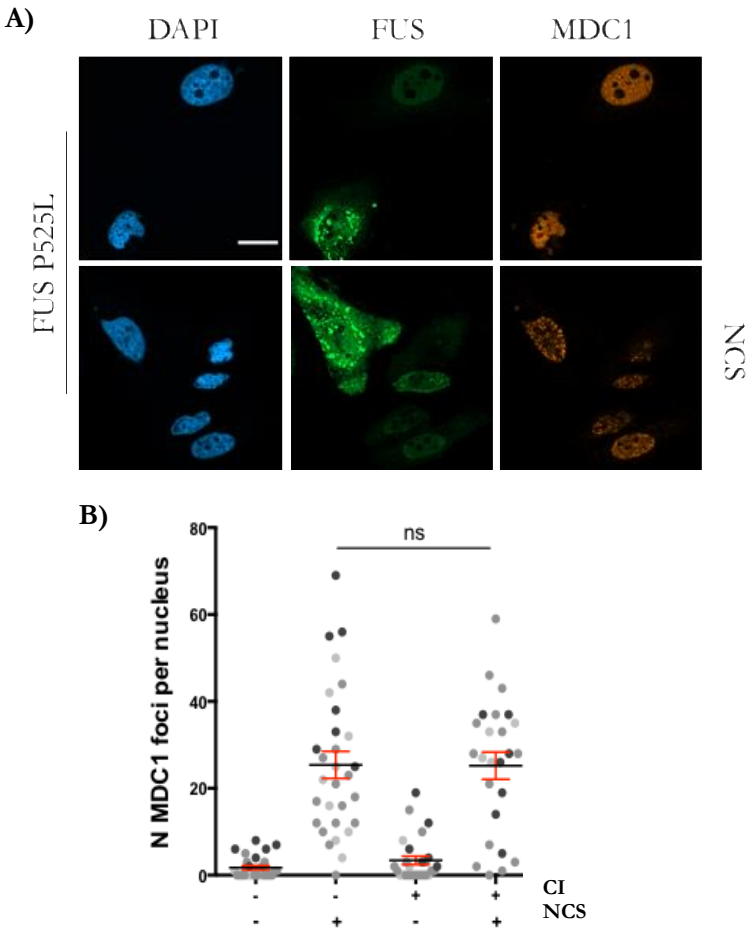


Figure 4.12. FUS CI do not affect MDC1 recruitment at site of damage. A. Imaging of HeLa cells expressing FUS-P525L immunostained for FUS and MDC1 untreated or treated with NCS in both undamaged and damaged condition**.** Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of MDC1 foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition.. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P value ≤ 0.05, ** P value ≤ 0.01, *** P value ≤ 0.001 ≤ 0.05.

These evidences indicate that the two DDR mediator proteins 53BP1 and MDC1 behave differently in cells bearing mutant FUS CI since the profound defect of 53BP1 foci formation is not reflected in a defect of formation of MDC1 foci. This further strengthen the relevance of our observation in the context of defining a defect of these cells in the activation of NHEJ DNA repair

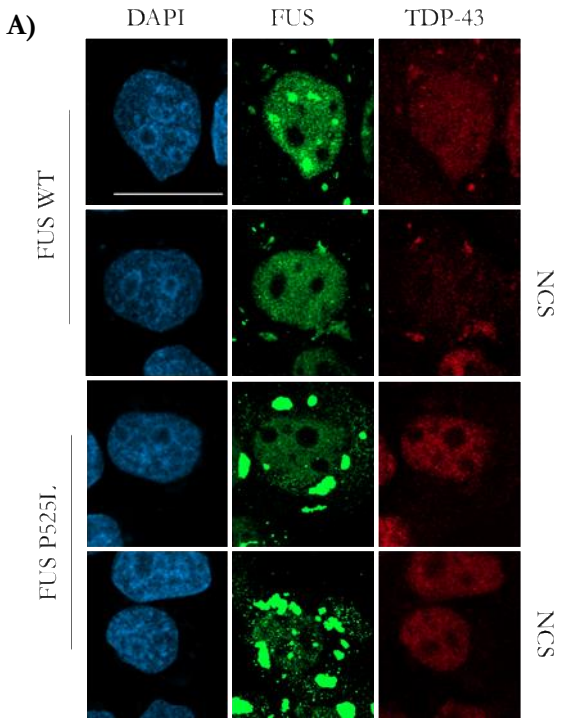
4.5. TDP43 DEPLETION DOESN'T AFFECT FUS P525L PHENOTYPE

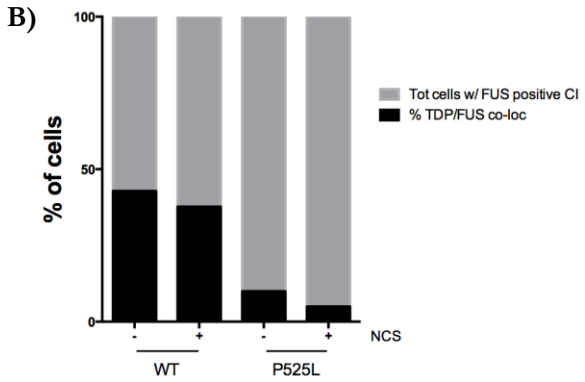
As widely reported, both TDP-43 and FUS are recruited into SGs as facultative components (Aulas and Vande Velde 2015). Although ALS-linked mutations in FUS promote its SGs localization, endogenous FUS is not required for SGs assembly (Aulas, Stabile, and Vande Velde 2012). Endogenous TDP-43 actively promotes SGs assembly, but not initiation, via G3BP1 binding and the number of SGs per cell is significantly reduced upon TDP43 knockdown (Aulas, Stabile, and Vande Velde 2012). Moreover TDP43 incorporation is known to stimulate the progression of liquid SG into a more solid fibrillar structure, believed to be toxic (Ratti et al. 2020) . Differently, FUS depletion does not affect SGs stability and both G3BP1 and TIA-1 protein levels remain unchanged upon FUS inactivation by siRNA (Aulas, Stabile, and Vande Velde 2012). In addition, FUS depletion does not reduce the expression of endogenous TDP-43 nor does it affects TDP-43 SGs localization (Aulas, Stabile, and Vande Velde 2012). In this regards, FUS and TDP-43 have been recently described as able to co-aggregates (Watanabe et al. 2020), however if these two proteins colocalize into SG or cytoplasmic inclusions is not fully characterized yet since some studies suggest that the two protein co-localize (Ikenaka et al. 2020), while other studies show that the two protein aggregates in a mutually exclusive fashion (Chen and Cohen 2019).

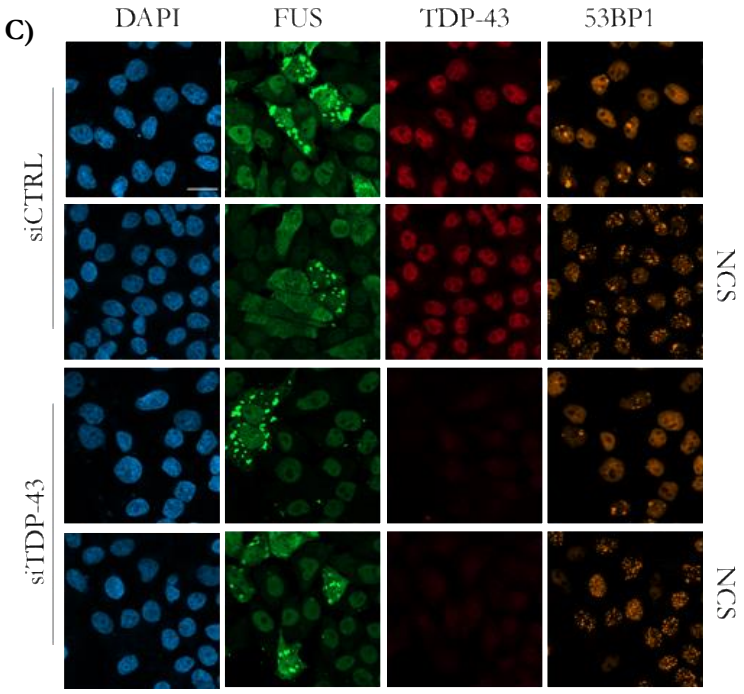
More recently, our lab characterized the role of TDP-43 positive inclusions in DDR activation observing that cells with TDP-43 cytoplasmic inclusions show γH2AX accumulation and DDR defects (https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwiaxKXg97HuAhXpxoUKHUKTDrAQFjAEegQIDBAC&url=https%3A%2F%2Fi

ris.unipv.it%2Fbitstream%2F11571%2F1243286%2F2%2FPhD%2520thesis%2520Brandi%2520low%2520quality.pdf&usg=AOvVaw1fxcH5TdzBv7dSAsM_xjul). Thus we tested if TDP-43 CI co-localize with the one of mutant FUS. As shown in figure (4.13.A-B) we observed that, in fact, most of the time TDP43 inclusions are negative for FUS and mutant FUS CI do not recruit TDP43, suggesting that these two facultative component of SG are often mutually exclusive.

In order to better asses if the phenotype observed upon mutant FUS-P525L CI could be explained by the recruitment of TDP43 into FUS CI, we tested TDP-43 depletion prior to mutant FUS overexpression and evaluated if DDR activation defect were abolished or maintained. As a marker for DDR activation we used 53BP1 foci formation in cells treated with NCS (Fig. 4.13.C-D). Importantly, TDP-43 depletion does not affect the amount of 53BP1 foci formed in cells with mutant FUS CI (Fig. 4.13.C-D) which still show fairly absence of 53BP1 foci (Fig. 4.13.C-D). These evidences confirm that the phenotypes observed upon mutant FUS CI formation is independent on the recruitment of TDP43 into SG or mutant FUS CI and is indeed caused mutant FUS-P525L expression.




B)
100
50
0
% of cells
Tot cells w/ FUS positive CI
% TDP/FUS co-loc
-
+
-
+
NCS
WT
P525L


C)
DAPI
FUS
TDP-43
53BP1
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siTDP-43
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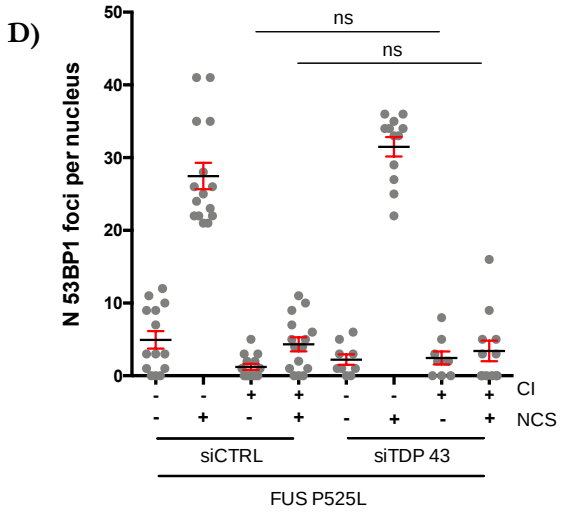


Figure 4.13. TDP43 depletion does not affect FUS P525L CI impact on 53BP1 foci A. Imaging of HeLa cells expressing FUS-P525L immunostained for FUS and TDP43 untreated or treated with NCS**.** Nuclei were counterstained with DAPI Scale bar 20µm. **B.** Quantification of co-localization between FUS and TDP43 at indicated condition. **C.** Imaging of HeLa cells expressing FUS-P525L immunostained for FUS, TDP43 and 53BP1 untreated or treated with NCS**.** Nuclei were counterstained with DAPI Scale bar 20µm. **D.** Quantification of 53BP1 foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition.. Error bars represent SEM among the population. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.5. FUS CYTOPLASMIC AGGREGATION TRIGGERS LOSS OF RNF168 NUCLEAR FOCI AND CONSEQUENT REDUCTION OF FK2-POSITIVE NUCLEAR SIGNAL

At site of DNA damage, MDC1 directly stimulates the recruitment of the E3 ubiquitin ligases RNF8 and then RNF168 to site of damage, which by mono and poly ubiquitinating H2A and H2AX can control both 53BP1 and BRCA1 recruitments. Indeed, along the DDR cascade pathway, chromatin ubiquitination represents a crucial step for the recruitment of downstream players. The major contributor is RNF168 which catalyse H2AX ubiquitination at Lysine 13 and 15 upon DNA damage induction thus ensuring the recruitment of different factors required for DNA repair (Mattiroli et al. 2012).

Since MDC1 foci were normal, we were eager to test if also for the ability of our cells to mount DDR foci for RNF168. Interestingly, by staining with an antibody against RNF168 cells expressing mutant FUS, we found that cells harbouring CI show a strong

reduction in the number of RNF168 foci if compared to cells without mutant FUS CI (Fig. 4.14A-B). It should be highlighted that, as previously shown (Doil et al. 2009) RNF168 forms nuclear foci also in undamaged condition, since chromatin ubiquitination is also important for transcription regulation (Fig. 4.14.A-B). Upon damage RNF168 foci are formed at damaged site and in fact in part co-localize with γH2AX foci. Importantly, cells with mutant FUS CI also present a defect in RNF168 foci formation also in undamaged cells. Intriguingly we noticed that the reduction of RNF168 nuclear level is associated with the appearance in the cytoplasm of small areas positive for RNF168 (Fig. 4.14.A-B).

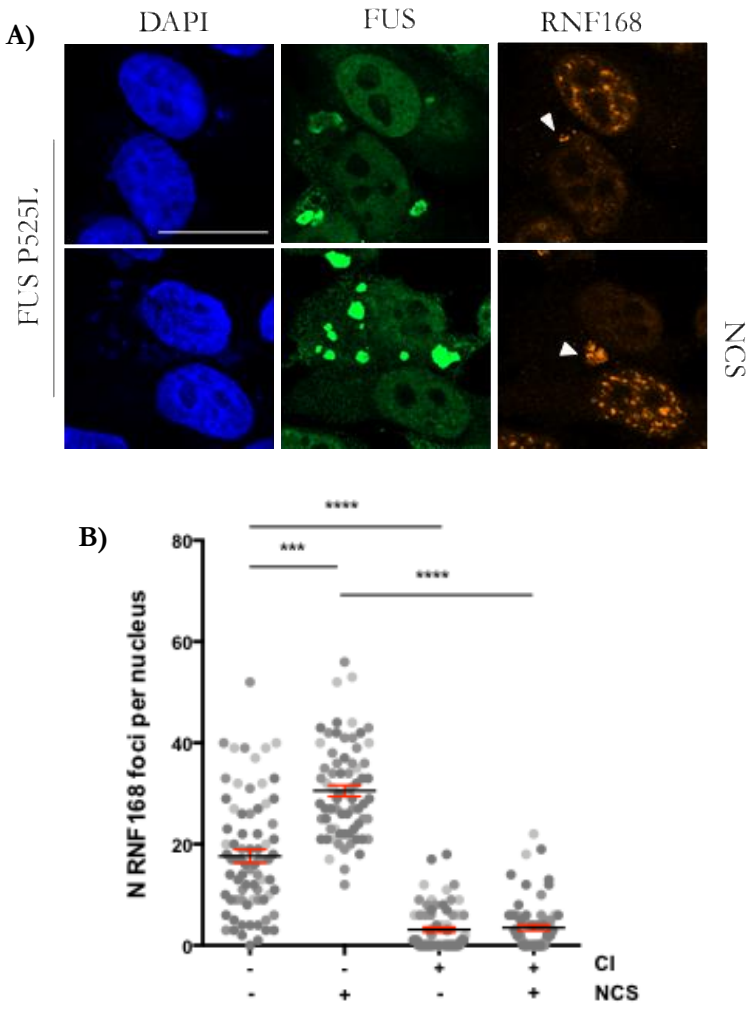
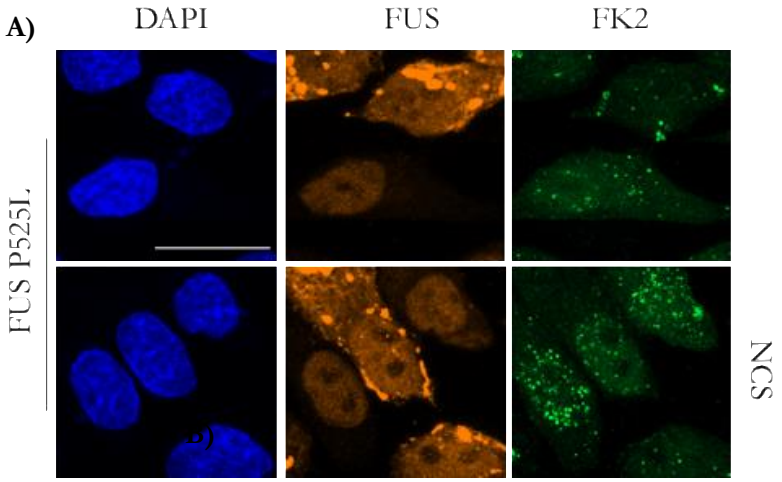


Figure 4.14. Cells with FUS positive CI show reduction of RNF168 nuclear foci A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained for FUS and RNF168 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of RNF168 foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

The absence of RNF168 nuclear foci should result in a strong reduction of the level of poly-ubiquitination in the nucleus of cells with CI. To address this point we stained cells with FK2 antibody which recognizes mono- and poly-ubiquitinylated residues (Fig. 4.15.A-B) and we confirmed that cells with mutant FUS CI have significantly less nuclear FK2-positive foci upon NCS induction (Fig. 4.15.A-B). These evidences strongly support the model that cells with mutant FUS CI present a strong defect in RNF168 dependent chromatin ubiquitination also functional to 53BP1 foci and DNA repair.



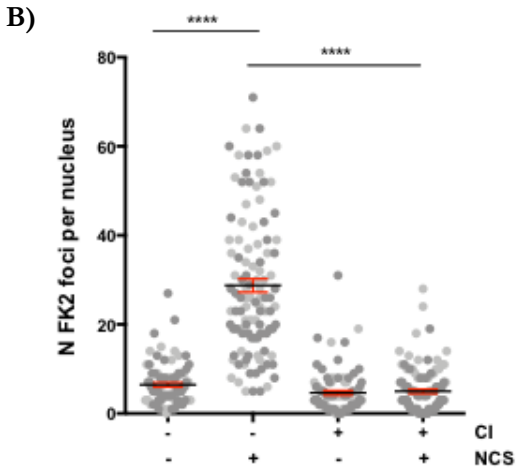


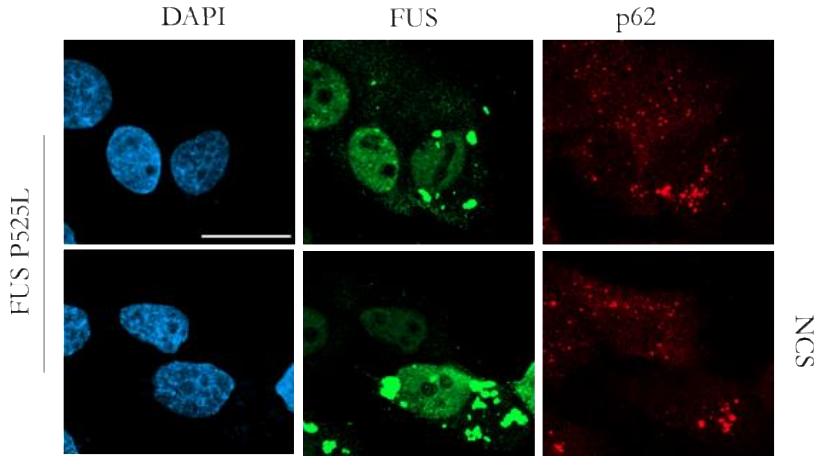
Figure 4.15. Cells harbouring FUS positive CI exhibits loss of FK2 foci upon DNA damage induction. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained for FUS and FK2 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20μm. **B.** Quantification of FK2 foci per nucleus measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.6. MUTANT FUS-P525L CI LEADS TO P62 ACCUMULATION AND SEQUESTRATION OF RNF168 INTO CYTOPLASMIC BODIES

The accumulation of misfolded proteins, one of the major hallmark in neurodegenerative diseases including ALS, is normally counteracted in our cells by autophagy-mediated clearance (Metcalf et al. 2012). Indeed, inefficient autophagy, or overloading of the autophagic flux, can lead to the accumulation of toxic protein aggregates and many evidences in literature suggest that defect in autophagy can be at the base of neurodegeneration (Fujikake, Shin, and Shimizu 2018)). Mutant proteins that accumulate in the cytoplasm and have the propensity to phase-separate into CI, such as FUS, put the autophagic process under stress. Has been reported that when autophagy is blocked the cargo protein p62 tend to accumulate and form p62-positive cytoplasmic bodies (Bjorkoy et al. 2005). Indeed, in the context of cells expressing FUS-P525L mutant form, Soo and colleagues showed increased levels of the p62 protein cytoplasmic fraction in mouse neuronal cells (Soo et al. 2015). Moreover, increased levels of p62 has also been observed in iPSC derived motorneurons which recapitulate the FUS cytoplasmic accumulations typical of ALS specimen (Marrone et al. 2019). Thus we investigated if also in our cellular

system the expression of FUS P525L leads to p62 accumulation and if this event is specific for cells exhibiting mutant FUS positive CI. Thus we stained our cells overexpressing FUS-P525L with an antibody against p62 and we clearly observed that indeed cells with mutant FUS CI show the formation of bright cytoplasmic bodies of p62 (Fig. 4.16.A). This observation is in line with what was recently reported in (Jakobi et al. 2020) where p62 accumulation is clearly identified by IF and is characterized by the formation of cytoplasmic bodies. Importantly, once again, the overexpression of FUS-P525L is not responsible *per se* for p62 accumulation, since its accumulation is occurring only in cells harbouring mutant FUS CI and not in the surrounding cells equally expressing FUS-P525L mutant isoform (Fig. 4.16.B).

A)



B)

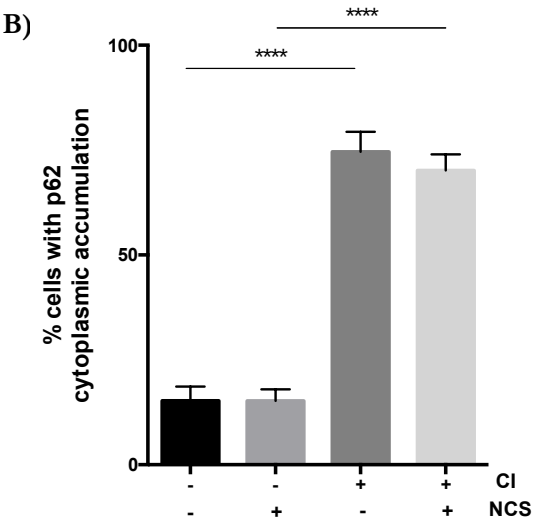


Figure 4.16. Cells with FUS CI show accumulation of p62. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained for FUS and p62in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm**. B**. Quantification of cells showing p62 accumulation measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

In the literature it has been reported that upon sodium arsenite treatment, a commonly used approach to induce SG formation, p62 co-localizes with FUS-positive SGs (Marrone et al. 2019). Therefore, we tested if p62 bodies co-localize with FUS CI in our cellular system, something we believed it was expected if we consider that p62 should clear FUS CI. Intriguingly, instead we observed that mutant FUS CI and p62 bodies co-exist in the same cytoplasm but never co-localize in the same plain (further details can be found in the next paragraph).

These results demonstrate that mutant FUS CI formation leads to p62 accumulation in characteristics cytoplasmic bodies of the same cell, however in areas distinct from FUS protein inclusions and SG. This might suggest that mutant FUS expression and possibly phase-separation causes an overloading of the autophagic machinery and thus an impairment of the process.

As described previously, we observed a strong reduction of RNF168 foci and nuclear signal in cells with mutant FUS CI in both undamaged and damaged cells. Importantly, we observed that the reduction of nuclear signal for RNF68 correlates with the appearance of a localized cytoplasmic signal (Fig. 4.17.A-B).

