UNIVERSITA' DEGLI STUDI DI PAVIA

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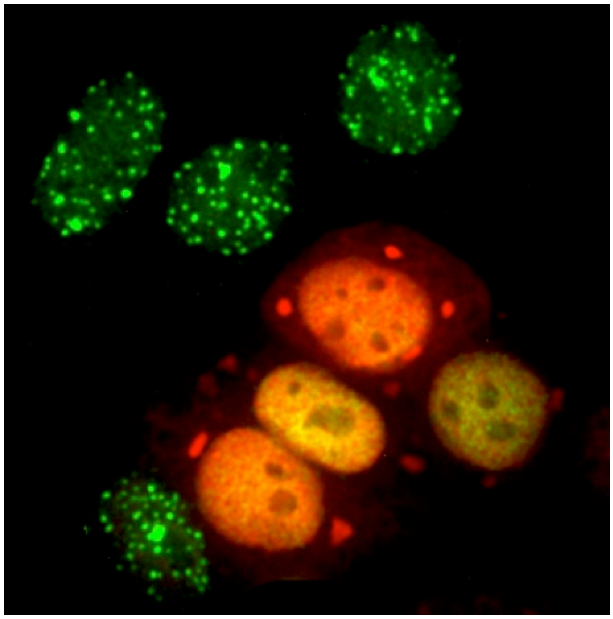

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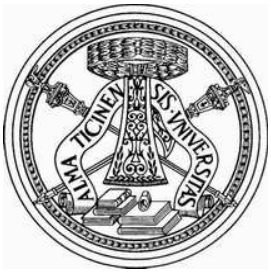
Ciclo XXXI - A.A. 2015-1028



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Supervisors: Dott. Sofia Francia and Prof. Antonio Torroni

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Abstract

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease affecting upper and lower motor neurons and leading to the progressive paralysis of almost all skeletal muscles and to death within 3-5 years from the diagnosis. Both sporadic and familial ALS forms (sALS and fALS) exist, the latter of which are dominantly transmitted and represent only 10% of all ALS cases. A certain fraction of both sALS and fALS forms is associated to mutations within genes implicated in RNA metabolism. One ALS-linked gene is TARDBP that encodes for the TDP-43 DNA/RNA binding protein. Moreover, common pathological hallmarks of both fALS and sALS are cytoplasmic neuronal inclusions containing TDP-43. For this reason ALS and the related disorder frontotemporal lobar degeneration are also known as TDP-43 proteinopathies.

ALS neurons accumulate oxidative DNA damage and DNA breaks, hazardous events that healthy cells are able to efficiently counteract by activating a set of molecular mechanisms known as DNA-damage response (DDR). A key role in DDR has been recently ascribed to a class of small non-coding RNAs named DNA damage response RNAs (DDRNAs) homologous to DSBs sites, whose biogenesis is dependent on the RNases DICER and DROSHA. Importantly, TDP-43 itself interacts with DICER and DROSHA boosting their enzymatic activity during small RNAs biogenesis. In this perspective, my PhD project aims to understand whether TDP-43 has a role in the DDR process and if its ALS-related mutations or its recruitment into cytosolic inclusions could hinder the efficacy of DDR. The results obtained indicate that TDP-43 inactivation prior to the induction of DNA breaks by a radiomimetic drug, leads to a defect in the accumulation of both upstream transducers and downstream mediators of DDR at sites of DNA damage, in the form of DDR foci. Even more evident was the DDR defect in cells forming TDP-43 containing cytoplasmic inclusions upon TDP-43 overexpression. TDP-43 positive inclusions were identified as a type of stress granules (SGs) since stained positive for well-known constitutive markers of such organelles. Importantly, in undamaged cells the recruitment of TDP-43 within SGs appeared *per se* genotoxic

and it was sufficient to induce chronic ATM and DNA-PK activation leading to a nuclear wide accumulation of the γH2AX DNA damage marker. Concomitantly we observed that cells recruiting TDP-43 into SGs show a dramatic decrease in DROSHA nuclear levels, according with the idea that DDR defects might be due to loss of DDRNA biogenesis. Furthermore, preliminary analysis performed in fibroblasts of fALS patients mutated on TARDBP, support the results obtained upon TDP-43 depletion, strengthening the idea that TDP-43 *per se* plays some role in DDR. Altogether these results prompt us to consider DDR alterations triggered by TDP-43 inclusions or dysfunction as a novel, relevant aspect driving ALS pathogenesis.

Abbreviations

53BP1: p53-binding protein 1

8-OHdG: 8-hydroxy 20-deoxy-guanosine

A-T: ataxia telangiectasia

A-TLD: A-T-like disease

AD: Alzheimer's disease

ALS: amyotrophic lateral sclerosis

ALS2: alsin

ALS4: ALS type 4

alt-NHEJ: Alternative non-Homologous end joining mechanism

AOA2: ataxia-ocular apraxia type 2

ASO: Antisense oligonucleotides

ATM: ataxia telangiectasia-mutated

ATR: ATM and Rad3-related

BER: base excision repair

BRCA1: breast cancer type 1 susceptibility protein

C9 ALS-FTD: C9orf72 ALS-FTD

***C9orf72*:** chromosome 9 open-reading frame 72

CDK: cyclin-dependent kinase

Cdk5: Cyclin-dependent kinase 5

CDKN1A: Cyclin Dependent Kinase Inhibitor 1A

CFTR: cystic fibrosis transmembrane conductance regulator

CNS: central nervous system

CPT: camptothecin

CSR: class-switch recombination

CTFs: C-ter fragments

CtIP: C-terminal binding protein

D-loop: displacement-loop

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

DDR: DNA damage response

Abbreviations

DDR: DNA-damage response

DDRNAs: DNA damage response RNAs

dilncRNAs: damage-induced long non-coding RNAs

diPLA: DNA damage in situ ligation Proximity Ligation Assay

diRNAs: DSB-induced RNAs

DMEM: Dulbecco's modified Eagle's medium

DNA-PK: DNA-dependent protein kinase

DNA-PKcs: DNA-PK catalytic subunit

dNTPs: deoxynucleotide triphosphates

DPR: dipeptide repeats proteins

DSBs: double-strand breaks

DTT: Dithiothreitol

EAAT2: excitatory amino acid transporter 2

EJCs: exon-junction complexes

EXO1: exonuclease 1

FTD: frontotemporal dementia

FTLD-U: frontotemporal lobar degeneration

FUS: fused in sarcoma

G3BP: Ras GTPase-activating protein-binding protein 1

GA/ GR/ PR/ PA/ GP: glycine-alanine/ glycine-arginine/ proline-arginine/ proline-alanine/ glycine-proline

GEF: guanine–nucleotide exchange factor

HDAC1: class I histone deacetylase

HDR: homology-directed repair

hMSCs: human mesencimal stem cells

hNFL: uman low molecular weight neuro filament protein

HR: homologous recombination

HS: Heat Shock

Ig: immunoglobulin