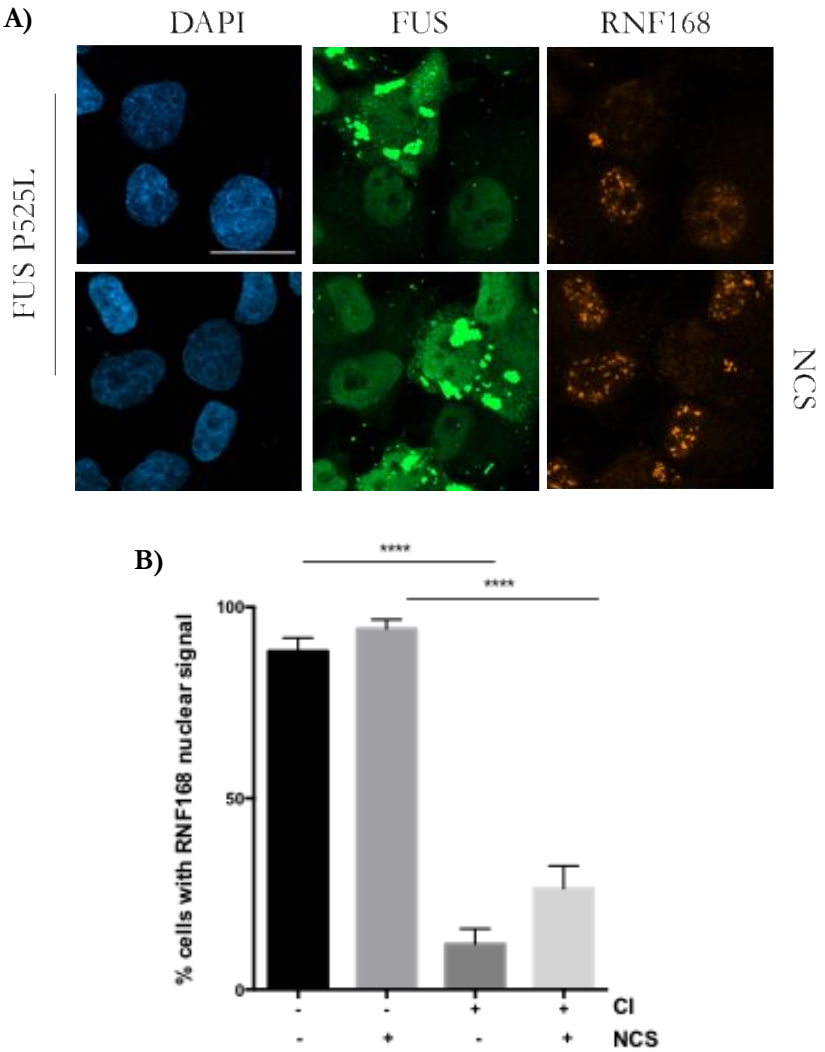
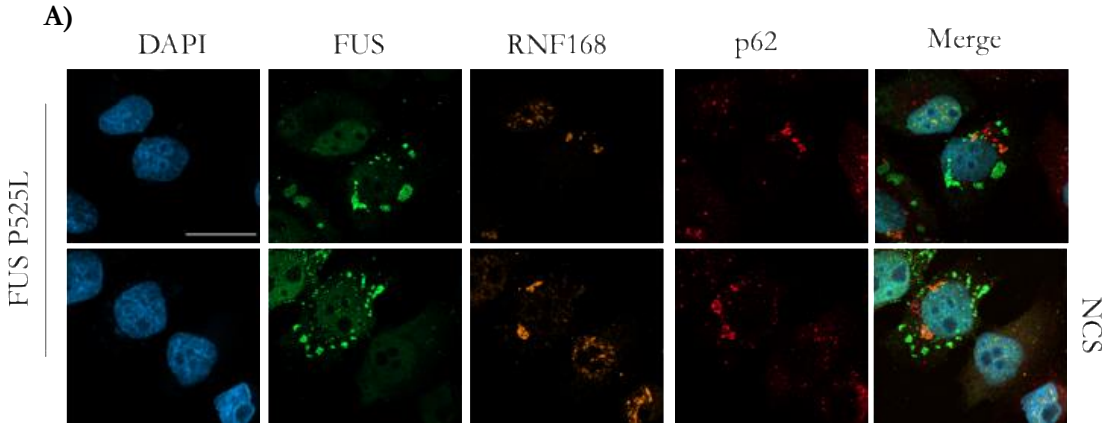


Figure 4.17. Cells with FUS CI show RNF168 nuclear depletion. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained for FUS and RNF168 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm**. B**. Quantification of cells showing RNF168 nuclear signal measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

It has been proposed that in cancer cells the LB domain within p62 protein interact with RNF168 (Wang et al. 2016) and in particular, the MIU1 motif of RNF168 is required for the binding to p62 (Wang et al. 2016). This interaction was shown to block RNF168 recruitment on the chromatin of damaged cells (Wang et al. 2016). Therefore, we asked if the signal that RNF168 staining gives in the cytoplasm of cells positive for mutant FUS CI, might indeed co-localize with p62 bodies.

Indeed by specific confocal acquisition of specific planes and 3D reconstructions of the cell nucleus we could measure the frequency of co-localization of RNF168 signal with p62 and FUS. Particularly, imaging acquisition for co-localization purposes has been carried out following the most recent guidelines (Jonkman et al. 2020) which includes specific technical microscope setting (e.g. reducing both pinhole and z-stack slices); in this regard more details can be found in the material and methods section.

We observed that cells with mutant FUS CI, RNF168 signal strongly co-localizes with p62-positive cytoplasmic bodies while it almost never merges with FUS signal of CI (Fig. 4.18.A-B). This observation suggests that upon autophagic pressure given by mutant FUS overexpression, p62 accumulates in bodies, which has the ability to cause the delocalization of RNF168 from the nucleus to the cytoplasm.



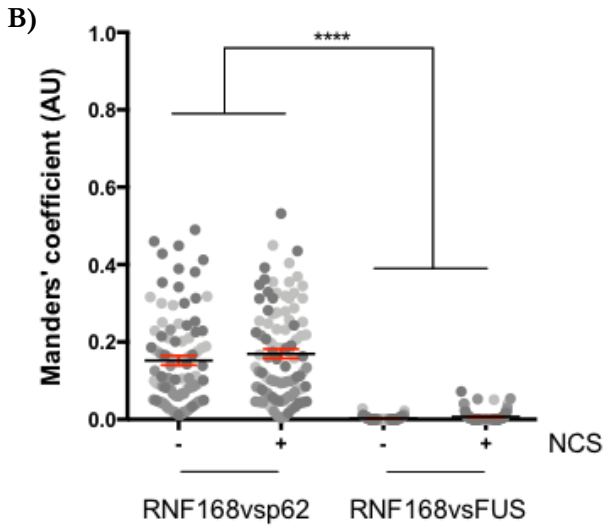


Figure 4.18. Cells with FUS CI show RNF168 nuclear depletion. p62 and RNF168 co-localize in the cytoplasm of cells with FUS CI. A. Imaging of HeLa cells overexpressing FUS-P525L and immunostained for FUS, RNF168 and p62 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of co-localization levels between RNF168 and FUS cytoplasmic signal and between RNF168 and p62 cytoplasmic signal measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS in each indicated condition. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

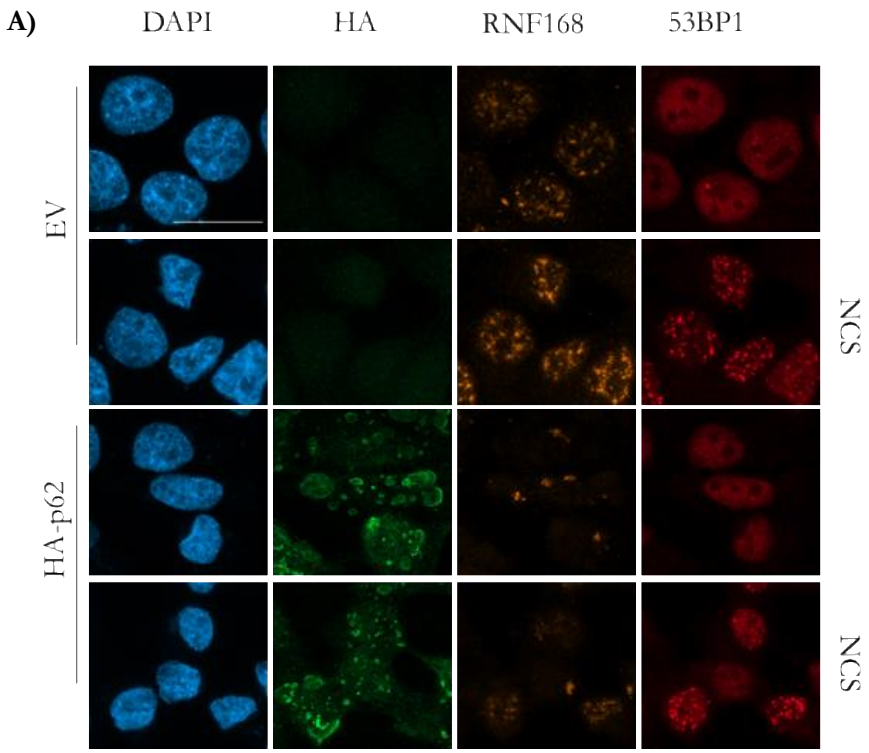
Overall these data intriguingly demonstrate that FUS CI formation stimulate p62 accumulation and RNF168 cytoplasmic de-localization. In addition, these analyses prove that the cytoplasmic sequestration of RNF168 is a novel and totally unexpected mechanism by which autophagy defect can alter RNF168 nuclear function in DDR and DNA repair.

4.6. HA-P62 OVEREXPRESSION STIMULATES RNF168 NUCLEAR DEPLETION AND 53BP1 FOCI LOSS AND IS ASSOCIATED WITH γ H2AX ACCUMULATION

A study in literature suggests that autophagy defect can damper 53BP1 foci formation by showing that p62 overexpression reduces RNF168 and 53BP1 recruitment at sites of damage while RNF8 is not affected (Wang et al. 2016). Thus, we investigated if HA-p62 overexpression in our cell system, by mimicking mutant FUS CI formation could lead to the same outcomes. Therefore, we transiently overexpressed HA-p62 in HeLa cells in the absence of mutant FUS overexpression. Intriguingly, we found that HA-p62 overexpression causes a clear reduction of RNF168 nuclear levels (Fig. 4.19.A-B) in both

undamaged and damaged condition suggesting that indeed p62 accumulation interfere with RNF168 nuclear localization. Moreover, 53BP1 foci upon DNA damage induction, are also reduced in cells overexpressing HA-p62 (Fig. 4.19.A-C) an observation that once again confirms that RNF168 DDR function is impaired.

Thus, HA-p62 overexpression per se recapitulates what we observed in cells experiencing mutant FUS CI strengthening the working hypothesis that mutant FUS CI are in fact altering RNF168 DDR functions by causing p62 accumulation.



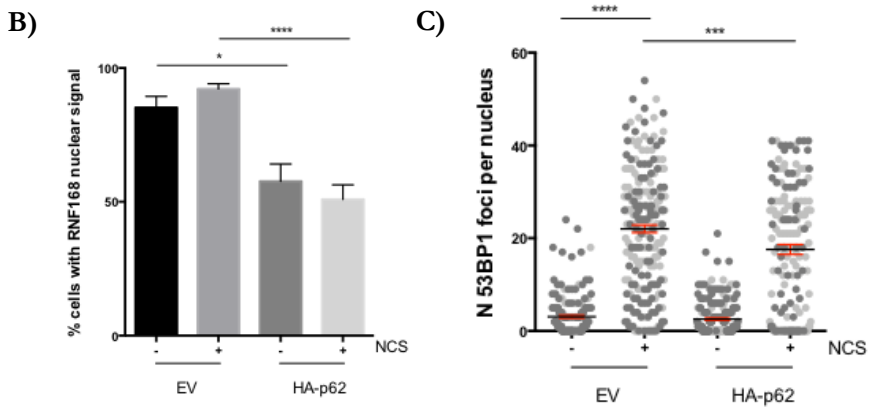
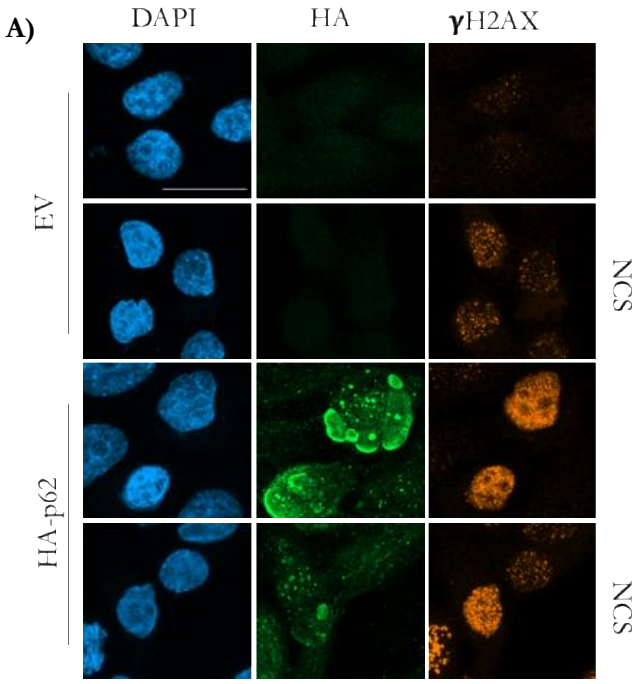


Figure 4.19. p62 overexpression affects DDR signalling. **A.** Imaging of HeLa cells overexpressing HA-p62 and immunostained for HA, RNF168 and 53BP1 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B-C.** Quantification of cells showing RNF168 nuclear depletion (B) and 53BP1 foci (C) measured in cells expressing HA-p62. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Taking in account that cells with mutant FUS CI also exhibit a strong γH2AX nuclear signal and physical DNA damage we wonder if upon HA-p62 overexpression the loss of proper RNF168-mediated signal may also cause DNA damage.

Interestingly, we observed that indeed HA-p62 overexpression induces γH2AX accumulation in comparison with the level of γH2AX present in EV-expressing cells (Fig. 4.20.A) as detected by the analyses of γH2AX mean intensity per nucleus (Fig. 4.20.B). i


A)
DAPI
HA
γH2AX
EV
HA-p62
NCS
NCS

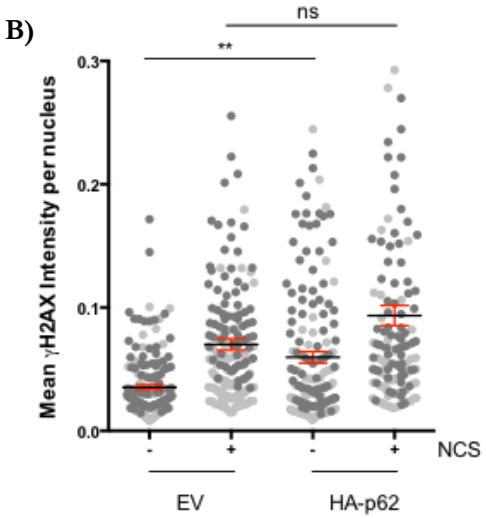
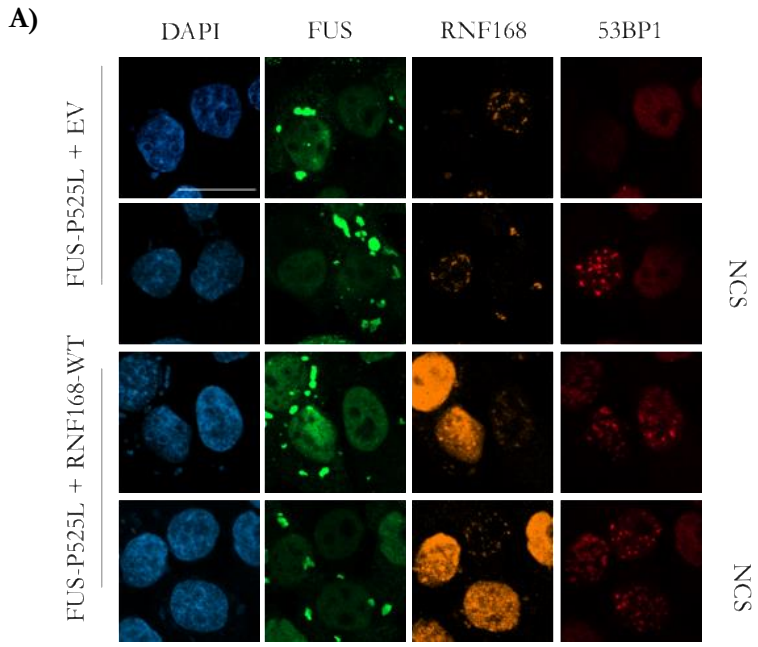

B)
ns
**
Mean γH2AX Intensity per nucleus
0.3
0.2
0.1
0.0
-
+
-
+
NCS
EV
HA-p62

Figure 4.20. p62 overexpression affects DDR signalling. **A. A.** Imaging of HeLa cells overexpressing HA-p62 and immunostained for HA, RNF168 and γH2AX in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of γH2AX nuclear signal measured in cells expressing HA-p62. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.7. RNF168 OVEREXPRESSION RESTORES 53BP1 FOCI, REDUCES γH2AX AND PATM BASAL HYPER ACTIVATION AND PARTIALLY RESTORES DROSHA NUCLEAR LEVELS IN CELLS WITH FUS CI

To further confirm the pivotal role of RNF168 nuclear loss in DDR deregulation observed upon FUS CI formation we attempt to restore RNF168 nuclear level by two approaches; RNF168 and RNF8 overexpression concomitantly with mutant FUS-P525L. To avoid chromatin alterations induced by RNF168 overexpression we reduced the amount of plasmid used in the transfection until we didn't observed any accumulation of heterochromatin foci by DAPI staining, and at the end RNF168 plasmid was transfected at the ratio of 1/5 respect to mutant FUS expressing plasmid. As a control, the FUS-P525L variant was transfected with the corresponding amount of EV. We observed that the FUS-P525L plus EV transfection reflects what previously observed where cells harbouring mutant FUS CI show loss of 53BP1 foci upon DNA damage induction (Fig. 4.21.A-B). Instead, we found that cells with mutant FUS CI that also express exogenous RNF168 show clearly detectable 53BP1 foci indicating that RNF168 overexpression can restore 53BP1 recruitment to DSB (Fig. 4.21.A-B). Through the magnified visualization where the RNF168 signal appears evident, we could clearly appreciate that the presence of exogenous RNF168 nuclear localization stimulates 53BP1 foci formation upon NCS treatment (Fig. 4.21.A-B). These evidences confirm that RNF168 nuclear depletion is a key player in the DDR mis-regulation, and its nuclear loss strongly affects 53BP1 recruitment to DSB.


A)
DAPI
FUS
RNF168
53BP1
FUS-P525L + EV
FUS-P525L + RNF168-WT
NCS
NCS

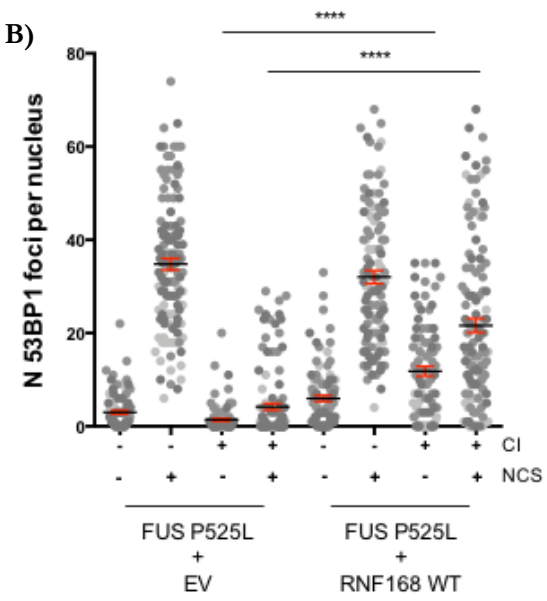
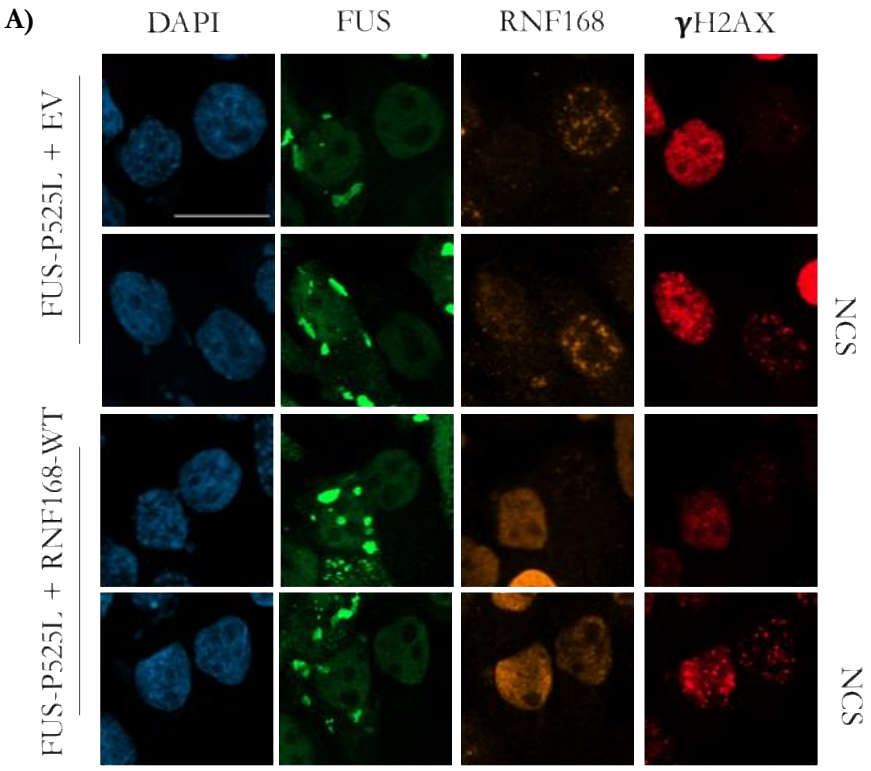

B)
****
****
N 53BP1 foci per nucleus
80
60
40
20
0
CI
NCS
FUS P525L + EV
FUS P525L + RNF168 WT

Figure 4.21. RNF168 overexpression restores 53BP1 foci in cells with mutant FUS CI. A. Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF168 and immunostained for FUS, RNF168 and 53BP1 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of 53BP1 foci measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Next we tested if RNF168 overexpression by restoring 53BP1 foci also enhances DNA repair and reduce γH2AX signal accumulation leading to a more physiological localization of this marker at distinctive DDR foci, also in damaged cells bearing mutant FUS CI. Thus cells transiently overexpressing RNF168 protein together with mutant FUS, were stained for the DNA damage marker γH2AX using as control condition cells transfected with an EV together with FUS-P525L as done previously. In these control conditions, γH2AX nuclear levels is significantly higher in cells with mutant FUS CI if compared to cells without CI, both upon damage and undamaged conditions (Fig. 4.22.A-B). Differently, the exogenous overexpression of RNF168 reduces γH2AX accumulation in cells with mutant FUS CI in undamaged cells and leads to the formation of γH2AX positive DDR foci in damaged cells (Fig. 4.22.A-B). Once again our data indicate that boosting RNF168 cellular protein level by expressing exogenous RNF168 can compensate to the loss of nuclear localization of this factor, thus allowing the activation of a functional DDR ultimately leading to better repair in cells harbouring mutant FUS CI.


A)
DAPI
FUS
RNF168
γH2AX
FUS-P525L + EV
FUS-P525L + RNF168-WT
NCS
NCS

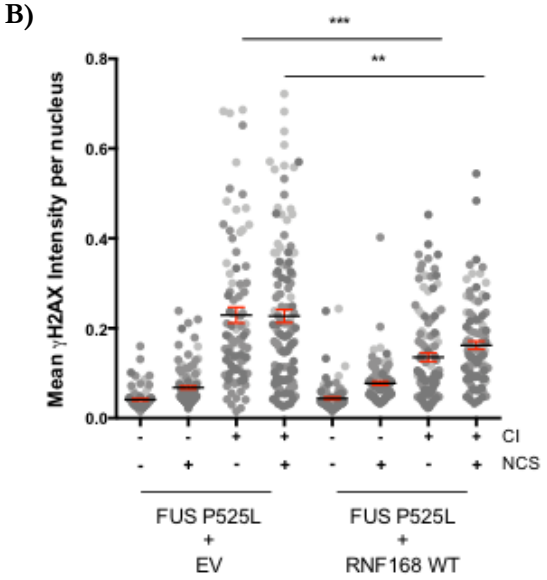

B)
***
**
Mean γH2AX Intensity per nucleus
0.8
0.6
0.4
0.2
0.0
CI
NCS
FUS P525L + EV
FUS P525L + RNF168 WT

Figure 4.22. RNF168 overexpression reduces γH2AX accumulation in cells with mutant FUS CI. A. Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF168 and immunostained for FUS, RNF168 and γH2AX in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of γH2AX nuclear signal measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Since the RNF168 co-expression with the FUS-P525L variant ameliorates γH2AX nuclear accumulation we wonder if this correlates also with a reduction in ATM hyper-activation observed in cells with FUS positive inclusions and could promote the formation of functional pATM foci in damaged cells. Accordingly, if cells expressing FUS-P525L and the EV showed the increased nuclear staining observed previously and the loss of pATM foci (Fig. 4.23.A-B), once exogenous RNF168 was expressed together FUS-P525L cells with CI show a significant reduction of diffused pATM nuclear signal but failed to sustain discrete pATM foci formation upon damage (Fig. 4.23.A-B). Indeed ATM activation occurs upstream to RNF168 in the DDR signalling cascade (Blackford and Jackson 2017).

This result may suggest that the primary recruitment of ATM at site of damage and its consequent activation take place without being retained. Thus rescue of proper DDR activation mediated by RNF168 significantly reduces its aberrant hyperactivation but do not restores ATM foci formation thus possibly reducing its ability to repair DNA.

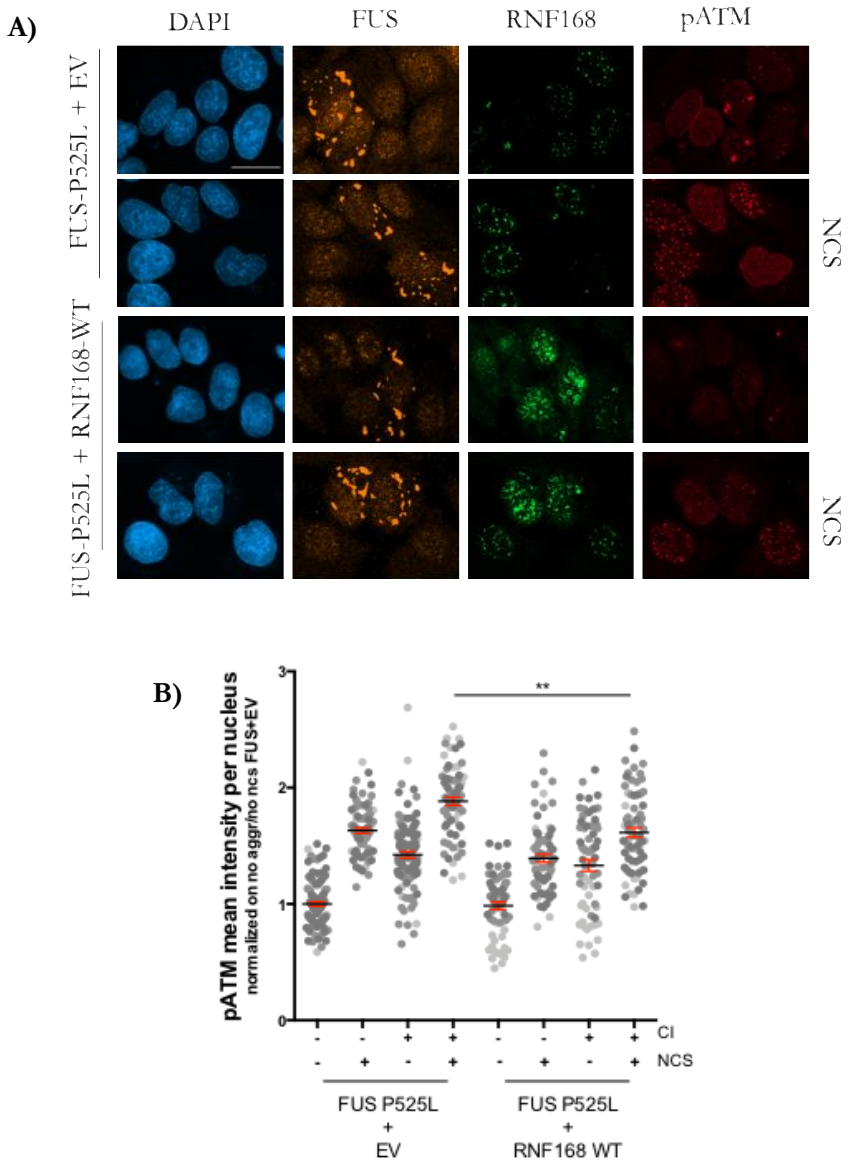
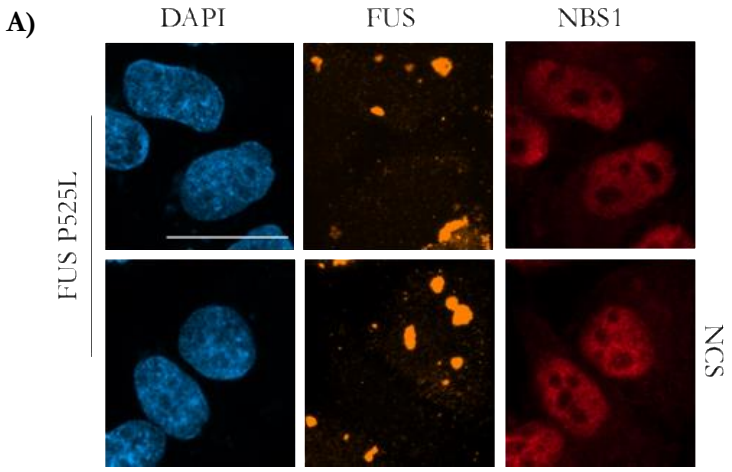


Figure 4.23. RNF168 overexpression reduces pATM hyper-activation in cells with mutant FUS CI. **A.** Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF168 and immunostained for FUS, RNF168 and pATM in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of pATM nuclear signal measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.8. CELLS MUTANT FUS CI HAVE REDUCED PROTEIN LEVEL OF DROSHA WHICH IS PARTIALLY RESCUED BY RNF168 OVEREXPRESSION

4.8.1. Cells bearing FUS CI have reduced DROSHA levels and impaired biogenesis of DDRNAs

Recently, our laboratory discovered that DROSHA and DICER RNA endonucleases are involved in DDR activation and foci generation and maintenance through the biogenesis of dilncRNAs and DDRNAs at site of damage (Francia et al. 2012; Michelini et al. 2017). We also showed that DROSHA and DICER control the secondary recruitment of DDR mediator factors such as 53BP1, while are dispensable for γH2AX marker accumulation (Francia et al. 2016). In addition our group have recently demonstrated that DROSHA is recruited at site of DNA damage very early in DDR cascade in a MRE11-RAD50-NBS1(MRN)-complex dependent manner but independently from ATM or DNAPK activation (Cabrini et al. 2021). Since we observed that DROSHA nuclear levels are reduced in cells with FUS CI we wonder if also the NBS1 (belong to the MRN complex) would be affected. We observed that NBS1 nuclear level is not affected in cells with mutant FUS CI showing comparable nuclear signals as the surrounding cells without FUS inclusions (Fig. 4.24.A-B).



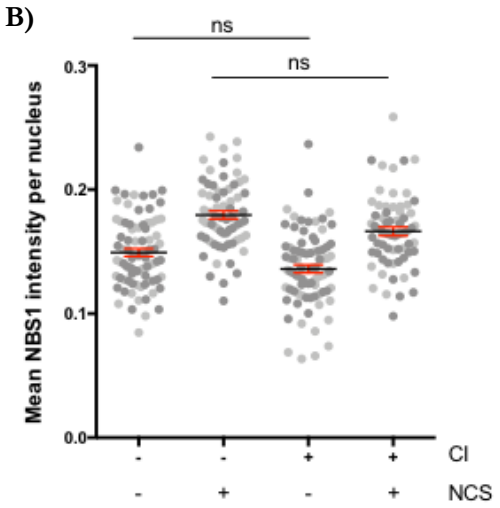
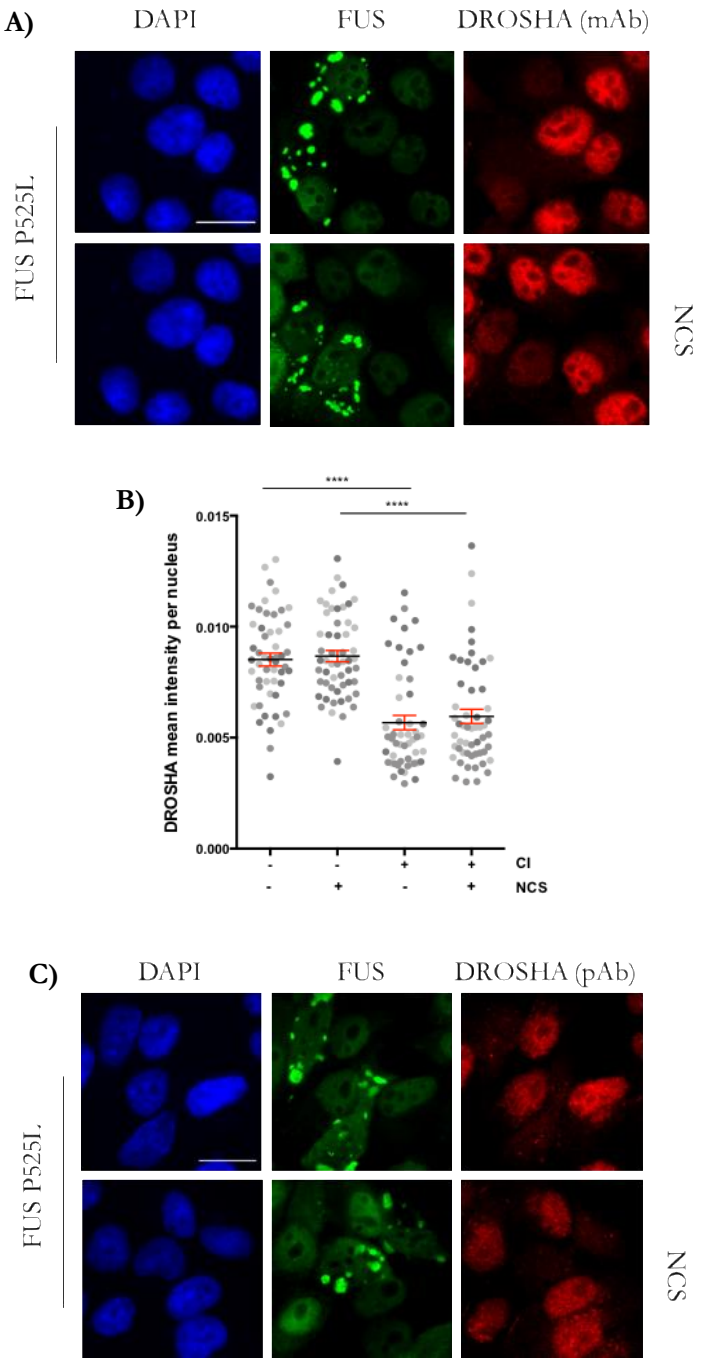


Figure 4.24. NBS1 nuclear levels are not affected in cells with FUS CI. **A.** Imaging of HeLa cells expressing FUS-P525L immunostained for FUS and NBS1 in basal conditions or upon DNA damage. Nuclei were counter-stained with DAPI. Scale bar: 20um. **B.** Quantification of NBS1 mean intensity in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Based on the observation that DDR foci formation is impaired in cells with mutant FUS CI, we wonder if DROSHA level is also altered. In fact we found that cells harbouring mutant FUS CI show a strong reduction in DROSHA nuclear levels in both basal condition and upon NCS treatment (Fig. 4.24 A-B-C-D). We performed two staining with different antibodies, one monoclonal (mAb) (Fig. 4.24A-B) and one polyclonal (pAb) (Fig. 4.24C-D), able to recognize the same portion of DROSHA protein (1-100aa), and both showed that DROSHA nuclear signal was strongly reduced, thus we exclude the hypothesis that one antibody was unable to recognize DROSHA in cells with mutant FUS CI (Fig. 19B-C). Intriguingly, we observed that DROSHA nuclear signal was even lower in cells exposed to DNA damage by NCS treatments suggesting that the experimental generation of additional DNA damage, in cells bearing mutant FUS CI further enhances DROSHA down-regulation. This result indicate that mutant FUS CI triggered by FUS-P525L overexpression severely impact on the regulation of DROSHA protein levels, however if this occurred at a transcriptional or translational level or result in a loss of DDRNAs biogenesis remained unknown.


A)
DAPI
FUS
DROSHA (mAb)
FUS P525L
NCS
B)
DROSHA mean intensity per nucleus
0.015
0.010
0.005
0.000
****
****
CI
NCS
C)
DAPI
FUS
DROSHA (pAb)
FUS P525L
NCS

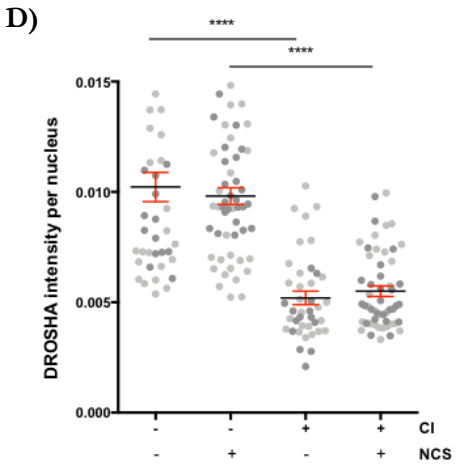


Figure 4.25. DROSHA nuclear levels are affected in cells with FUS CI. **A-C.** Imaging of HeLa cells expressing FUS-P525L immunostained for FUS and DROSHA moncoclonal (A) or polyclonal (C) antibody in basal conditions or upon DNA damage. Nuclei were counter-stained with DAPI. Scale bar: 20um. **B-C.** Quantification of DROSHA mean intensity in cells expressing FUS-P525L separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Next we wonder if the nuclear depletion of DROSHA observed in cells with mutant FUS CI is associated with reduced levels of mature DDRNAs. To this end, we took the advantage of an engineered cell system (I-HeLa111) carrying the consensus sites for the meganuclease I-SceI flanked by LAC operon repeats sequences and with the potential of expressing the I-SceI enzyme in a inducible fashion thus allowing the generation of a single DSB in a traceable locus (Lemaitre et al. 2014). DDRNAs were detected through strand-specific quantitative RT-PCR (qRT-PCR) designed for small RNA molecules (Miscript technology) as previously described (Gioia et al. 2019). Interestingly we observed that overexpression of FUS P525L mutant protein strongly reduces the level of DDRNAs biogenesis upon DSB generation by I-SceI induction (Fig. 4.26), thus suggesting that DROSHA downregulation observed in cells with mutant FUS CI also leads to loss of DDRNAs processing.

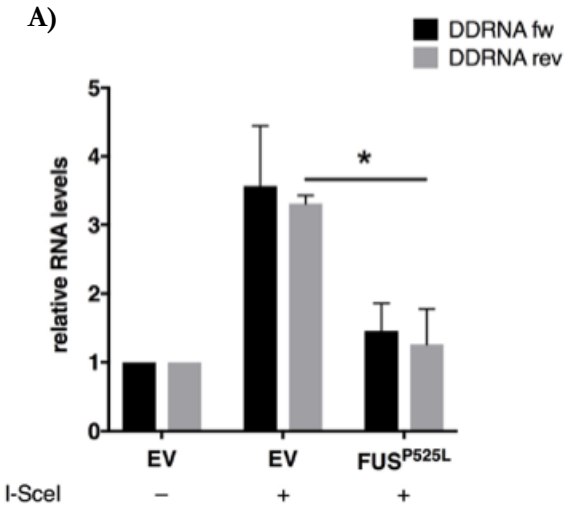


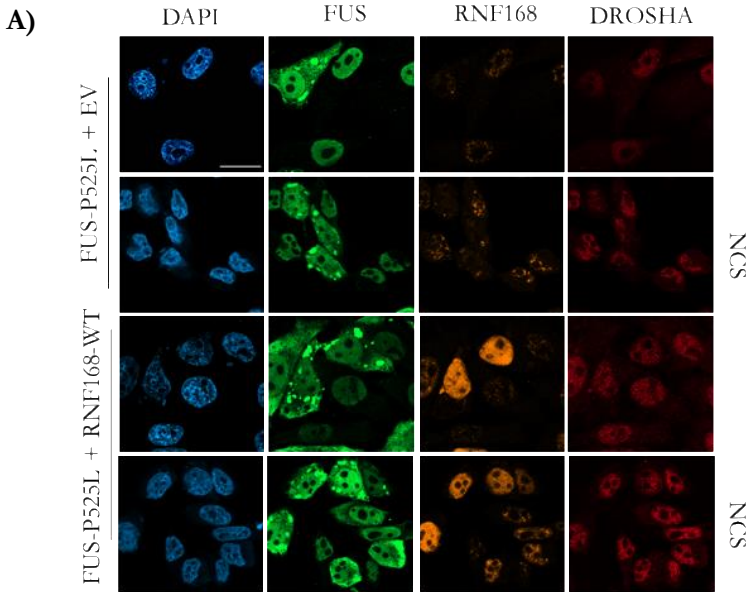
Figure 4.26. FUS P525L overexpression leads to reduced DDRNAs detection. **A.** Quantification of DDRNAs levels in I-HeLa111 overexpressing FUS P525L along with EV at indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Collectively, these data suggest that mutant FUS CI result in unique DROSHA down regulation, which is associated with reduced DDRNA biogenesis. Differently, in the same cells nuclear level of components of the MRN complex is not affected.

4.8.2. RNF168 rescues DROSHA nuclear protein levels in cells with mutant FUS CI

Finally we asked if DROSHA nuclear depletion in both undamaged and damaged condition could also be partially rescued by RNF168. Indeed, an interdependency between RNF168 and DROSHA action in DDR was recently published by another group showing that DROSHA inactivation by siRNA causes reduced DDR foci for RNF168 (Fig. 4.27.A-B) (Lu et al. 2018). Importantly we collected identical results in previous study. However, in the context of cells bearing mutant FUS CI the investigated relationship is inverted since we tested if RNF168 up-regulation by giving a more proficient DDR could also restore DROSHA levels. Unexpectedly, indeed in cells co-expression of RNF168 and FUS-P525L mutant protein, DROSHA nuclear levels are partially reverted to normal despite the presence of mutant FUS CI in absence or in presence of DNA damage induction (Fig. 27.A-B).

Thus we could show that the restoration of RNF168 nuclear level is beneficial for genome integrity and can somehow also restore DROSHA stability in cells with mutant FUS CI.



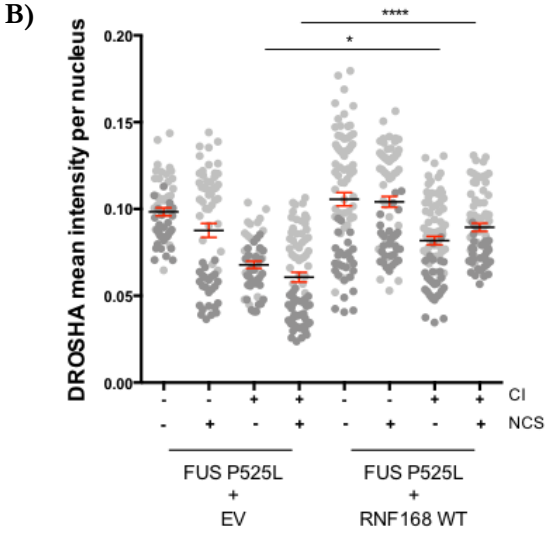


Figure 4.27. RNF168 overexpression partially rescues DROSHA nuclear levels in cells with mutant FUS CI. A. Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF168 and immunostained for FUS, RNF168 and DROSHA in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of DROSHA nuclear signal measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.9. RNF8 CO-EXPRESSION WITH MUTANT FUS LEADS TO RESCUE OF RNF168 NUCLEAR LEVEL THUS STIMULATING 53BP1 FOCI, REDUCE γH2AX AND PATM BASAL HYPERACTIVATION IN CELLS WITH MUTANT FUS CI

As we mentioned in the introduction, as soon as DSB occurs, a complex signalling cascade of events is activated and ATM-mediated phosphorylation of γH2AX allows the recruitment of MDC1 and in turn of the E3 ubiquitin ligases RNF8 (Kolas et al. 2007; Mailand et al. 2007) required for RNF168 signalling amplification. At damaged site, RNF8 stimulates H2A histones mono-ubiquitination, which represents the event fuelling RNF168 recruitment, thus controlling the addition of K63-linked poly ubiquitin chains on H2A and H2A.X histone variants (Doil et al. 2009; Stewart et al. 2009). As described in the introductive section, this histone modification catalysed by RNF8 first and then RNF168 is crucial for the recruitment and the retention of 53BP1 at DSB sites.

We previously showed that MDC1 foci are unaffected while RNF168 and 53BP1 foci are impaired in cells with mutant FUS CI. Since RNF8 acts in DDR downstream to MDC1 but upstream to RNF168 we asked whether RNF8 overexpression could stimulate the recruitment of RNF168 thus also DDR signalling and DNA repair in cells with mutant FUS CI. To address this point we overexpress the GFP-RNF8 WT protein together with FUS P525L mutant isoform and we evaluated RNF168, 53BP1, pATM foci and γH2AX nuclear level in cells with mutant FUS CI. We found that cells with FUS CI, that also incorporate GFP-RNF8 expressing plasmid, show a minor rescue of RNF168 positive foci and this event occurs in both undamaged and damaged conditions (Fig. 4.28.A-B).

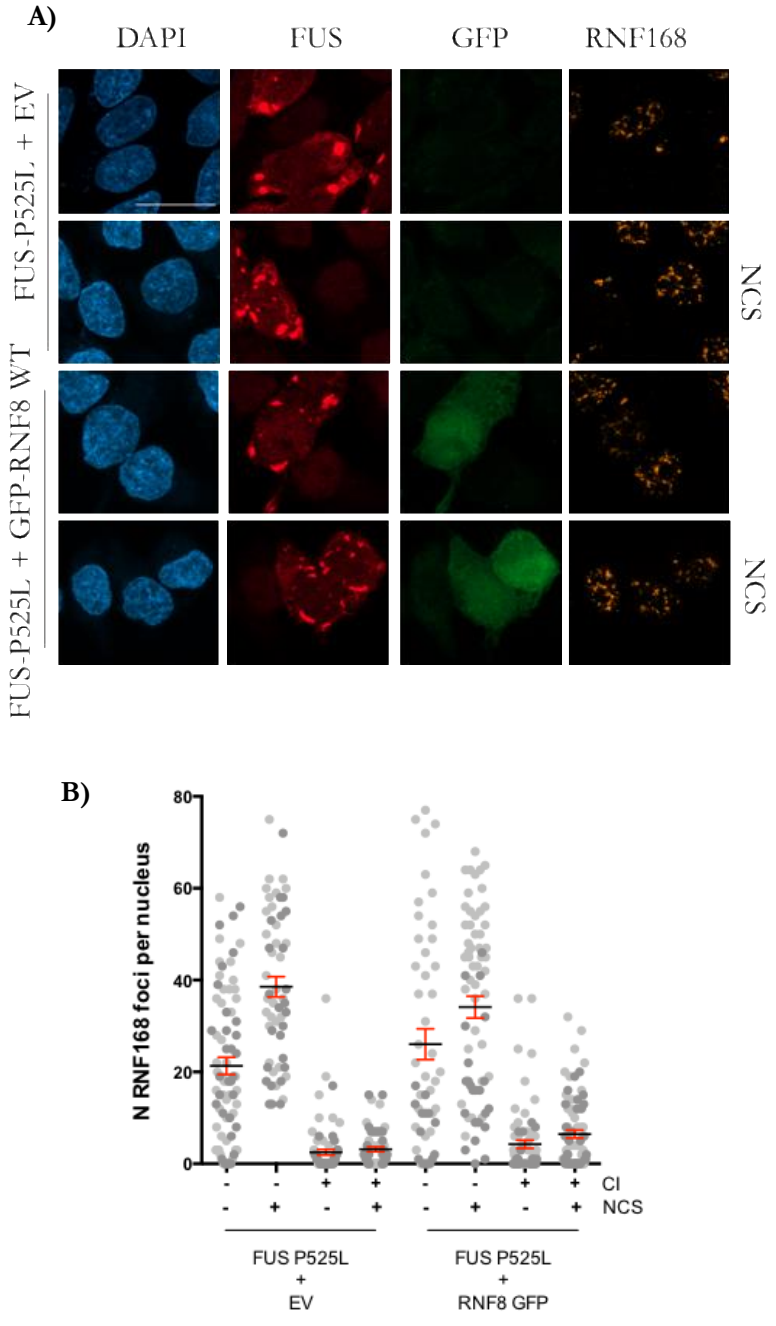
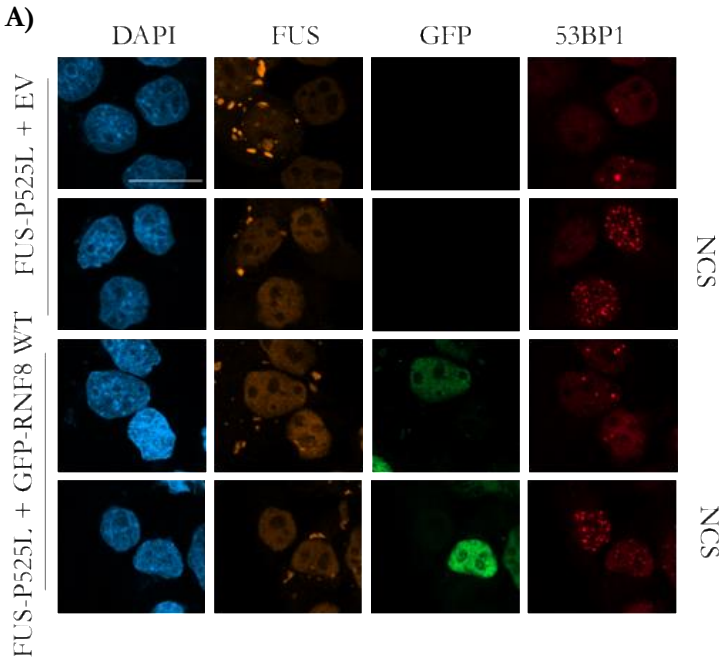


Figure 4.28. RNF8 overexpression stimulates restore of RN168 nuclear foci in cells with mutant FUS CI. A. Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF8 and

immunostained for FUS, RNF8 and RNF168 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of RNF168 foci measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Then, we evaluated 53BP1 foci rescue in the same experimental settings. Interestingly, we observed that cells with FUS CI that express GFP-RNF8 clearly show detectable 53BP1 foci which appear in number similar to the one of surrounding cells without FUS CI in damaged conditions (Fig. 4.29.A-B) suggesting that the overexpression of GFP-RNF8 can stimulates 53BP1 foci through the rescue of RNF168 functionality.



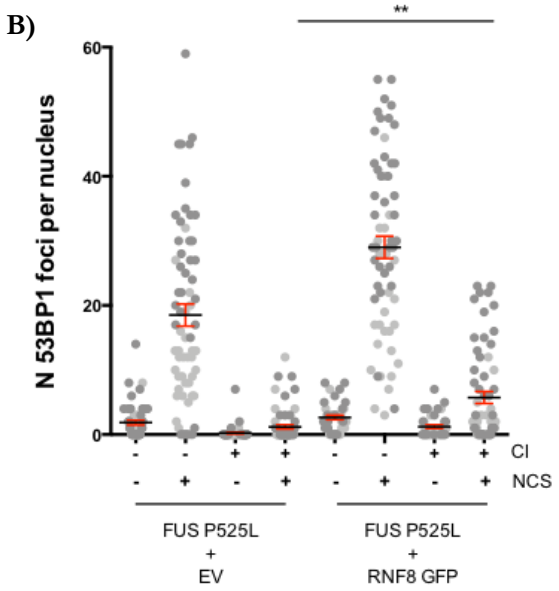
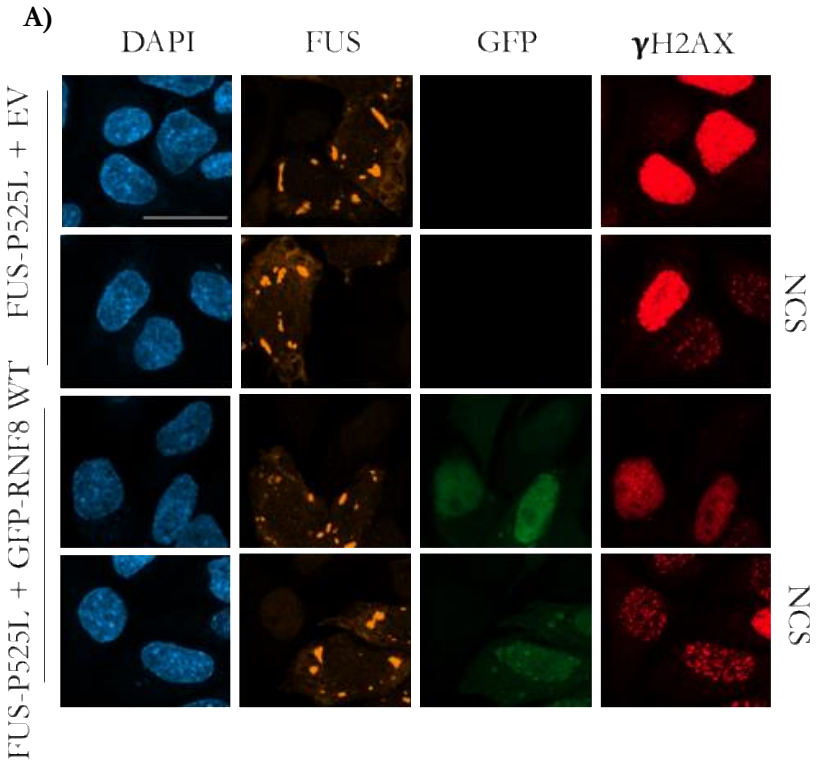


Figure 4.29. RNF8 overexpression stimulates restore of 53BP1 nuclear foci in cells with mutant FUS CI. A. Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF8 and immunostained for FUS, RNF8 and 53BP1 in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of 53BP1 foci measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

Finally, we evaluated if the correct activation of the signalling cascade could also stimulate a positive feedback loop leading to better repair thus resulting in γH2AX reduction in cells with FUS CI. Indeed, as observed for RNF168, we found that the overexpression of GFP-RNF8 in cells with mutant FUS CI stimulates the reduction of γH2AX pan-nuclear signal (Fig. 27D-E).


A)
DAPI
FUS
GFP
γH2AX
FUS-P525L + EV
FUS-P525L + GFP-RNF8 WT
NCS
NCS

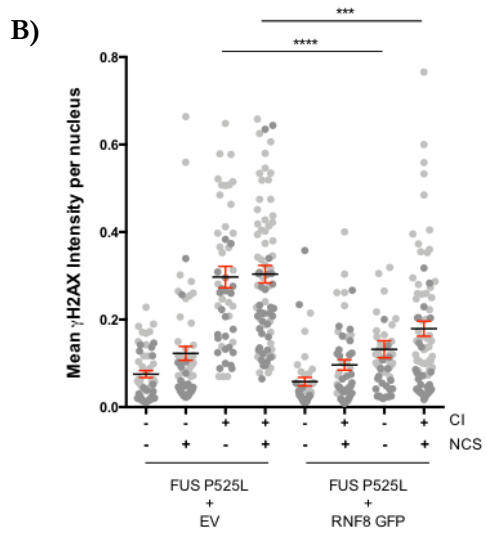


Figure 4.30. RNF8 overexpression stimulates restore of γH2AX nuclear foci in cells with mutant FUS CI. A. Imaging of HeLa cells overexpressing FUS P525L plus EV or plus RNF8 and immunostained for FUS, RNF8 and γH2AX in untreated and NCS-treated conditions in order to induce DNA damage. Nuclei were counterstained with DAPI Scale bar 20 μm. **B.** Quantification of γH2AX nuclear intensity measured in cells expressing FUS P525L. Error bars represent SEM from three independent experiments, discernible by the different colour of spots. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

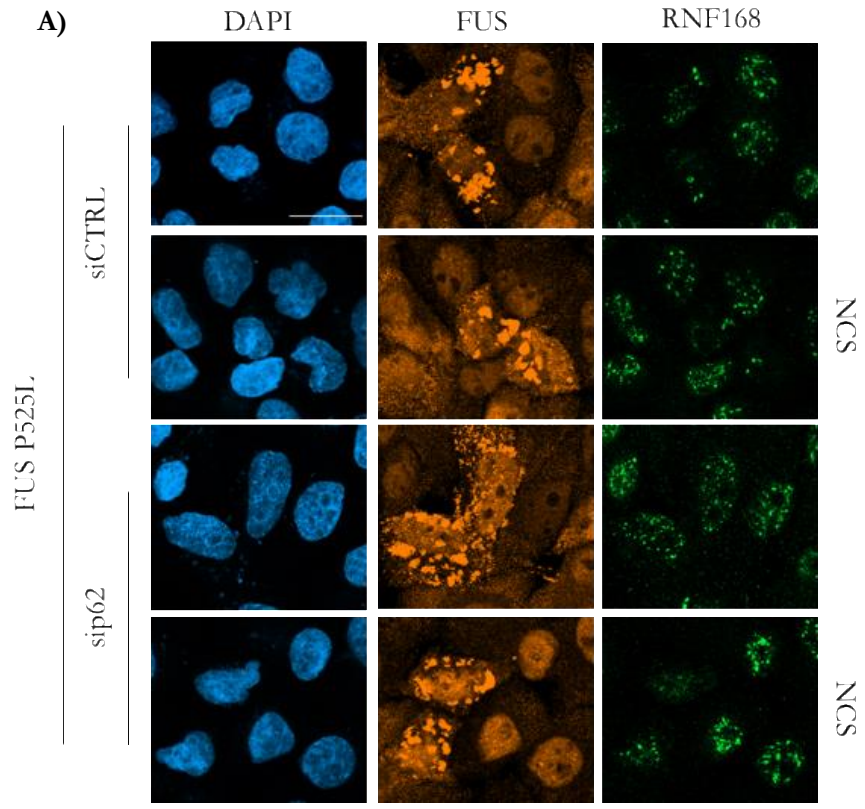
Collectively our data suggest that the RNF8 overexpression in cells experiencing mutant FUS CI can stimulate RNF168 activity, rescuing its recruitment to DDR foci thus also promoting 53BP1 foci formation and reduces γH2AX accumulation. To our knowledge, it is the first time that somebody shows that RNF8 upregulation can strengthen the cellular response to DNA damage.

4.10. P62 DEPLETION RESTORES RNF168 NUCLEAR SIGNAL AND 53BP1 FOCI IN CELLS WITH MUTANT FUS-P525L, THUS REDUCING γH2AX AMOUNT

The accumulation of p62 and its co-localization with cytoplasmic RNF168 in cells with mutant FUS CI lead us to speculate that p62-dependent RNF168 sequestration from the nucleus is one key event that damper DDR activation and DNA repair in these cells. Thus, we tested if p62 inactivation by siRNA, prior to transfection with mutant FUS expressing plasmid, could restore DDR functions in cells with FUS positive CI. Thus, we knocked down p62 by a pool of siRNA, 48h prior the FUS-P525L transfection and then

we stained for RNF168, to assay if p62 depletion could rescue of nuclear RNF168 levels and thus, possibly, DDR functions. As a control, cells were transfected in parallel with a set of 4 siRNAs with a scrambled sequences (siCTRL) and the efficiency of p62 depletion has been evaluated by western blotting. (Fig. 4.30.D).

As previously showed, we observed that cells transfected with siCTRL and harbouring mutant FUS CI show RNF168 cytoplasmic signal and a concomitant reduction of RNF168 nuclear foci. Excitingly, p62 depletion restores RNF168 nuclear localization in cells with mutant FUS CI in both damaged and undamaged conditions (Fig. 4.30.A-B-C). This important observation strongly suggests that p62 accumulation induced by mutant FUS CI is the event responsible for RNF168 nuclear depletions and sequestration into the cytoplasm and that p62 inactivation might be beneficial to restore DDR functions.



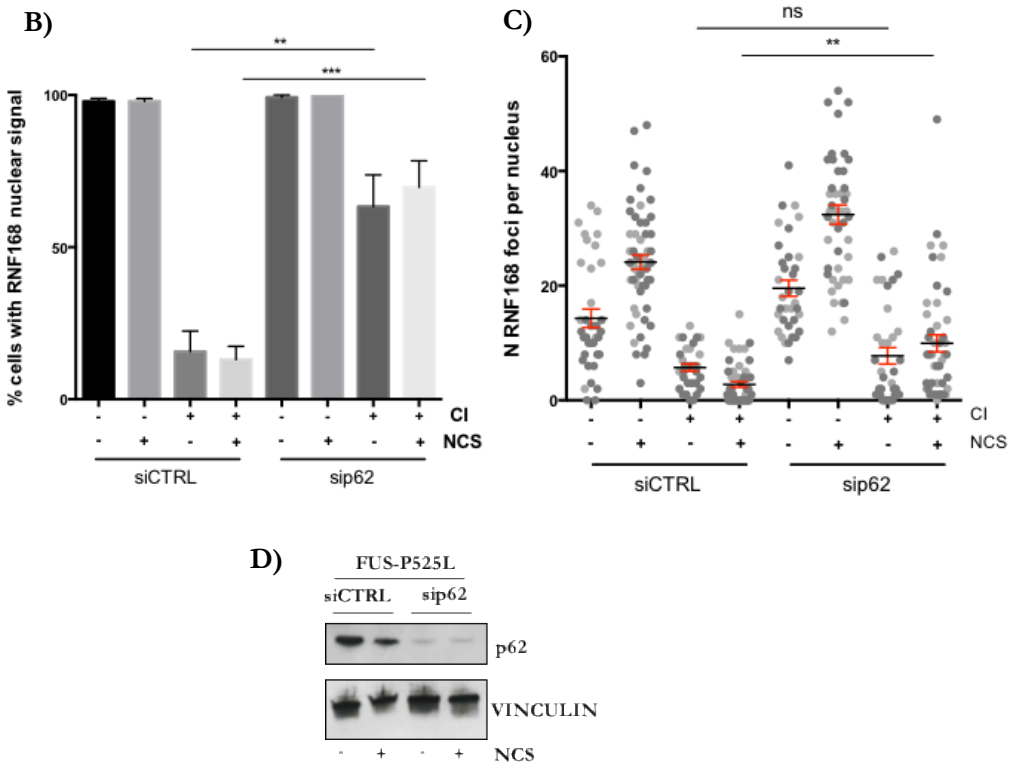


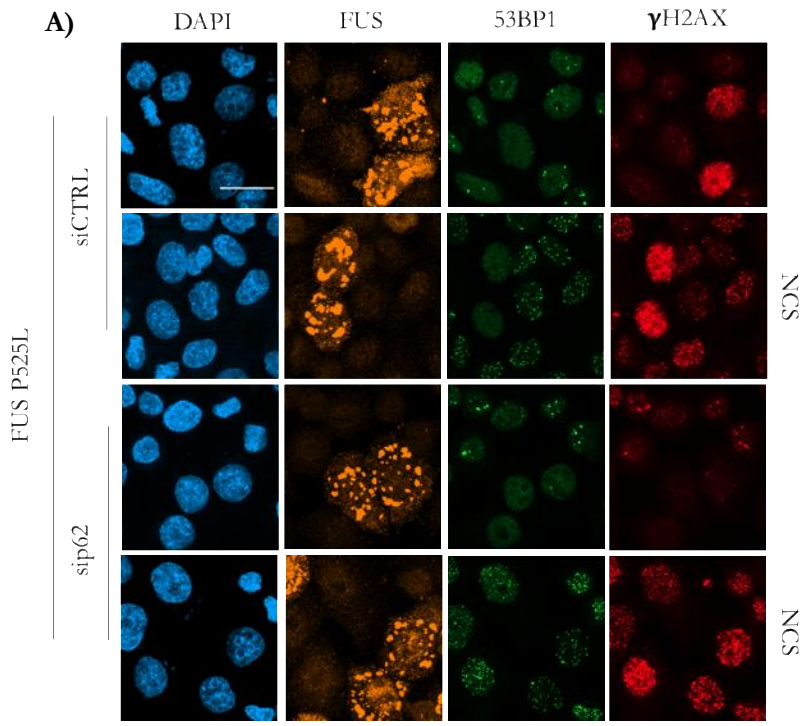
Figure 4.31. p62 depletion rescues RNF168 nuclear signal and DSB localization in cells with FUS CI. **A.** Imaging of HeLa cells expressing FUS-P525L immunostained for FUS, and RNF168 in basal conditions or upon DNA damage induction in both siCTRL and sip62 transfection. Nuclei were counter-stained with DAPI. Scale bar: 20um. **B.** Quantification of percentage of cells showing RNF168 cytoplasmic signal measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition. (C) Quantification of number of RNF168 foci per nucleus in cells expressing FUS-P525L by separating cells with FUS inclusions from cells without, in each indicated condition. Error bars represent SEM from two independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001. **D.** Western blotting oh HeLa cells treated with siCTRL and sip62 48h prior FUS-P525L transfection.

Thus, we next investigated if p62 knockdown result also in 53BP1 partial foci restoration in cells with mutant FUS CI. Importantly, we could confirm that damaged cells bearing mutant FUS CI and transfected with siCTRL show the previously observed phenotype of loss of 53BP1 foci but differently, cells depleted for p62 re-acquire the ability to mount proper 53BP1 foci (Fig. 4.31.A-B). Thus, we can confirm that p62 knockdown rescuing

RNF168 nuclear levels, is beneficial for the proper recruitment of 53BP1 at DSBs and a proficient DDR activation ultimately allowing DNA repair (Fig. 4.31A-B).

Indeed, we noticed and measured that also the level of γH2AX accumulation was significantly reduced in cells knocked down for p62 despite bearing mutant FUS CI, suggesting that DNA repair functionality has been restored (Fig. 4.28A-B). Intriguingly, we could appreciate that upon p62 knock-down, γH2AX signal is not anymore pan-nuclear but appears organized in clear detectable DDR foci similarly to the staining observed in cells without CI (Fig. 4.31.A-C). This last information confirms that the pan nuclear γH2AX observed indeed originate from DNA damage generation due to a defect in DDR factor recruitment and DNA repair and not a spurious signal associated with alteration of chromatin status, an event that can also activate ATM as previously described (Burgess et al. 2014).

These data indicate that p62 knockdown is beneficial for proper DDR activation in cells harbouring mutant FUS CI since it stimulates the rescue of 53BP1 recruitment at site of damage and consequent reduction of redundant γH2AX accumulation.



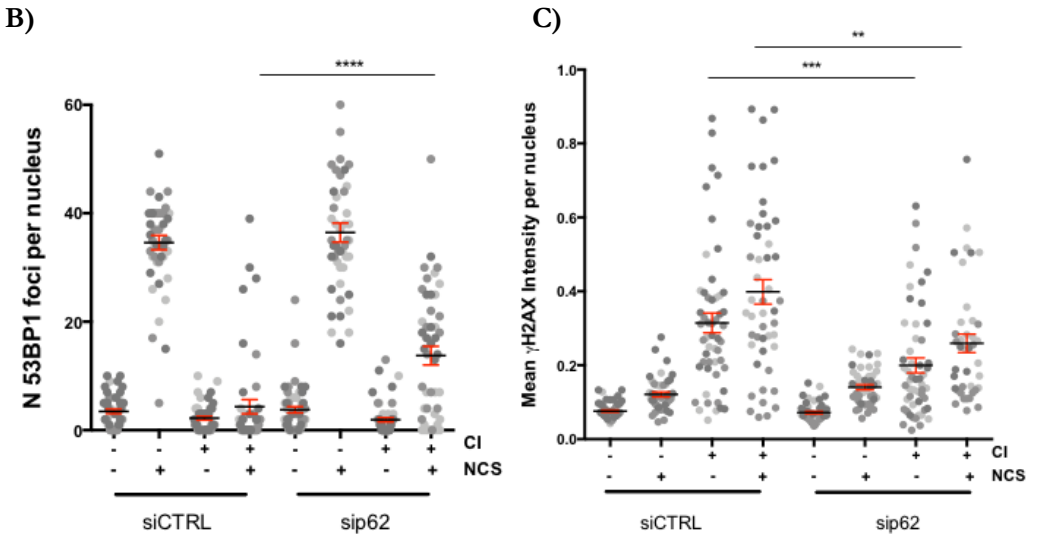


Figure 4.32. p62 depletion rescues DDR activation in cells with FUS CI. A. Imaging of HeLa cells expressing FUS-P525L immunostained for FUS, 53BP1 and **γ**H2AX in basal conditions or upon DNA damage induction in both siCTRL and sip62 transfection. Nuclei were counter-stained with DAPI. Scale bar: 20um. **B.** Counts of 53BP1 foci and **γ**H2AX mean intensity **(C)** measured in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

We previously observed that the γH2AX accumulation is ATM dependent and its inhibition reduces the strong γH2AX nuclear signal in cells with mutant FUS CI. We observed that p62 knockdown also restores proper γH2AX localization in clear detectable DDR foci. Thus, we evaluated if p62 depletion could restore also pATM foci in cells with mutant FUS CI. Accordingly, we stained cells knocked down for p62 and expressing mutant FUS with an antibody able to recognized the phosphorylated form of ATM (Ser 1981) (Fig. 4.22A). Intriguingly, we observed that pATM mean intensity per nucleus is significantly lower in cells knocked down for p62 respect to cells transfected with CTRL siRNA, still in condition of presence of mutant FUS CI, suggesting that the aberrant ATM autophosphorylation and activation is reduced (Fig. 4.32.A-B). Nevertheless, we observed that the p62 inactivation does not significantly rescues the ability of cells to form pATM foci (Fig. 4.32.A-C). Therefore we believe that p62 accumulation directly impinge on the RNF168 nuclear levels with a strong effect on 53BP1 foci and ultimately on DNA repair, thus reducing also ATM hyperactivation and γH2AX accumulation, nevertheless do not significantly restore pATM localization at site of damage, a recruitment which is upstream to RNF168 dependent signalling pathway.

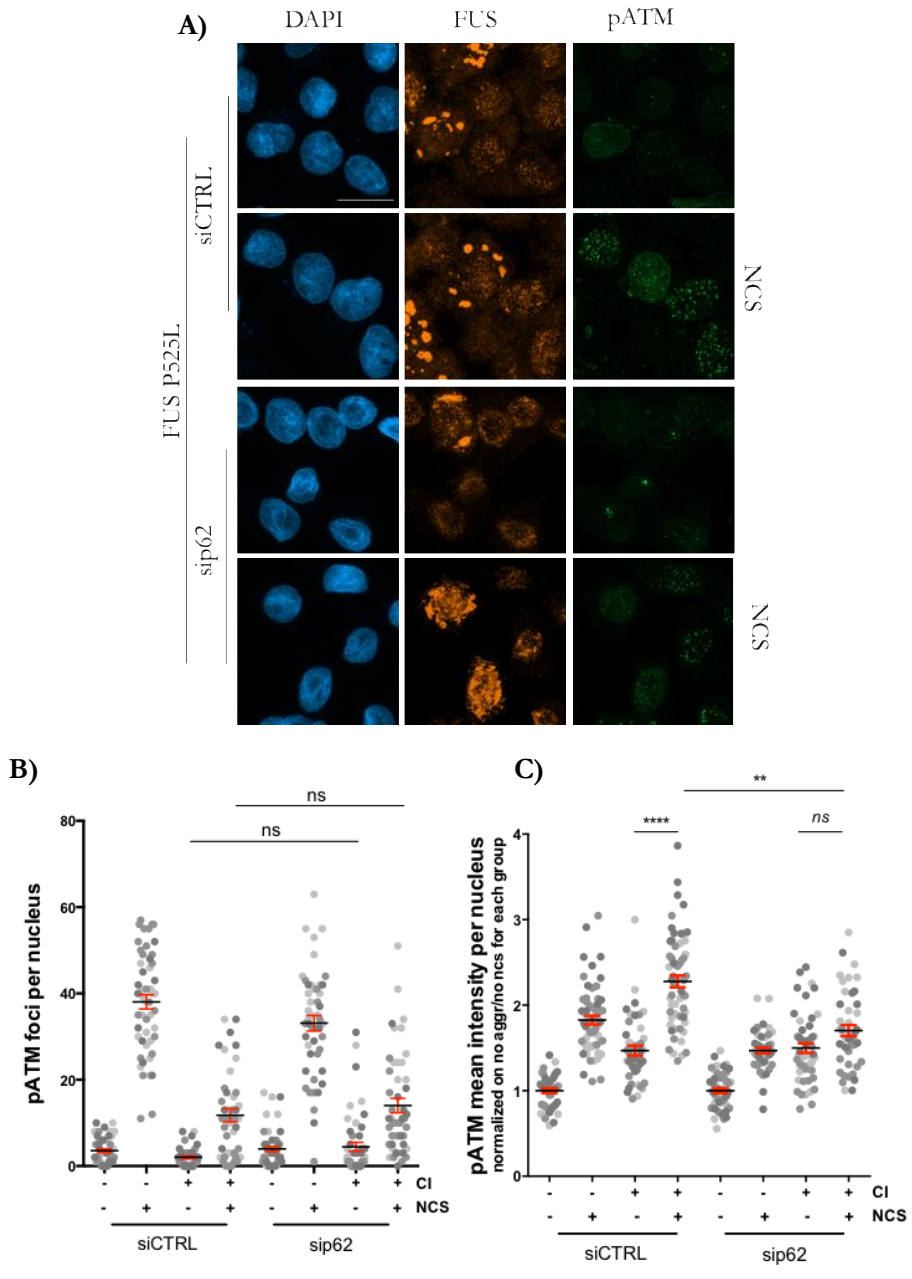
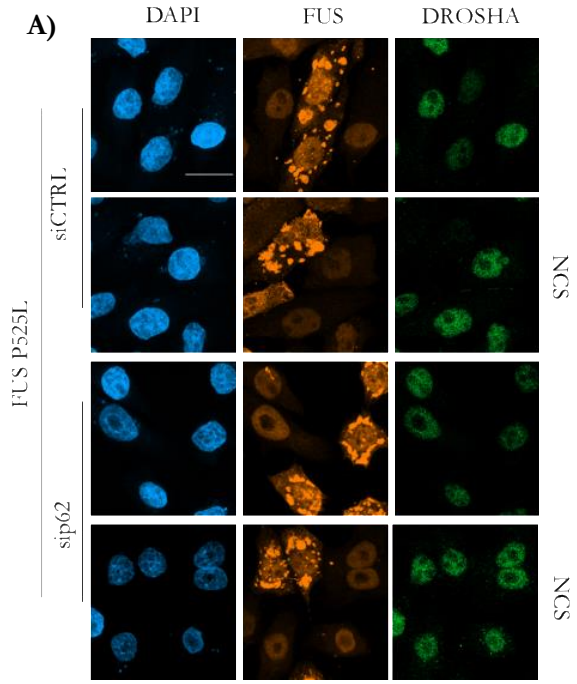

A)
DAPI
FUS
pATM
siCTRL
sip62
FUS P525L
NCS
NCS
B)
pATM foci per nucleus
ns
ns
CI
NCS
siCTRL
sip62
C)
pATM mean intensity per nucleus
normalized on no aggr/no ncs for each group
**
****
ns
CI
NCS
siCTRL
sip62

Figure 4.33. p62 depletion rescues DDR activation in cells with FUS CI. **A.** Imaging of HeLa cells expressing FUS-P525L immunostained for FUS and pATM in basal conditions or upon DNA damage induction in both siCTRL and sip62 transfection. Nuclei were counter-stained with DAPI. Scale bar: 20um. **B.** Counts of pATM foci and pATM mean intensity (C) in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.11. p62 DOWN REGULATION RESCUES DROSHA NUCLEAR PROTEIN LEVELS IN CELLS WITH MUTANT FUS CI

It has been observed that autophagy regulates DROSHA neuronal levels in Spinal Muscular Atrophy (SMA) (Goncalves et al. 2018). Therefore, we asked if autophagy deregulation in cells bearing mutant FUS CI could also control DROSHA protein level in our cellular system mimicking ALS proteinopathies. With this in mind we investigated if p62 depletion would restores DROSHA nuclear levels in cells bearing mutant FUS CI and as a consequence accumulation of p62 in cytoplasmic bodies, indicative of autophagy block. Thus we stained for DROSHA in cells expressing FUS P525L and knocked down for p62. As previously observed, upon control transfection with siCTRL, cells harbouring mutant FUS CI show significant reduction of DROSHA nuclear levels in both undamaged and damaged condition (Fig. 4.33.A-B). Instead, we found that p62 inactivation by siRNA knockdown ameliorates DROSHA nuclear depletion in both undamaged and upon NCS-mediated DNA damage induction (Fig. 4.33.A-B). Intriguingly we observed that in damaged cells DROSHA level was better rescued by p62 knockdown, again supporting the idea that DNA damage also modulate DROSHA protein level in a p62 dependent fashion. This set of data support the notion that DROSHA protein level might be regulated by p62 accumulation and autophagy block and that its down-regulation contribute to DDR impairment observed in cells with mutant FUS CI.


A)
DAPI
FUS
DROSHA
FUS P525L
siCTRL
sip62
NCS
NCS

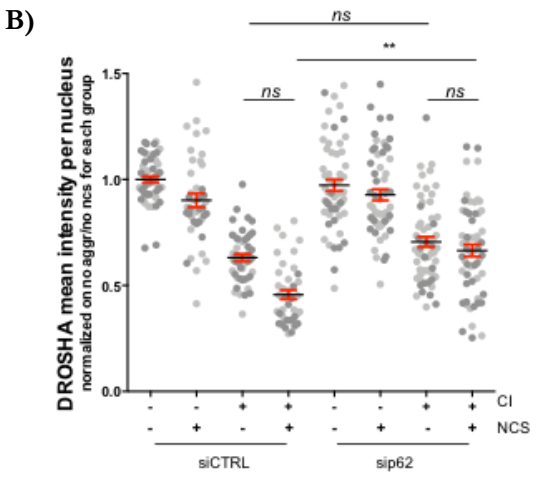

B)
ns
**
ns
ns
DROSHA mean intensity per nucleus
normalized on no aggr/no ncs for each group
1.5
1.0
0.5
0.0
CI
NCS
siCTRL
sip62

Figure 4.34. p62 depletion rescues DROSHA nuclear levels in cells with with FUS CI. **A.** Imaging of HeLa cells expressing FUS-P525L immunostained for FUS and DROSHA in basal conditions or upon DNA damage induction in both siCTRL and sip62 transfection. Nuclei were counter-stained with DAPI. Scale bar: 20um. **B.** Counts of DROSHA nuclear mean intensity in cells expressing FUS-P525L and separating cells with FUS inclusions from cells without FUS inclusions, in each indicated condition. Error bars represent SEM from three independent experiments. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001, **** P-value ≤ 0.0001.

4.12. P62 DOWN REGULATION STIMULATES THE SURVIVAL OF CELLS HARBOURING FUS P525L POSITIVE CI

As for all neurodegenerative diseases, ALS is characterized by the progressive death of neuronal cells (Taylor, Brown, and Cleveland 2016). As mentioned above ALS patient carrying FUS mutations show a significative increase of marker of DNA damage γH2AX in neurons (Naumann et al. 2018). Above we show that importantly, p62 down regulation can rescue 53BP1 foci, a DDR signalling reactivation, which reflects a reduction of γH2AX. In this scenario we wondered if upon p62 inactivation, a better DDR signalling could also stimulate the survival of cells with mutant FUS CI. This would point to a potential strategy for intervention in ALS, at least at the level of proof of concept. Thus we were very intrigued by the possibility that p62 inactivation might allow the survival of cells with mutant FUS proteinopathies and we monitored the survival of cells bearing mutant FUS CI after 24, 48 and 72 hours post transfection by counting the percentage of these cells in the population upon p62 knockdown or control siRNA transfected cells. Importantly, the percentage of cells bearing mutant FUS P525L CI is significantly higher in condition of p62 silencing (Fig. 4.34.). This result enhances the speculation that p62 could be directly involved in the formation of FUS CI since its depletion significantly increases the percentage of cells with those CI. We believe that p62 has a dual effect: in one hand it mediates the proper clearance of misfolded proteins thus its down regulation increases the amount of cells with FUS CI; on the other hand, the fact that p62 inactivation allows proper DDR signalling and reduced DNA damage accumulation in cells with FUS CI supports the survival of these cells. Both events explain the increase percentage of cells tolerating mutant FUS CI.

We observed that at 72h cells with CI decreased in both siCTRL and sip62 conditions and this could be due to the fact that cells devoid of CI can still proliferate until the end of the experiment while we showed that cells bearing CI are arrested. As expected, we indeed noticed that cells knocked down for p62 proliferate less than control cells. Thus to avoid any misinterpretation of our data due to different cells confluence we seeded cells differently (25% less in control condition respect to sip62 transfected cells). After 72h cells transfected with siCTRL show a comparable confluence to cells transfected with

sip62 allowing us to evaluate in a similar culture condition if sip62 stimulates the survival of cells with FUS CI.

This result strongly supports the model that cells with mutant FUS CI accumulate p62 protein which sequesters in the cytoplasm RNF168 and negatively impact on DDR signalling and DNA repair, ultimately causing cell death. Nevertheless, p62 inactivation is sufficient to reduce cell lethality allowing the survival of cells with FUS positive CI at different time points.

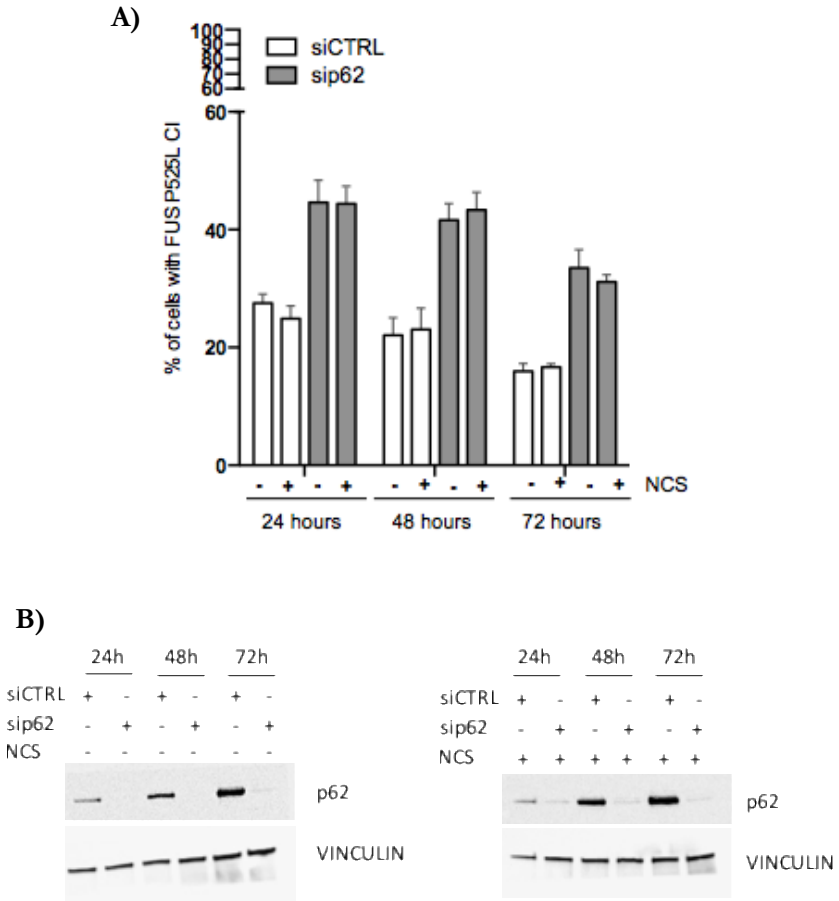


Figure 4.35. p62 depletion stimulates the survival of cells with FUS CI. A. Quantification of percentage of cells (calculated on the total population) harboring FUS CI at indicated time and treatments. Error bars represent SEM from three independent experiments. **B.** Western blotting oh HeLa cells treated with siCTRL and sip62 at indicated time where the last 24h cells were transfected with FUS-P525L and treated with NCS for 20 minutes.

5. DISCUSSION

Protein aggregation results in toxic effects especially when it occurs in neuronal districts and causes progressive cellular loss of function in a context were regeneration is limited (Lee et al. 2011). In fact, protein aggregation is one of the predominant hallmark of neurodegenerative diseases including AD, PD, HD and ALS (Ross and Poirier 2004). Disease-causing mutations play a pivotal role in protein aggregation by stimulating the alteration of protein stability or solubility, negatively impacting on the tendency of the protein to behave in a prion-like fashion (Lee and Yu 2005).

Various RNA-binding proteins involved in ALS-neurodegeneration like FUS, hnRNPA1 and TDP43 show a pronounced propensity to self-assemble (Maharana et al. 2018). Thanks to the interaction with RNA and component of stress granules (SG), these factors can undergo LLPS into condensate which may eventually lead to the formation of more solid amyloid like fibrils often very toxic for the cell (Aguzzi and Altmeyer 2016). While in normal condition the equilibrium between liquid and amyloid-like state is thinly regulated, disease-related mutations exacerbate the conversion in pathological amyloid aggregation or reduce the ability of the cell to clear these structure, thus causing cell death and neurodegeneration (Ramaswami, Taylor, and Parker 2013).

As discussed in the review I also contributed to write (Pessina et al. 2020), SGs are cytoplasmic membrane-less organelles (MLOs) believed to exploit the main function of slowing down mRNA translation and guarantee cell survival under stressful conditions (Protter and Parker 2016). Beyond their physiological relevance, SGs are becoming object of interest due to a proposed connection with the pathogenesis of various neurodegenerative diseases, including ALS /FTD spectrum. Often, indeed, these diseases harbour SG components co-localizing with TDP-43- and FUS-positive inclusions in patients neurons (Alberti and Dormann 2019; Wolozin and Ivanov 2019).

FUS is one of the better characterized intrinsically disordered RBPs involved in neurodegeneration. Mutations in both prion like domain and nuclear localization signal enhance FUS conversion from liquid to solid deposits (Guerrero et al. 2016). Particularly, the ALS- linked FUS P525L mutation strongly stimulates the recruitment into SGs in different cell system (Lenzi et al. 2015; Lo Bello et al. 2017; Marrone et al. 2018). More recently, also chaperone proteins have been associated with SGs dynamics (Liu et al. 2020) and interestingly, the phosphorylated form of Hsp27, present upon stress, is

actively recruited in FUS P525L-positive CI and the overexpression of phospho-Hsp27 strongly mitigates the amount of cells with FUS positive CI (Liu et al. 2020).

When nuclear DNA is damaged, cells promptly activate a concerted signalling cascade of DDR in order to recognize the damage and coordinate its repair. FUS has been acknowledged as an important player in DNA damage repair. FUS is recruited to DNA lesions by interacting with PAR chains (Mastrocola et al. 2013) and this interaction facilitates the compartmentalization of damage DNA into liquid structures (Pessina et al. 2020; Naumann et al. 2018). Moreover, FUS was shown to play a direct role in DNA repair since it promotes the recruitment of XRCC1/ligase III repair complex to damaged chromatin and it is involved in chromatin changes since it interacts with HDAC1 at DSBs (Pessina et al. 2020; Wang et al. 2013).

Recently our group and other showed that DDR foci are in fact LLPS compartment driven by the interaction of 53BP1 with chromatin and RNA (Kilic et al. 2019; Pessina et al. 2019) thus suggesting that altered FUS LLPS in ALS-linked mutation, might also altered the formation of reversible and functional liquid DDR foci. Importantly, DNA damage generation has been shown to change the interactomes of FUS (Kawaguchi et al. 2020). This may also imply that DNA damage generation could establish interaction of mutant FUS with other key DDR components possibly leading to their sequestration into more fibrillary-like solid condensate inactivating them. Many evidences have shown that FUS-NLS mutations are associated with the significant increase of DNA damage both *in vitro* (Naumann et al. 2018; Wang, Guo, et al. 2018) and *in vivo* (Qiu et al. 2014) suggesting that DNA repair is strongly impaired in presence of FUS-NLS mutations, including FUS P525L. Importantly, this occurs also in condition of heterozygosis, suggesting that more than a loss of function this mutation causes a gain of toxic function as also described for other ALS linked mutations (Farg et al. 2017). However, the mechanism by which cells harbouring FUS P525L mutation and its cytoplasmic de-localization impact on DDR signalling and DNA repair is poorly understood. In the present PhD thesis we used HeLa cells transfected with a plasmid expressing FUS-P525L mutation, or WT as control to tackle the molecular mechanism by which the formation of mutant FUS CI can lead to loss of genome integrity. After testing different cell lines, we noticed that HeLa cells are the best tool to investigate DDR in cells bearing mutant FUS CI since they have a good transfection efficiency and grow in monolayer with a fairly flat morphology, thus allowing good imaging quality of both nucleus and cytoplasm. Indeed, different groups before us used HeLa as a cellular model system to study the impact of mutant FUS CI on different aspect of cellular metabolism, relevant for ALS (Baron et al. 2013; Dormann et al. 2010; Liu et al. 2020). Importantly, the fact that HeLa are tumour cell line allows a better

tolerance of high amount of DNA damage, thus the possibility for us to study the molecular mechanism behind its formation in living cells. We also generated a stable cell line bearing an inducible FUS mutated gene under doxycycline control, however the percentage of cells bearing CI observed in this inducible system for the expression of mutant FUS, was even lower than the one obtained by transient transfection, thus reducing the number of cells useful for our analyses. We are aware of using only one cell system could represent a limitation of the present study however we could refer to a strong literature supporting the validity of this approach (please see before) and we worked on the establishment of motorneurons cell lines derived from human IPSc although we are still managing rule out several technical issues in order to use them for our purposes. Moreover, upon FUS P525L transfection only 20-30% of cells show formation of FUS CI thus limiting the application of any biochemical (e.g. western blots) or functional (e.g. reporter assays to measure DNA repair) approaches. In this regard, we mainly used single cell imaging approaches in order to better characterize the impact of FUS CI on DDR.

In line with previous literature, the ectopically expression by transient transfection of a plasmid expressing FUS P525L in HeLa cells, significantly enhances the formation of FUS CI compared to FUS WT expressing cells and, most importantly, they co-localize with two well established stress granule markers thus mimicking the behaviour of FUS CI detected in tissues of ALS patients and giving us the chance to further investigate DDR-related phenotypes in a cell system that reproduces some aspects of the pathology.

Firstly, we noticed that cells bearing mutant FUS CI present high level of nuclear DNA damage marker γH2AX. Importantly, the presence of the mutant protein is not sufficient to induce genotoxicity, while only the formation of CI induces it. After less than 24 hour of FUS P525L expression, roughly 20-30% of cells present mutant FUS CI and specifically these cells accumulate γH2AX in basal condition, meaning in the absence of exogenous DNA damaging treatment. This suggests that an acute DNA damage is generated endogenously. Importantly, FUS-P525L expressing cells present a subpopulation with an higher tail moment respect to FUS WT expressing cells as detected by comet assay in neutral condition in order to separate DNA fragments induced by DSB independently from the presence of SSBs (Olive and Banath 2006), demonstrating the presence of physical DNA damage in a fraction of nuclei, which nicely reflects the amount of cells harbouring FUS positive CI. Moreover, γH2AX signal of cells with FUS CI was drastically reduced after inhibiting ATM and DNA-PK, and not ATR, indicating that kinases activated by DSB are indeed the ones responsible for H2AX

phosphorylation in these cells. Consistently, the diffuse and nuclear wide activation of ATM in cells with mutant FUS CI was also confirmed by directly analysing the distribution of pATM. Others in the literature have reported that, in addition to be required for γH2AX generation within discrete DDR foci around individual DSBs (Harper and Elledge 2007; Lavin 2008; Meek, Dang, and Lees-Miller 2008), ATM and DNA-PK kinases can be responsible for the generation of pan nuclear γH2AX signal after the clustering of DNA lesions. Thus the observation that γH2AX is associated with generation of physical DNA damage does not exclude the possibility that also chromatin conformation changes could be responsible for diffused ATM activation and consequent γH2AX spreading. Indeed ATM activation has been reported to occur also upon chromatin compaction (Burgess et al. 2014). This aspect is under study in our laboratory at the moment.

We spent some time trying to address the possibility that γH2AX signal was due to replication stress or activation of the apoptotic programme, but our results described above strongly indicate that this is not the case.

Importantly, the treatment with the DNA damaging agent NCS, a radiomimetic drug used to generate DNA DSBs (Banuelos et al. 2003; Kuo, Meyn, and Haidle 1984; Segal-Raz et al. 2011) revealed that in cells with mutant FUS positive CI, DDR activation, as detected by DDR foci formation, was compromised at different levels. Indeed, the accumulation within DDR foci of both ATM and the downstream DDR mediator 53BP1 was selectively impaired in cells harbouring mutant FUS CI. By confocal analyses of a single plane, we confirmed that the formation of mutant FUS CI do not result in clearance of endogenous nuclear FUS, suggesting that the loss of DDR foci in these cells is not due to the loss of function of endogenous FUS and is instead more likely explained by a gain of toxic function, a model often formulated to explain other phenotypes associated with ALS pathogenesis (An et al. 2019; Sharma et al. 2016). Indeed, the incorporation of FUS into CI appear to cause genome toxicity related to impairment of DDR signalling and DNA repair.

In recent year my group discovered a novel class of small ncRNA defined DDRNAs that are directly involved in the first steps of DDR activation (Francia et al. 2012). Particularly, DDRNAs are processed by DICER and DROSHA endonucleases, which are historically involved in miRNAs biogenesis. Moreover, in one hand FUS is involved in DROSHA complex (Gregory et al. 2004) and facilitates DROSHA loading at chromatin thus stimulating miRNA biogenesis in neuronal cells (Morlando et al. 2012). On the other

hand, defects in miRNAs generation were widely reported in ALS (Emde et al. 2015). In this scenario, it would be interesting to address if both DROSHA and DICER are compromised in terms of proteins levels, activity or localization in cells with FUS positive CI. Indeed, a possible explanation is that the alteration of the expression level or activity of both endonucleases affects DDRNA biogenesis and thus DDR activation and DDR foci formation in cells with FUS positive CI. Previously in fact we observed that ATM diffuse activation was induced upon acute treatment with DRB, a transcription inhibitor that block DDRNA biogenesis (Michelini et al. 2017). Accordingly, a decrease in DICER activity has been already associated to the generation of SGs including TDP-43 (Emde et al. 2015). Thus we analysed DROSHA and DICER cellular level in cells bearing mutant FUS CI. None of these factors nevertheless were recruited into mutant FUS positive CI and DICER cytoplasmic levels were unaffected (data not shown). Instead, nuclear level of DROSHA was strongly reduced in bearing mutant FUS positive CI. The mechanism by which DROSHA expression level is reduced still remains uncertain. One possible explanation correlates DROSHA decrease with activation of the p38 MAPK and calpain protease, or to proteasome-mediated degradation under stress (Ye et al. 2015). However we didn't observed any difference in terms of p38 MAPK activation thus ruling out the possibility that DROSHA was degraded by calpain (data not shown). Moreover, proteasome inhibition by MG-132 does not rescue nuclear DROSHA levels upon transfection suggesting that DROSHA protein is not degraded in a proteasomal dependent fashion. Treatment with MG132 is also known to inhibit 53BP1 formation thus impeding us to use this approach for DDR studies (Hu et al. 2014). Possibly, DROSHA could be degraded through the autophagy pathway, as reported for Spinal Muscular Atrophy (SMA) motor neurons (Goncalves et al. 2018) and this could be responsible also for the cell retention of SGs, since their resolution is mediated by autophagy (Buchan et al. 2013) and indeed autophagy is impaired in ALS (Protter and Parker 2016) and we showed that p62 inactivation can partially restore DROSHA nuclear signal. Nevertheless, the reduction of DROSHA nuclear levels suggests that DDRNAs biogenesis could be strongly dampened in cells harbouring FUS positive CI upon FUS P525L overexpression. To address this question we set up a dedicated protocol able to detect DDRNAs in cells transfected with FUS P525L taking advantage of specific cell system where the site of damage is sequence specific (Lemaitre et al. 2014; Soutoglou et al. 2007). By this tool, we observed a significant reduction of DDRNAs upon cut induction in cells expressing FUS P525L compared with control cells, thus confirming that indeed DROSHA nuclear levels reduction observed in cells with mutant FUS positive CI negatively impact on DDRNAs synthesis that in turn impairs 53BP1 formation in those cells.

The prompt activation of DSB repair mechanisms is extremely important to avoid chromatin rearrangements, which are associated with tumorigenesis, aging and aberrant development of the nervous and immune systems (Jeggo and Lobrich 2007; McKinnon and Caldecott 2007). Among the DDR cascade signalling, the histone ubiquitination mediated by RNF168 represents a crucial step and stimulates the recruitment of downstream factors like 53BP1 and BRCA1, required for activation of DNA repair pathways (Stewart et al. 2009). The pivotal role of histone ubiquitination-RNF168 mediated in DDR is represented by the RIDDLE syndrome, a severe disease characterized by radio sensitivity, sysmorphic features, immunodeficiency and also learning difficulties (Stewart et al. 2007). The RIDDLE syndrome shares few neurological phenotypes with A-T although cells derived from patient affected by this syndrome show predominantly reduction of 53BP1 and BRCA1 at site of damage while MDC1 and NBS1 are still recruited (Stewart et al. 2007). Particularly, RNF168 nuclear depletion leads to a significant reduction of nuclear FK2 foci and consequent impairment of 53BP1 recruitment to DSB upon IR (Stewart et al. 2009). In our study we observed that cells harbouring mutant FUS CI show reduced capacity to mount distinguishable FK2 nuclear foci and remarkable absence of 53BP1 foci that possibly suggests loss of the correct chromatin ubiquitination, which in turn affects the activation of DNA repair mechanism. Indeed, cells with FUS positive CI present a significant RNF168 nuclear depletion compared to cells without CI and noteworthy, upon DSBs induction, cells do not show detectable RNF168 foci likely leading to impairment of the DDR signalling. Moreover, we did not observe a reduction in MDC1 foci in agreement with the fact that the DDR defects are RNF168-mediated that occur downstream MDC1.

Many evidences correlate neurodegeneration and autophagy pathway dysfunctions (Fujikake, Shin, and Shimizu 2018; Nixon 2013). Particularly in ALS patients the accumulation of autophagosomes in the cytoplasm of spinal cord neurons suggests that the autophagy dysfunctions are involved in the pathophysiology of ALS (Sasaki 2011). In this regards, the expression of FUS P525L mutation is associated with the inhibition of autophagosome formation and the accumulation of p62 (Soo et al. 2015). Besides, the accumulation of cytoplasmic protein aggregates or autophagy defects are associated with p62 accumulation (Korolchuk, Menzies, and Rubinsztein 2009; Wang et al. 2016). Interestingly our results clearly show that the p62 accumulation is an event strictly related to the formation of FUS positive CI.

A plethora of cellular events appears to be affected in ALS disease including defects in misfolded protein clearance by macro-autophagy(Walker and El-Khamisy 2018). The cargo protein p62 plays a crucial role in macro-autophagy by binding to misfolded

proteins and targets them for final degradation (Bjorkoy et al. 2005). Recent evidences indicate that in cancer an increased level of p62 negatively regulate DNA repair pathway by depleting nuclear levels of RNF168 (Wang et al. 2016). The LIM-binding (LB) domain of p62 regulates the role of this protein in inflammation response and through NF-κB factor (Feng and Longmore 2005) and more recently Wang and colleagues show that the p62-LB domain is able to bind to the MIU domain of RNF168 which is also essential for its E3 ligase activity and this event result in RNF168 sequestration and DDR signalling impairment (Wang et al. 2016). The overexpression of p62 leads to reduced RNF168 nuclear signal, 53BP1 foci reduction (Wang et al. 2016) and in this thesis we also show that p62 overexpression leads to γH2AX accumulation. Intriguingly, we observed that cells harbouring FUS positive CI upon FUS P525L expression show a significant accumulation of p62 in the cytoplasm. Besides, RNF168 show a peculiar behaviour in cells with FUS positive CI since it is distinctly detectable in cytoplasmic bodies instead of being localized in the nucleus as occurs in the surrounding cells without CI. In this regards, our results indicate that the RNF168 cytoplasmic signal preferably co-localizes with p62 bodies while remarkably it never co-localizes with FUS positive CI.

To address the key role of RNF168 in this model, we overexpressed RNF168 with FUS P525L in the attempt to complement the lack of nuclear RNF168 in cells with FUS positive CI. Indeed, the rescue of RNF168 nuclear level obtained by its overexpression in cells with FUS CI showed a clear rescue of 53BP1 foci upon exogenous DNA damage and as a consequence the re-activation of proper DNA repair significantly ameliorates γH2AX accumulation. The significant reduction of pATM diffused signal suggests that the restoration of functional DDR signalling and possibly DNA repair is sufficient to block the chronic stimulation of the ATM activity. In addition, RNF168 overexpression restores also DROSHA nuclear levels restoring also DDRNAs biogenesis in cells with FUS positive CI. Similar results were obtained by overexpression of RNF8 suggesting that also RNF8 might be affected in cells bearing mutant FUS CI

The functional link between DROSHA and RNF168 have been recently put forward by a study from Martin Bushell laboratory which shows that DROSHA knockdown leads to a reduction in RNF168 foci (Lu et al. 2018). This indicates that also in our cellular system DROSHA depletion induced by formation of mutant FUS CI could co-operates in reducing RNF168 foci formation. On the other hand, nobody has proposed yet if also the reverse is real meaning if RNF168 level can regulate DROSHA nuclear localization. Intriguingly it has been recently proposed that DGCR8, a co-factor of DROSHA is a target of USP51, a de-ubiquitinase enzyme counteracting RNF168 activity in DDR

(https://www.researchsquare.com/article/rs-47767/v1). To address if DROSHA nuclear depletion observed in cells with FUS CI could be responsible for RNF168 loss we tested whether DROSHA-flag overexpression in cells with FUS CI could rescue RNF168, 53BP1 foci formation and reduce γH2AX. However, we observed that cells with FUS CI that overexpress DROSHA-flagged version do not show rescue of RNF168 or 53BP1 foci with consequent detection of high level of γH2AX in those cells (data not shown). These results suggested us that restoration of DROSHA level per se is not sufficient to restore RNF168 nuclear level, possibly because the sequestration of RNF168 by p62 bodies is still active and stronger in these cells.

In the attempt of restoring RNF168 protein level in cells with mutant FUS CI, we knocked down the p62 and excitingly we observed that p62 depletion indeed significantly restores RNF168 nuclear foci consequent 53BP1 foci rescue and reduction of γH2AX nuclear signal in cells with FUS positive CI. Moreover, p62 downregulation also ameliorates pATM hyper activation in those cells possibly as a result of reduced DNA damage accumulation. Instead, we couldn't detect any rescue of pATM foci, a result in line with the literature showing that RNF168 acts downstream to ATM (Stewart et al. 2009). Importantly, p62 knock down also restore DROSHA nuclear levels in cells with FUS positive CI, particularly upon DNA damage induction supporting the model by which DROSHA might be depleted in a autophagy-dependent fashion, especially in damaged cells. We have already planned to measure DDRNAs levels in cells expressing FUS P525L in the context of p62 depletion which might also stimulates DDRNAs biogenesis.

Finally, we tested if the restoration of proper DDR signalling and reduction of γH2AX upon p62 depletion could stimulate the survival of cells harbouring FUS CI. Importantly, this evidence could indicate that p62 inactivation can reduce neuronal cell death in ALS patient with FUS P525L mutations. Thus, we counted the amount of cells with FUS CI at 24, 48, and 72h after p62 depletion, all receiving 24h of FUS P525L overexpression. Excitingly, we observed that p62 downregulation significantly stimulates the survival of cells with FUS CI at all three time points. Indeed, p62 depletion leads to an increase of cells with FUS CI in the population, which are double in percentage compared to control cells. These results strongly support the p62 inactivation could enhance survival of cells affected by FUS CI.

Overall our data suggest that different aspects of ALS pathogenicity like protein aggregation, autophagy defects and DNA damage accumulation are intrinsically

connected and as a whole contribute to disease progression. Our conclusions are also in line with what previously reported by Walker and colleagues in the context of *C9orf72* repeats expansions which negatively impact on ATM signalling, a defect that can be ameliorated by p62 depletion and RNF168 overexpression (Walker et al. 2017). Apparently, autophagy defects leading to DNA repair inefficiency and DNA damage accumulation can be considered as new common features of ALS pathology not only restricted to familiar ALS cases bearing the mutations FUS P525L.

This novel point of view open to a wide spectrum of potential therapeutically approaches also taking the advantage of the deeply knowledge of DDR signalling obtained in the context of cancer. Indeed, recently drugs that act as specific autophagy activator have been proposed as a promising therapeutically methodology for counteracting protein aggregation in neurodegenerative diseases and our study suggests that targeting autophagy could also enhance DNA repair efficiency leading to neuronal cell survival.

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ABBREVIATIONS

53BP1: p53-binding protein 1

A-T: Ataxia Telangiectasia

AD: Alzheimer's disease

ALS: Amyotrophic Lateral Sclerosis

alt-NHEJ: Alternative non-Homologous end Joining mechanism

AOA2: Ataxia-ocular Apraxia Type 2

ARS: Sodium Arsenite

ASOs: Antisense Oligonucleotides

ATLD: rare A-T like disease

ATM: Ataxia Telangiectasia-mutated

ATR: ATM and Rad3-related

BER: Base Excision Repair

BRCA1: Breast Cancer Type 1 Susceptibility protein

C9orf72: Chromosome 9 open-reading frame 72

Cdk5: Cyclin-dependent kinase

CDKN1A: Cyclin-Dependent Kinase Inhibitor 1A

CHK1/CHK2: checkpoint kinases

CMA: chaperone mediated autophagy

CtlP: C-terminal binding protein

D-loop: Displacement-loop

DAPI: 4'-6'-Diamidino-2-phenylindole

DDR: DNA Damage Response

DDRNAs: DNA Damage Response RNAs

diRNAs: DSB-induced RNAs

dlincRNAs: Damage-induced long non-coding RNAs

dNTP: Deoxynucleotide Triphosphates

DPR: Dipeptide Repeats Protein

DSBs: Double-Strand Breaks

dsDNA: Double Strand DNA

fALS: Familiar ALS

FTD: Frontotemporal Dementia

FUS: Fused in Sarcoma

GEF: Guanine-Nucleotide Exchange Factor:

HR: Homologous Recombination

IDR: Intrinsically Disorder Regions

IR: Irradiation

L3MBTL2: Lethal(3)malignant brain tumor-like 2 protein

LCDs: Low Complexity Domains

LLPS: Liquid-liquid phase separation

MDC1: Mediator of DNA damage checkpoint protein

miRNA: micro-RNA

MMEJ: Microhomology-mediated end-joining

MMR: Mismatch Repair

MRN: MRE11-RAD50-NBS1 complex

NCI: Neuronal CI

ncRNA: non-coding RNAs

NER: Nucleotide excision repair

NHEJ: Non Homologous End Joining mechanism

PAR: Poly ADP-ribose

PARP1/2: Poly(ADP)ribose Polymerase 1/2

PD: Parkinson's disease

PIKKs: phosphatidylinositol 3-kinase-like protein kinases

PTMs: Post Translational Modifications

RBPs: RNA Binding Proteins

RNAPII: RNA Polymerase II

RNF8: Ring Finger Protein 8

RNF168: Ring Finger Protein 168, E3 Ubiquitin Protein Ligase

RNP: Ribonucleoprotein

ROS: Reactive Oxygen Species

RPA: Replication protein A

sALS: Sporadic ALS

SGs: Stress Granules

SSA: Single-Strand Annealing

SSBR: Single-Strand Break Repair

SSBs: Single-Strand Breaks Double-Strand Breaks

ssDNA: Single Stranded DNA

tASOs: Telomeric Antisense Oligonucleotides

TCR: T-cell Receptor

TDP-43: Tar DNA Binding protein 43

TIAR: TIA-1 related protein

tDDRNAs: Telomeric DDRNAs

tncRNAs: Telomeric Non-Coding RNA

UV: Ultraviolet

APPENDIX

Some parts of this thesis are contained in the Future Review "DNA Damage triggers a new phase in neurodegeneration" published in Trends in Genetics, 2020.

I have performed most of the experiments presented in this thesis although few results were generated by other colleagues in my group. Particularly, Comet assay and DDRNAs detection (Fig. 4.4 and 4.26) were performed by Dr Ubaldo Gioia (IFOM, Milan). Confocal acquisition and post-imaging analysis of co-localizations (Fig. 4.2 and Fig. 4.18) were generated by Dr. Anna Garbelli (IGM-CNR, Pavia). Western blot analysis and quantification showed in Fig. 4.5 (E-F)) and Fig 4.34. were carried out by Francesca Esposito (IGM-CNR). Martina Battistoni (IGM-CNR, Pavia) performed BrdU assay showed in Fig. 4.6 (C). Finally, Claudia D'Urso (IFOM, Milan) performed MDC1 staining presented in Fig. 4.12.

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Feature Review

DNA Damage Triggers a New Phase in Neurodegeneration

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Subcellular compartmentalization contributes to the organization of a plethora of molecular events occurring within cells. This can be achieved in membraneless organelles generated through liquid–liquid phase separation (LLPS), a demixing process that separates and concentrates cellular reactions. RNA is often a critical factor in mediating LLPS. Recent evidence indicates that DNA damage response foci are membraneless structures formed via LLPS and modulated by noncoding transcripts synthesized at DNA damage sites. Neurodegeneration is often associated with DNA damage, and dysfunctional LLPS events can lead to the formation of toxic aggregates. In this review, we discuss those gene products involved in neurodegeneration that undergo LLPS and their involvement in the DNA damage response.

Highlights

Intracellular compartments can assemble as membraneless organelles through a demixing process known as 'liquid–liquid phase separation' (LLPS).

DNA damage response foci are membraneless structures fueled by LLPS of some DNA damage response factors and are modulated by noncoding transcripts synthesized at DNA damage sites.

Several forms of neurodegeneration are associated with, and possibly caused by, dysfunctional LLPS events, ultimately leading to the accumulation of toxic solid-like structures.

Emerging evidence links factors involved in LLPS events and neurodegeneration with the cellular response to DNA damage.

Being Liquid Helps to Focus

In response to a stimulus or an insult, molecular interactions and enzymatic reactions have to occur promptly within the cell. Equally quickly, once the stimulus ends, interactions have to disengage. Thus, cells constantly face the demanding task of organizing a variety of simultaneous molecular reactions, often in close spatial proximity, yet preserving their selectivity. To address this challenge, cells evolved compartments to facilitate spatiotemporal control of biological reactions. It is emerging that, in addition to confining specific events within organelles delimited by a lipid membrane, cells also exploit the properties of membraneless organelles (MLOs), coherent structures that can compartmentalize and concentrate selected molecules, thus favoring reactions. In the past few years, MLOs have been referred to with various terms, such as 'condensates' or 'droplets,' but essentially they are the result of demixing events due to **liquid–liquid phase separation (LLPS)** (see Glossary). The formation of MLOs relies on dynamic interactions among little structured proteins and/or nucleic acids, held together by weak intermolecular bonds, which generate a surface tension sufficient to induce phase separation [1]. Compared with a lipid membrane, surface tension between two phases has the advantage of insulating MLO content from molecules with different biophysical properties, yet granting transience and reversibility [2]. Protein components of MLOs generally contain **intrinsically disordered regions (IDRs)** or **low-complexity domains (LCDs)**, which are more prone to phase separate through their flexibility, lack of predetermined structure, and ability to engage multivalent weak interactions [3]. Although there is ample evidence that LCDs are able *per se* to phase separate *in vitro*, they are usually embedded as part of proteins also containing structured domains [2], raising the question whether such structured regions modulate their LLPS properties. This has recently been investigated for hnRNPA1, an RNA-binding protein (RBP) composed of a folded, structured module and an LCD [3]. Differently from the isolated LCD that undergoes LLPS at high salt concentrations, the full-length protein phase separates only at low ionic concentrations, and thanks to the interaction of its LCD with the RNA recognition motifs (RRMs), suggesting that folded regions in a protein contribute to modulation of LLPS [3]. Recently, a molecular grammar of phase separation has been proposed, indicating that the percentage and position of specific

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amino acids in the protein sequence can predict the ability of a protein to undergo LLPS more accurately than its disordered structure [4]. For example, tyrosine residues in **prion-like domains (PrLDs)** and arginine residues in folded RNA-binding domains establish multivalent interactions; thus, proteins with structured domains enriched in specific amino acids can be more prone to LLPS than a disordered region devoid of them [4].

Examples of MLOs in the nucleus are paraspeckles, bodies formed by the ubiquitously expressed long noncoding RNA NEAT1, involved in gene expression regulation [5]; Cajal bodies, centers of assembly and modification of spliceosomal **small nuclear RNPs** [6]; nucleoli, sites of ribosomal RNA transcription and assembly [7]; histone locus bodies, where histone mRNAs are transcribed [8]; and promyelocytic leukemia bodies, involved in multiple processes of genome homeostasis, including **homologous recombination** at telomeres, in some cancer cell lines [9]. MLOs in the cytoplasm include **processing bodies (P-bodies)**, condensates of enzymes involved in mRNA decay and microRNA (miRNA)-induced mRNA silencing, and **stress granules (SGs)**, which function mainly as reservoirs of untranslated mRNA (Box 1). Some MLOs are constitutive, such as nucleoli, whereas others are transient and need to be efficiently resolved to avoid pathological stabilization, as, for instance, SGs in neurodegeneration [10–12]. Although the number of reported examples of MLOs constantly increases, a need for more rigorous analyses and a careful choice of the techniques used to identify and define LLPS in the cell has recently been invoked [13].

Identification and analysis of MLOs in cells is in fact not always an easy task, and the simple model of a homogeneous MLO has proved to be inadequate to capture the variety of cellular MLOs. Indeed, some MLOs may exhibit multilayered structures with distinct coexisting liquid phases, thanks to different surface tensions, generating 'droplet within droplet' architectures [2,14], with some having partially solid-like portions [4]. A recent report that condensate can undergo a structural transition from a droplet-like to a hollow vesicle-like form, characterized by a rim and an internal lumen, seems consistent with this notion [15].

Box 1. Stress Granules

Stress granules (SGs) are cytoplasmic membraneless organelles (MLOs) with the main function of slowing down mRNA translation thought to guarantee cell survival upon different insults, such as viral infections, oxidative stress, heat shock, and increased osmolarity [64]. Fundamental constituents of SGs are polyadenylated RNAs, stalled translation complex, and various RNA-binding proteins (RBPs) which bear intrinsically disordered regions (IDRs) that promote extensive weak intermolecular interactions [65]. The RBPs T cell internal antigen 1 (TIA1) and RAS GTP-activating protein-binding protein 1 (G3BP-1) are constitutive SG components, intrinsically able to undergo liquid-liquid phase separation (LLPS) and considered the minimal set of factors needed to nucleate the formation of these MLOs in response to stress [140,141].

SG nucleation is initiated upon polysome dissolution following translation arrest. This exposes mRNA to TIA1 and G3BP-1 and favors their accumulation on the RNA [65]. SG then recruits additional intrinsically disordered RBPs, ultimately promoting phase separation [14,29]. Importantly, this process is reversible, and, once stress is resolved, recovery of translation causes an ATP-dependent SG disassembly [60,64,141].

Beyond their physiological relevance, SGs are becoming objects of interest due to a proposed connection with the pathogenesis of various neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) [60]. Affected tissues of the ALS/FTLD spectrum often harbor TDP-43- and FUS-positive inclusions colocalizing with SG components [10,65,140]. However, although SGs devoid of TDP-43 are reversible, TDP-43 incorporation makes them more persistent after stress removal. It has been reported that TIA1 mutations, in a subset of patients with ALS, are accompanied by less dynamic TDP-43-containing SGs [142]. A recent study found that mitoxantrone, a planar compound known to interact with RNAs, prevents the inclusion of TDP-43 into SGs, thus representing a potential therapeutic strategy for ALS/FTD [143].

Also, the microtubule-associated protein tau was observed to colocalize with TIA1 at SGs in both animal models and postmortem brain tissues of subjects with Alzheimer disease [63]. It has been proposed that interactions between SGs and MAPT, possibly due to the high RNA content present within SGs, stimulate MAPT LLPS and aggregations [66].

Glossary

Homologous recombination: the most accurate DSB repair process, active in the S–G_2 phases of the cell cycle because it uses homologous sequences on duplicated sister chromatids for precise repair.
Intrinsically disordered region (IDR): protein region that lacks a fixed and well-defined 3D structure.
Liquid–liquid phase separation (LLPS): a process by which a solution of proteins and/or nucleic acids condensates into liquid-like droplets that coexist with the surrounding diluted phase. LLPS can consist of three main steps: (i) nucleation, defined as the time required for the new phase to segregate; (ii) growth, the expansion of condensate dimension; and (iii) coarsening, which describes the increase of the average size and reduction of total number of droplets over time.
Low-complexity domains (LCDs): portion within a protein sequence characterized by the presence of short repeats of a few amino acid residues resulting in reduced amino acid variability compared with an average composition, often characterized by structural disorder.
Multivalency: tendency of different biologic polymers, such as proteins and RNAs, to form multiple noncovalent interactions that sharply increase the reciprocal binding avidity compared with the corresponding monovalent interactions.
Nonhomologous end joining (NHEJ): mechanism of DSB repair predominant in the G_1 phase of the cell cycle that involves the direct ligation of DNA ends having little or null homology, thus resulting in an error-prone process during which nucleotides can be lost or gained at the ends prior to ligation.
Pi interactions: a class of noncovalent chemical interactions associated with π (pi) systems, comprising several subtypes: pi-pi, cation-pi, C-H(pi), and so forth. All of them are attractive interactions due to the polarization of the electron cloud in pi systems mainly occurring either between adjacent aromatic residues or between the pi system of an aromatic residue and a nearby cation.
Poly(ADP-ribose) (PAR): a linear or branched polymer of adenosine diphosphate ribose synthesized from NAD^+ cofactor on target proteins by PARPs in response to DNA damage. PAR chains are removed by PAR glycohydrolase.

Given the potentially metastable nature of phase-separated MLOs, their liquid-like reversible condensed state can eventually evolve toward a more rigid, less dynamic state. This is a consequence of the formation of stronger ionic interactions between molecules, often organized in intermolecular beta sheets, leading to the formation of stable aggregates [16,17]. These phenomena have a relevant impact on pathologies caused by proteinopathies [10]. Intriguingly, an important role in the assembly of MLOs and in the regulation of their stability has emerged for RNA molecules. Local synthesis or recruitment of RNA molecules often controls condensation of MLOs [4,18].

The Role of RNA in Phase Transition

Even before the realization that MLOs result from LLPS events, several of them were reported to assemble in an RNA-dependent manner [19]. Nucleolar RBPs associated with RNA of transcriptionally active rDNA loci were known to form the nucleation point for nucleoli *in vivo* [20]. Indeed, transcriptional inhibition has been shown to result in the loss of nucleolar structural organization and delocalization of several nucleolar components [21]. Conversely, triggering ectopic transcription or concentrating RNA locally is sufficient to favor the formation of nuclear bodies, including paraspeckles, histone locus bodies, Cajal bodies, and nuclear stress bodies [22,23]. Still, several observations also point to an RNA-mediated increase in fluidity of MLOs, which counteracts a more unsafe liquid-to-solid phase transition [10,24]. This suggests that RNA can modulate the viscoelasticity of MLOs, inducing LLPS but preventing their more rigid stabilization. How this occurs remains to be fully understood [25].

As mentioned before, the forces driving the formation of LLPS are a set of intermolecular weak interactions that, acting as pulling forces, induce the demixing of a subset of molecules from a homogeneous solution [2,4,14]. These interactions are often favored by polymeric molecules such as RNA or DNA and by the similarly highly negatively charged **poly(ADP-ribose) (PAR)** chains [4,26]. Thanks to their repetitive and charged nature, these polymers act as concentrating agents and nucleation points that initiate phase separation of proteins [4,26]. In the case of SGs (Box 1), LLPS is strictly dependent on RNA. In particular, single-stranded, ≥250-nt-long RNA molecules were shown to boost **Ras GTPase-activating protein-binding protein 1 (G3BP-1)** phase transition in SGs, apparently regardless of any sequence specificity [27]. In more general terms, proteins containing IDRs often bear RNA-binding domains, too, leading to a cooperative effect favoring LLPS [4]. RNA promotes phase separation in a dose-dependent manner and, intriguingly, sometimes in a sequence-specific fashion, suggesting that LLPS condensates might bear specific densities based on the sequence of the RNA retained [28]. RNA concentration acts by lowering viscosity inside droplets, thus promoting more dynamic interactions and liquidity [10]. At super-stoichiometric ratios, RNA can trigger the formation of vesicle-like condensate with a lumen inside [15].

Nonetheless, it appears that there are exceptions, and *in vitro* studies with the P-granule RBP LAF-1 demonstrated that its phase separation is controlled by the combination of length, number of ionic interactions per molecule, and concentration of RNA molecules. Indeed, short RNA molecules decrease LAF-1 droplet viscosity, whereas the same mass of a longer RNA increases it [29]. Importantly, long G/C-rich transcripts have been found to trigger the formation of solid aggregates with toxic biological properties [30]. Beyond RNA avidity, other aspects determinant for LLPS are protein–protein interactions and multimerization. Nevertheless, the hierarchy of these components during LLPS is still unknown. A recent study characterized networks of RNA–protein interactions in reconstituted cytoplasmic SGs and P-bodies and contributed to better identifying what defines the composition and miscibility of MLOs [31]. It was observed that the IDR of G3BP-1 is dispensable for LLPS of SG (Box 1), whereas its dimerization and RNA-binding domains are essential. Upon RNA exposure, specific protein interaction networks involving G3BP-1 act as nodes of nucleation for

Post-translational modifications (PTMs): covalent chemical modifications that can control structure, function, and stability of the targeted polypeptide. Coordinated series of PTMs, involving phosphorylation, methylation, acetylation, ubiquitination, SUMOylation, and PARylation, allow several cellular pathways, including transcription and DDR to take place in a coordinated fashion. PTMs occurring at IDRs of various proteins tune their capacity to condensate.

Prion-like domain (PrLD): a low-complexity region found in RNA-binding proteins that undergoes liquid phase transitions that drive ribonucleoprotein granule assembly associated with the neurodegenerative disorder.

Processing bodies (P-bodies): a type of constitutive cytoplasmic ribonucleoprotein granules generated by LLPS, enriched in translationally repressed mRNA and proteins, mainly involved in mRNA decay. P-bodies can share components with SGs and are often placed nearby in the cytoplasm.

Ras GTPase-activating protein-binding protein 1 (G3BP-1): One of the main constitutive components of SGs essential for their condensation upon several type of stressors.

Ring finger protein 168 (RNF168): E3 ubiquitin protein ligase involved in histone and nonhistone protein ubiquitination in DDR signaling and transcriptional regulation. Particularly during DDR activation, RNF168 mediates the polyubiquitination at lysine 13 and lysine 15 on H2A and H2AX histone variants, necessary for the recruitment of 53BP1 and BRCA1.

R-loop: three-stranded nucleic acid structure constituted by an RNA-DNA hybrid and one displaced DNA strand generated by stalled transcription. It may influence transcription, DDR, and DNA repair. When R-loops are not properly resolved, they can lead to chromosomal instability.

Stress granules (SGs): cytosolic ribonucleoprotein granules generated through LLPS upon stress-dependent interruptions of the initial phases of protein synthesis. SGs are constituted by polyadenylated RNAs, stalled translation complexes, and various RBPs. Their composition strictly depends on both the cell type and the kind of stress inflicted, except for some constitutive components such as G3BP1 (Box 1).

phase separation, but this depends on the number of protein–RNA interaction interfaces that must be equal or greater than three (**multivalency**) [31]. According to this model, the binding of protein partners increasing the valence of G3BP-1–containing nodes favors SG assembly, whereas interactor proteins that decrease the overall multivalence reduce LLPS. Thus, competition between protein networks may represent a general mechanism by which cells tune LLPS of different MLOs [31], but much remains to be learned about how RNA controls viscosity and dynamics of condensates, including the contribution of RNA length and sequence.

Phase Separation Regulates the DNA Damage Response

When nuclear DNA is damaged, cells promptly activate a concerted signaling cascade called the 'DNA damage response' (DDR) (Box 2) in order to recognize the damage and coordinate its repair. The DNA damage sensor protein complex MRN (Box 2) was recently reported to recruit the RNA polymerase II (RNAPII) transcriptional machinery at DNA DSBs and promote, starting from exposed DNA ends, the bidirectional synthesis of damage-induced long noncoding RNAs (dilncRNA) (Box 3); these RNAs can be further processed by DROSHA and DICER endoribonucleases into shorter RNAs named 'DNA damage response RNA' (DDRNA) (Box 3) [32–34]. Promptly upon generation of DSBs, the Ataxia-Telangiectasia Mutated (ATM) kinase-dependent phosphorylation of the histone variant H2AX (known as γH2AX when phosphorylated) next to the DSB acts as a chromatin scaffold for the accumulation of several DDR factors, including 53BP1, a largely disordered protein.

Intriguingly, 53BP1 has been shown to accumulate at DSBs in a manner controlled by RNA [24,32–34] and to phase separate [24,35]. By fluorescence recovery after photobleaching analysis of 53BP1 foci in cells expressing 53BP1-GFP, it was observed that they display liquid-like behavior, as shown by a fast and homogeneous recovery within seconds after bleaching [24]. Viscosity of

Box 2. DNA Damage Response and Repair

Double-strand breaks (DSBs) are toxic DNA lesions that may lead to cellular senescence, apoptosis, or chromosomal instability. Therefore, cells have evolved a coordinated surveillance system called the 'DNA damage response' (DDR) to detect the damage and send signals to stop normal cell cycle activities, prioritizing DNA repair [144,145]. DSBs are repaired by two main repair pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR) [146].

Upon DNA damage, exposed DNA ends are immediately recognized by two protein complexes: the Ku70-Ku80 heterodimer (Ku) and MRE11-RAD50-NBS1 (MRN) [147,148]. MRN functions as a platform for the activation, through autophosphorylation, of ATM, a phosphatidylinositol 3-like kinase, which in turn phosphorylates the histone variant H2AX at its Ser139, named γH2AX. The γH2AX signal spreads for hundreds of kilobases from the DSB [149]. γH2AX is bound by the DDR factor MDC1, forming a scaffold that promotes a number of post-translational modifications on the chromatin, including histone ubiquitination and methylation, leading to the recruitment of several additional DDR factors, including 53BP1 [50,150]. Mono- and polyubiquitination at the site of damage are mainly deposited by RNF8 and RNF168 E3 ubiquitin ligase [151]. Several copies of these factors are recruited at individual lesions, leading to the formation of microscopically detectable foci. In particular, 53BP1 recruitment at DSB counteracts DNA nucleolytic degradation required for HR, thus orienting DNA repair toward NHEJ.

The formation of DDR foci in the nucleus prompts a set of actions that decides the fate of a cell. If DNA damage is repaired, this causes DDR foci resolution, but in case of irreparable damage, persistent DDR foci consequently form and enforce a permanent proliferation arrest known as 'cellular senescence' [152].

In NHEJ, the DNA-end binder Ku recognizes and stabilizes exposed DNA ends and recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) complex that coordinates the activities of NHEJ repair factors. Among these, specialized ligases seal the DSB [148]. A number of accessory factors support and regulate NHEJ, including 53BP1.

HR instead requires the formation of single-stranded DNA (ssDNA) at the DSB through a process called 'DNA end resection.' In eukaryotes, resection is initiated by MRE11, the MRN subunit retaining nuclease activity [147]. This nuclease function of MRE11 acts in concert and is regulated by the presence of Ku and replication protein A [153,154]. The recombinase RAD51 is then loaded onto the ssDNA generated to form a nucleoprotein filament that invades the homologous DNA region that is used as a template for new synthesis of the resected DNA [148].

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Box 3. DNA Damage Response and RNA

There is a complex cross-talk between DNA damage response (DDR) and RNA metabolism [155]. Mounting evidence indicates the direct involvement of noncoding RNAs (ncRNAs) generated locally in DDR activation [155,156]. Indeed, the formation of a double-strand break (DSB) triggers the assembly of a functional RNA polymerase II (RNAPII) complex by recruiting the complete preinitiation complex, MED1, and CDK9 [24]. Importantly, this recruitment depends on the MRE11-RAD50-NBS1 complex [33], providing a mechanistic link between the DDR machinery and the assembly of the transcriptional apparatus at the DSB.

RNAPII loading at DSBs results in the transcription of a few kilobases long ncRNA molecules named 'damage-induced long noncoding RNAs' (dilncRNA) [33]. In S/G_2 phase cells, dilncRNA can form RNA-DNA hybrids at resected DNA ends, a structure that favors the recruitment of homologous recombination (HR) factors [30,157]. dilncRNA can be further processed into small ncRNAs called 'DNA damage response RNAs' (DDRNA) by the RNA machinery factors DROSHA and DICER [32]. A study in *Arabidopsis thaliana* and human cells showed that, upon DSB generation, small RNAs are produced in an ATR-, DICER-, and AGO2-dependent manner [156] and act in association with AGO2 protein [158] and take part in HR and nonhomologous end joining repair [157]. A recent study based on deep sequencing of endogenous RNA generated at multiple DSBs mostly occurring at ribosomal DNA loci in human cells identified two populations of damage-induced ncRNA: one expressed at low level and required for DNA repair and a secondary amplified population loaded onto Argonaute proteins [160]. Once generated, DDRNAs act locally at the site of break through base pairing with complementary dilncRNA [30]. RNA synthesis and DDRNA–dilncRNA annealing support the nucleation of DDR foci downstream of H2AX phosphorylation [36]. Indeed, preventing dilncRNA and DDRNA synthesis by transient RNAPII inhibition or preventing DDRNA–dilncRNA pairing by the use of specific antisense oligonucleotides (ASOs) is sufficient to suppress DDR foci formation and DNA repair, demonstrating that dilncRNA and DDRNA, rather than the mere recruitment of the transcriptional machinery, modulate DDR foci formation and stability [33]. Consistently, DDR foci are sensitive to RNA degradation, similarly to other MLOs, such as nucleoli and SGs [32,40,41].

dilncRNA and DDRNA are induced by *de novo* transcription also at damaged or dysfunctional telomeres [34] with important implications for the use of ASOs with telomeric sequences as a potential therapeutic approach for the treatment of pathologies associated with telomere dysfunction, as demonstrated in a mouse model of Hutchinson-Gilford progeria syndrome [137].

53BP1 foci is similar to that of glycerol. Live-cell analysis of 53BP1 individual foci showed that the biophysical properties of 53BP1 foci, such as their morphology and their dynamics (nucleation, growth, and coarsening), display features characteristic of liquid droplets. In addition, accurate surface tension measurements of 53BP1 foci demonstrated values similar to those reported for P-granules. Importantly, dilncRNA/DDRNA inhibition by antisense oligonucleotide (ASO) disrupts 53BP1 foci following the physical laws of a liquid, not a solid, object, further demonstrating their liquid nature. Finally, incubation of chromatinized linear DNA fragments with extracts from cells expressing 53BP1-GFP generates GFP-positive liquid droplets in a manner dependent on H2AX and transcription of the DNA template. Both *in vivo* and *in vitro*, 53BP1 foci are sensitive to chemicals reported to dismantle LLPS events [24].

Experiments in HeLa and U2OS human cells, in NIH2/4 murine cells, and in an *in vitro* system recapitulating the coordinated recruitment of DDR factors at chromatinized DNA ends, mimicking DSBs, demonstrated that neither γH2AX nor RNA is individually sufficient to support the formation of 53BP1 foci, which instead depends on the presence of both [24,36]. 53BP1 foci formation is also dependent on H2AK13/15ub histone modifications, generated by the E3 ubiquitin ligase **ring finger protein 168 (RNF168)** and methylation of H4K20me2, a modification constitutively present on chromatin that becomes accessible upon damage [37,38]. It is possible that the deposition of **post-translational modifications (PTMs)** on the chromatin surrounding the DSB and the synthesis of dilncRNA and DDRNA, although to a great extent independent events, can mutually support each other because some PTMs have been shown to depend on transcription [39] and to have a role in transcription next to DSBs [33]. Although the different contributions of chromatin modifications and dilncRNA/DDRNA remain to be studied, it is possible that modified chromatin may act as a beacon for the initial recruitment of DDR factors, whereas locally generated dilncRNA/DDRNA may act by retaining them, thus modulating the progression of nucleation, growth, and condensation of DDR foci [24] (see Figure 1 for schematic representation).

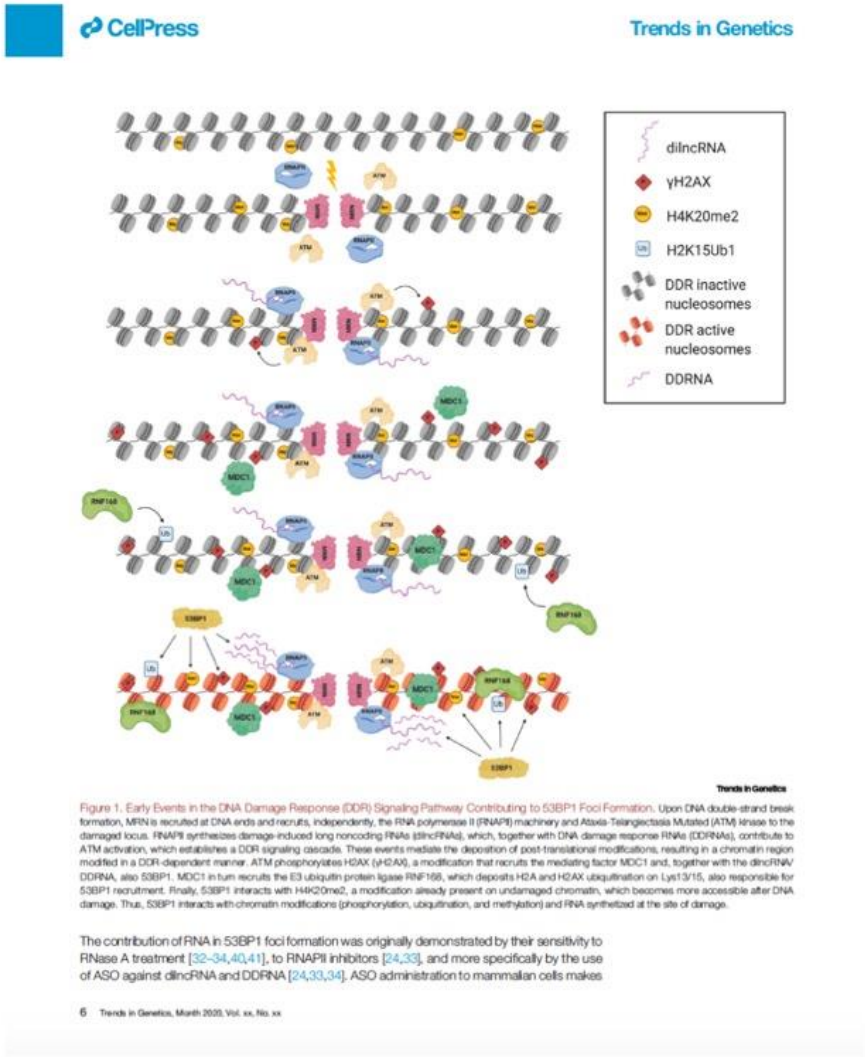

Figure 1. Early Events in the DNA Damage Response (DDR) Signaling Pathway Contributing to 53BP1 Foci Formation. Upon DNA double-strand break formation, MRN is recruited at DNA ends and recruits, independently, the RNA polymerase II (RNAPII) machinery and Ataxia-Telangiectasia Mutated (ATM) kinase to the damaged locus. RNAPII synthesizes damage-induced long noncoding RNAs (dilncRNAs), which, together with DNA damage response RNAs (DDRNAs), contribute to ATM activation, which establishes a DDR signaling cascade. These events mediate the deposition of post-translational modifications, resulting in a chromatin region modified in a DDR-dependent manner. ATM phosphorylates H2AX (γH2AX), a modification that recruits the mediating factor MDC1 and, together with the dilncRNA/DDRNA, also 53BP1. MDC1 in turn recruits the E3 ubiquitin protein ligase RNF168, which deposits H2A and H2AX ubiquitination on Lys13/15, also responsible for 53BP1 recruitment. Finally, 53BP1 interacts with H4K20me2, a modification already present on undamaged chromatin, which becomes more accessible after DNA damage. Thus, 53BP1 interacts with chromatin modifications (phosphorylation, ubiquitination, and methylation) and RNA synthetized at the site of damage.

The contribution of RNA in 53BP1 foci formation was originally demonstrated by their sensitivity to RNase A treatment [32–34,40,41], to RNAPII inhibitors [24,33], and more specifically by the use of ASO against dilncRNA and DDRNA [24,33,34]. ASO administration to mammalian cells makes

already formed 53BP1 foci disappear. Importantly, all these events can be recapitulated in an *in vitro* system of reconstituted damaged chromatin [24].

At its C-terminus, 53BP1 has been found to interact, directly or indirectly, with dilncRNA and DDRNA through its Tudor domain [33], a conserved motif of 50 amino acids found in several RNA-associated proteins [42]. Consistently, the Tudor domain not only is an important part of the minimal region of 53BP1 necessary for its recruitment to DDR foci but also undergoes LLPS [35,43]. This domain is, perhaps surprisingly, at the carboxy-terminal end of 53BP1 and thus distinct from the largely disordered amino-terminal portion. By taking advantage of optogenic systems to study LLPS in cells expressing fluorescent 53BP1, a recent study identified the segments of 53BP1 mainly controlling its liquid-like behavior [35] and confirmed that the unstructured N-terminus is dispensable for droplet coalescence, strengthening the notion that disordered regions may not always cause LLPS. Instead, the oligomerization domain (OD) and the BRCA1 C-ter domain (BCRT), as well as most of the C-terminal region rich in tyrosine and arginine residues, were all required for droplet formation, highlighting the relevance of multivalency in LLPS. In particular, the OD, fairly conserved among 53BP1 orthologs, has been widely reported to exert a crucial role for 53BP1 homodimerization, DSB recognition, and LLPS droplet formation [35,43]. Mutagenesis studies demonstrated that deletion of the OD resulted in abrogation of both 53BP1 recruitment to DNA damage sites and droplet formation [35,43]. The minimal focus-forming region of 53BP1 hosts a GAR motif that, upon methylation at its arginine residues by PRMT1 methyltransferase, has been suggested to confer 53BP1 DNA-binding abilities [44], although this has been challenged in later studies [43]. Intriguingly, the segment comprising the 53BP1 GAR motif has also been demonstrated to take part in LLPS along with the OD [35]. In the same way, the C-terminal tandem BRCT domains, besides mediating 53BP1–p53 interaction [45], were also shown to positively contribute to 53BP1 liquid demixing [35] (see Figure 2 for schematic representation).

In summary, the ability of 53BP1 to undergo LLPS seems to be dependent on a number of elements, namely RNA and deposition of phosphate, ubiquitin, and methyl groups on chromatin (see Figure 3 for schematic representation). In addition, weaker **pi interactions** (pi–pi and cation–pi interactions) provided by amino acid residues are important and can be tuned through PTMs to modulate viscosity of 53BP1 and accessibility to damaged chromatin. Indeed, the 53BP1 C-terminus is enriched in arginine, tyrosine, and lysine residues able to form pi–pi and cation–pi interactions and to regulate 53BP1 oligomerization and binding to RNA, DNA, and PTMs of damaged chromatin. Intriguingly, tyrosine residues and their interactions with arginines have been found to be essential for phase separation of FET proteins (FUS, EWSR1, TAF15) [46]. Interestingly, FUS, an RBP involved in transcription regulation and RNA metabolism, was the first reported example of a protein undergoing LLPS at DSBs [47]. It does so in a PAR-dependent manner [47]; PAR chains are deposited quickly in proximity to DSBs by poly(ADP-ribose) polymerases (PARPs), and, given their similarity with polymeric RNA structure and shared negative charge, it is tempting to imagine that they also favor LLPS. Indeed, PARs have also been shown to promote a chromatin environment permissive for transcription and RNAPII recruitment [48], although the role of poly(ADP-ribosylation) on transcription DSBs is likely complex, with evidence demonstrating that it can recruit transcriptional repressive elements such as Polycomb, components of the nucleosome remodeling and deacetylase complex, and the macroH2A1.1 histone variant [49]. Thus, it is possible to imagine a potentially coordinated set of events in which an early and local PARylation occurring in the region immediately flanking the damage stimulates RNAPII activity, contributing to LLPS events within DDR foci, while the following spreading of PAR chains on damaged chromatin instead reduces surrounding gene transcription.

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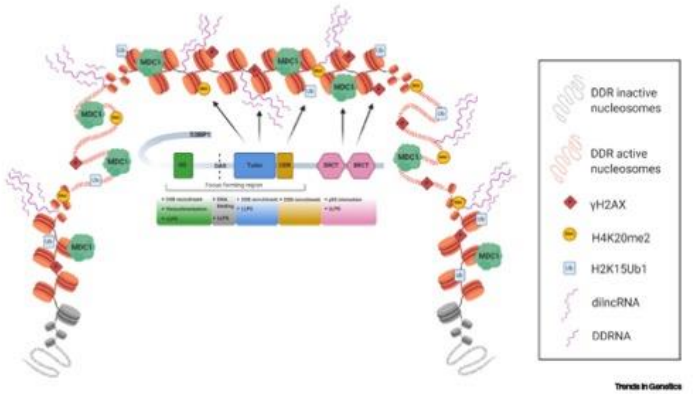

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Figure 2. C-terminal Region of 53BP1 With Different Domains and Their Functions Highlighted. DNA damage response (DDR) active chromatin provides multiple sites of interaction with 53BP1, which not only recruits it but also drives its retention and accumulation at sites of damage, thus facilitating its liquid–liquid phase separation (LLPS). Several domains and motifs present in the 53BP1 C-terminus contribute to its behavior: The oligomerization domain (OD), the glycine-arginine rich (GAR) motif, the Tudor domain, and the ubiquitination (Ub)-dependent recruitment (UDR) region compose the minimal focus forming region of 53BP1 and are crucial for its recruitment to DNA double-strand breaks (DSBs) and/or for its liquid behavior. The tandem BRCT domain, which mediates interaction with p53, also plays a role in 53BP1 LLPS by enhancing its ability to form droplets. Dimethylated histone 4 on lysine 20 (Me), a constitutive chromatin modification which becomes unmasked following DNA damage, contributes to 53BP1 recruitment via interaction with the Tudor domain.

Although the role of 53BP1 in DNA repair has been addressed in several studies [50], the study of the contribution of 53BP1 LLPS to DNA repair would benefit from a specific inhibitor. However, so far, LLPS inhibitors are fairly blunt tools, unable to target events specifically, and thus one can only speculate that the DNA repair defects, **nonhomologous end joining (NHEJ)** in particular, observed upon LLPS inhibition may be ascribed to 53BP1 known functions, namely long-range DNA repair events and inhibition of DNA end resection, although a role played by other DDR factors also undergoing demixing cannot be excluded.

Overall, the interplay between DNA damage, RNA, and phase separation of RBPs provides a hint of how mechanisms and players described in the previous text may be relevant in pathological events.

Phase Separation in Neurodegenerative Diseases: A DNA Damage Connection

A number of intrinsic and extrinsic causes may trigger phase transition from a liquid-like to a less dynamic, solid-like state with the consequent formation of pathological aggregates in the cell [10]. Accumulation of such aberrant structures is a hallmark of several neurodegenerative disorders, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Parkinson's disease (PD) [12,51]. The potential contribution of DDR activation to the regulation of this transition and the impact that formation of solid aggregates has on the maintenance of genome stability are discussed in the following portion of this review.

Accumulation of DNA lesions and defects in DNA damage repair have been observed in the nervous system of individuals affected by neurodegeneration. Over the last two decades, mounting evidence has indicated the potential contribution of genome maintenance mechanisms to the onset and progression of neurological degenerative diseases [52–55]. Specifically, mutations predisposing to ALS were shown to alter DDR activation, impair repair, and increase DNA damage accumulation in motor neurons derived from patients induced pluripotent stem cells and in spinal cord tissues [56–58]. Although a causative role of DSB in the etiopathology of AD and PD remains to be conclusively demonstrated, multiple lines of evidence clearly indicate that increased levels of DSBs and/or their imperfect repair are associated with these disorders [53]. Indeed, primary cells cultured from patients with HD presented an elevated sensitivity to different DNA damage sources [55]. Hence, unsurprisingly, factors found to be altered in neurodegenerative pathologies are often also involved in DDR.

Altered LLPS Is Involved in Neurodegeneration

ALS

ALS is a severe progressive syndrome characterized by a loss of motor neurons that leads to the death of patients. Frequent mutations associated with the risk of developing ALS fall within genes encoding for proteins involved in RNA metabolism, including TDP-43, FUS, Ataxin-2, and hnRNPA1 [59]. Intriguingly, all these proteins contain IDRs and bear the ability to phase separate, as by observed in *in vitro* experiments [16,60–62]. Although most of these factors exert their roles mainly within the nucleus, they also take part in the assembly of SGs (Box 1) [63,64].

FUS is one of the better characterized intrinsically disordered RBPs involved in neurodegeneration. Its IDRs are prone to aggregate like prions; therefore, they are collectively called 'prion-like domain' (PrLD) [65]. Mutations in its PrLD or nuclear localization signal enhance FUS conversion from liquid to solid deposits [66]. FUS controls many aspects of RNA biogenesis, ranging from transcription to RNA processing [67]. For example, it has been implicated in miRNA maturation because it promotes DROSHA recruitment on nascent miRNA primary transcripts [68]. Besides its multiple functions in RNA biology, FUS has been acknowledged as an important player also in DNA damage repair and in telomere maintenance [69,70]. Indeed, FUS is readily recruited to DNA lesions by interacting with PAR chains, accumulating at damaged chromatin [71]. FUS–PAR interaction facilitates the compartmentalization of damaged DNA into liquid-like structures. This process *in vivo* is thought to favor DNA damage resolution [47,72]. Indeed, FUS was shown to play a direct role in DNA repair by promoting the recruitment of the XRCC1/ligase III repair complex to damaged chromatin, and its nuclear loss caused DNA ligation defects and accumulation of single-stranded DNA damage [73]. The role of FUS in DNA repair may also involve chromatin changes because FUS was reported to interact with HDAC1 at DSBs [74]. The liquid state of FUS, in turn, seems to be directly controlled by DDR kinases because phosphorylation of FUS by DNA-PK, a protein directly involved in DNA repair, prevents its liquid-to-solid state transition and formation of fibril-like structures *in vitro* and in cells [75]. However, the protective effect of DNA-PK–mediated phosphorylation of FUS on its LLPS and nuclear functions is controversial because other reports showed that DNA-PK stimulated nuclear clearance of FUS by inducing its translocation to the cytoplasm and initiating a pathological, solid-like transition [76,77]. Most intriguingly, the very same mutations turning liquid-like FUS into pathological fibrillar deposits [16,78] have been correlated with defective DDR and DNA damage accumulation in ALS motor neurons [73,77]. These results therefore suggest that neurotoxicity of FUS aggregates, observed in patients with ALS, may be ascribed to their detrimental impact on genome maintenance.

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in the presence of RNA [97,98,100]. Importantly, the capacity to undergo LLPS is crucial for tau to exert its functions in microtubule polymerization [99]. Nevertheless, mutations or their hyperphosphorylation may turn tau liquid droplets into stable aggregated forms, thought to contribute to AD pathogenesis [97]. The nuclear functions of tau have only recently been explored. A protective role against DNA damage has been proposed for tau because its loss was found to sensitize neuronal cells to different kinds of DNA-damaging events [101,102]. Interestingly, DDR activation has been reported to promote nuclear translocation and dephosphorylation of tau, suggesting that DDR signaling engagement could counteract the neurotoxic assembly of hyperphosphorylated tau [101–103]. However, it remains to be established if chronic DNA damage generation and thus persistent DDR activation may act differently, possibly negatively, on the physiology of cells and if the formation of tau tangles found in AD is the cause or the consequence of impaired DDR and damage accumulation.

Another piece of evidence linking LLPS, DNA damage, and neurodegeneration involves α-synuclein, an intrinsically disordered DNA- and RNA-binding protein that is the main component of Lewy bodies, a hallmark of neuropathology in PD [104]. Recent evidence describes its ability to condensate into liquid-like droplets both *in vitro* and in cells [105]. Importantly, a familial mutation associated with early PD onset, as well as oxidative stress, seems to favor α-synuclein liquid-to-solid transition over time. Such findings, along with the observations that α-synuclein colocalizes with DDR foci and that its loss leads to DSB accumulation in mouse cortical neurons [106], together with the fact that defective repair is sufficient to elicit α-synuclein stress [107], suggest an intriguing link between pathological α-synuclein and defective DNA damage repair.

HD

In HD pathogenesis, nucleotide repeat expansion plays a causative role. Specifically, the extent of CAG repeat amplification, occurring in the first exon of the huntingtin gene (*HTT*), determines both the severity and the age of onset of HD [108]. A well-known consequence of this trinucleotide expansion is the production of a mutant truncated huntingtin protein (mHTT) that contains a long tract of glutamines (polyQ) [109]. Recently, a novel mechanism involving LLPS has been proposed for conferring pathogenicity to mHTT [110]. The propensity of mHTT to undergo LLPS depends on the polyQ length when the polyQ tract expands beyond the threshold length associated with HD such that the liquid-like assemblies of mHTT are irreversibly converted into ordered fibrillar structures *in vitro* and in cells [110]. Intriguingly, aberrant DDR activation and DNA repair defects reported in HD correlate with the expression of polyQ-containing mHTT [111–113]. Mechanistically, mHTT, but not its wild-type form, was shown to directly interact *in vivo* with Ku70, NHEJ factor [111], and hamper DNA-PK activity, thus inhibiting DNA repair and causing DSB accumulation in neurons [111]. These findings highlight the importance of the ability of polyQ-rich IDR of mHTT in helping neuronal cells to sense and resolve DNA damage. Besides mHTT, uninterrupted amplification of CAG repeats in *HTT* can encode for additional polypeptides, characterized by long stretches of the same residue, an extreme example of low-complexity proteins [114], that are associated with cell death and tend to accumulate within dense aggregates in the brain of patients with HD [115]. Nevertheless, CAG repeats *per se* could contribute to the onset of HD, possibly more than polyQ-rich peptides [116]. Whether such repeats and/or polypeptides could affect DNA damage repair is yet to be investigated.

A Role of RNA Phase Separation in DDR in Neurodegeneration

Although protein-only LLPS events are most frequently discussed, it has lately become evident that RNA not only is a critical player in regulating phase separation of ribonucleoparticles and several cellular MLOs [117,118] but also retains the intrinsic ability to undergo gelation *in vitro* in the absence of protein cofactors [30]. Intriguingly, it has been shown that repetitive G/C-rich RNA

molecules, such as those transcribed from *HTT* and *C9ORF72* genes, are *per se* prone to form liquid-like condensates. Strikingly, this tendency occurs only when the number of repeats exceeds the threshold that corresponds to the onset of the disease [30,119]. Furthermore, overexpression of RNAs containing a pathological number of G_4C_2 repeats was sufficient to promote SG assembly independently of their coding properties [119], pointing to the possibility that toxic RNA molecules could be responsible for the pathology, regardless of their translation products. A potential explanation for the pathogenicity of the G_4C_2 repeat-containing RNAs in ALS has recently been attributed to their harmful effects on genome integrity [56]. Such aberrant RNAs indeed have the tendency to form R-loops [120], which are observed accumulating at the *C9ORF72* locus in ALS motor neurons, ultimately driving DSB accumulation and consequent neurodegeneration [56].

Concluding Remarks

MLOs are useful tricks that cells exploit to compartmentalize biological activities and multitask in an efficient manner. However, their tendency to become solid-like aggregates with time or accelerated by mutations or other events impairs proper regulation of their metastability, posing a potential threat to the life of cells and the organism as a whole. Why, then, did evolution select such systems? It may be another example of antagonistic pleiotropy, previously also invoked to explain the lack of selection of genes with detrimental effects during aging [121,122]; thus, MLOs may have a complex set of functions and consequences. Liquid-like organelles are particularly abundant in the nucleus [123]. In this context, the phase separation of 53BP1 at DSBs is intriguingly emerging as a new MLO. It has been reported that DSBs trigger both chromatin compaction [124,125] and chromatin relaxation [126], transcription burst [24,33,127], and transcription inhibition [128]. Because multiple compartments with different surface tensions may coexist, transcription burst and inhibition may be regulated in different compartments. It is possible that the role of LLPS at sites of damage is to orchestrate the occurrence of these different biochemical reactions, seemingly at the same time and at the same genomic locus (see Outstanding Questions). Indeed, the dynamic and fluid compartments associated with active transcription, formed in proximity to the break, are in agreement with the notion that elements of active promoters or enhancers do phase separate [129] and accumulate at DNA lesions [24].

It is noteworthy that RNA generation at DSBs and its role in fueling LLPS within DDR foci may also contribute to isolating individual DNA lesions and preventing dangerous translocations and unscheduled recombination events by controlling immiscibility of different DDR foci generated from different RNA sequences. Only when damage persists may heterochromatin-associated PTMs be deposited and transcription suppressed, potentially allowing persistent DDR foci to merge [130]. Intriguingly, if, as reported for LAF-1 [26], RNA length can influence droplet viscosity, with long RNA increasing it and short RNA reducing it, it is tempting to imagine dilncRNAs and DDRNAs, their shorter processed forms, being able to differentially modulate DDR foci properties, perhaps over time.

Dysfunctional phase transition of key MLOs, proteins, and RNAs has been acknowledged as a major driving force in the development of neurodegenerative diseases [10]. Therefore, molecular approaches aimed at reverting toxic liquid-to-solid conversion of factors involved in such pathologies are now being explored as potential therapeutics. Intriguingly, some of the drugs found to be effective at restoring physiological LLPS also target crucial factors in DDR and DNA repair. For example, DNA-PK inhibition was shown to decrease cytoplasmic FUS accumulation and recover proper FUS localization at damaged DNA [76,77]. Boosting PARylation by administrating PAR glycohydrolase inhibitors to iPSC-derived motor neurons carrying FUS cytoplasmic aggregates also rescued FUS nuclear localization and restored DNA repair, ultimately counteracting motor

Outstanding Questions

How does LLPS affect DDR signaling and neurodegeneration?

What is the interplay between transcription and chromatin PTM deposition during LLPS at sites of damage?

What is the link between the aberrant liquid-solid transition of MLOs and genome damage during neurodegeneration?

Can small molecules be identified to specifically inhibit selected LLPS events, despite often involving unstructured proteins?

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neuron degeneration [77]. However, inhibiting PARylation has proved effective at restoring proper TDP-43 LLPS in *Drosophila* and mammalian cells, without disturbing SG formation [131]. Recently, molecules able to target LLPS *per se* have attracted the attention of academics and biotechnology companies, and some interesting candidate molecules have been identified and been shown to have promising therapeutic effects in neurodegenerative models [132,133]. Enoxacin, a small molecule recently found to stimulate DDR activation and NHEJ-mediated repair by increasing RNA-assisted 53BP1 nucleation at DSBs [134], has also been shown to ameliorate defects in neuromuscular functions of ALS mouse models [135]. Finally, the use of ASOs disrupting toxic condensation of G/C-rich RNAs associated with repeat expansion disorders, such as *C9ORF72* and *HTT* transcripts [30,136], represents another promising opportunity, currently already pursued by some biotechnology companies, for the treatment of these diseases [132]. The efficacy of this approach mirrors that observed for 53BP1 foci disruption by ASO against dilncRNA/DDRNA [24,33,34,137].

The exact etiology of sporadic neurodegenerative disorders remains elusive. Liquid-to-solid transition and dysfunctional DDR and DNA damage accumulation are often observed in these pathologies. Here, we have discussed how DDR and LLPS reciprocally control each other. However, whether altered DDR regulation is the cause or the consequence of pathological aggregate formation remains to be firmly established. Neurodegeneration is tightly intertwined with aging and generally manifests at late stages during the human lifespan [138]. It is well documented that irreparable DNA damage and persistent DDR engagement play causative roles in cellular senescence and aging [139]. This suggests that disorders in DDR may accelerate the formation of toxic aggregates and DNA damage retention, ultimately accelerating neuronal cell death. Therefore, future efforts are needed to shed light on the mechanisms leading to DNA damage accumulation in the context of neurodegeneration.

Author contributions

F.P., U.G., So.F., and F.d'A.d.F. structured the review. F.P., U.G., O.B., St.F., and So.F. wrote the text. M.C. integrated the text during revision. F.P., St.F., and So.F. generated Figures 1 and 3. M.C., St.F., and So.F. generated Figure 2, and O.B.

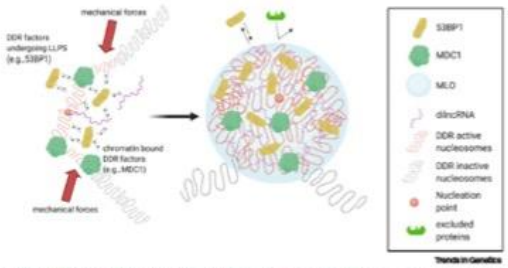

Figure 3. RNA and Modified Chromatin Promote Membraneless Organelle (MLO) Formation. Starting from the nucleation point (orange sphere) provided by the DNA double-strand break, the synthesis of damage-induced long noncoding RNAs (dilncRNA/DNA damage response RNA [DDRNA]) and different chromatin modifications establishes the chemical forces that promote the accumulation and retention of DNA damage response (DDR) factors, some of which undergo liquid–liquid phase separation (LLPS) and generate an MLO.

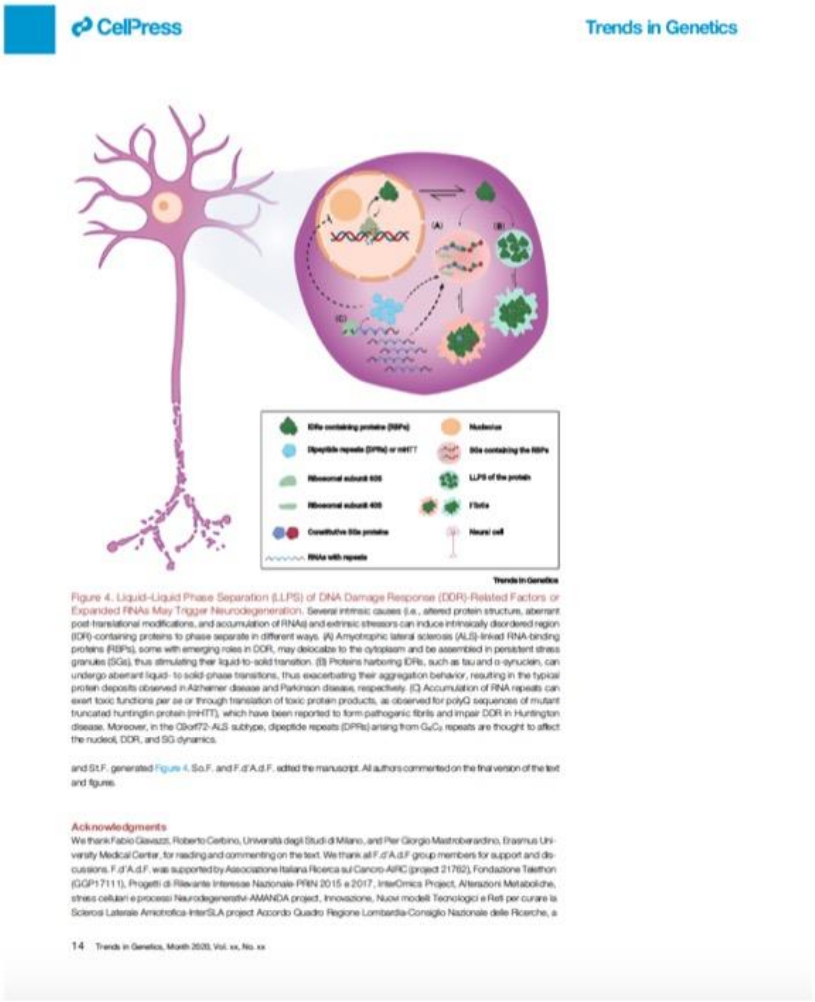

Figure 4. Liquid–Liquid Phase Separation (LLPS) of DNA Damage Response (DDR)-Related Factors or Expanded RNAs May Trigger Neurodegeneration. Several intrinsic causes (i.e., altered protein structure, aberrant post-translational modifications, and accumulation of RNAs) and extrinsic stressors can induce intrinsically disordered region (IDR)-containing proteins to phase separate in different ways. (A) Amyotrophic lateral sclerosis (ALS)-linked RNA-binding proteins (RBPs), some with emerging roles in DDR, may delocalize to the cytoplasm and be assembled in persistent stress granules (SGs), thus stimulating their liquid-to-solid transition. (B) Proteins harboring IDRs, such as tau and α-synuclein, can undergo aberrant liquid- to solid-phase transitions, thus exacerbating their aggregation behavior, resulting in the typical protein deposits observed in Alzheimer disease and Parkinson disease, respectively. (C) Accumulation of RNA repeats can exert toxic functions *per se* or through translation of toxic protein products, as observed for polyQ sequences of mutant truncated huntingtin protein (mHTT), which have been reported to form pathogenic fibrils and impair DDR in Huntington disease. Moreover, in the C9orf72-ALS subtype, dipeptide repeats (DPRs) arising from G_4C_2 repeats are thought to affect the nucleoli, DDR, and SG dynamics.

and St.F. generated Figure 4. So.F. and F.d'A.d.F. edited the manuscript. All authors commented on the final version of the text and figures.

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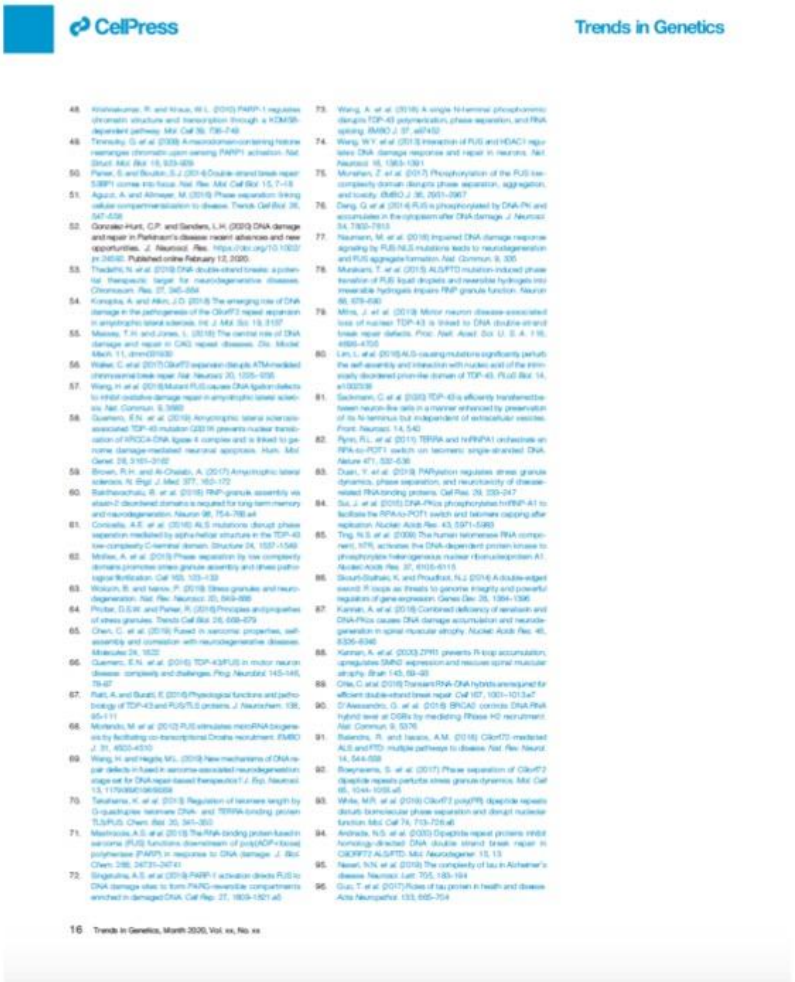

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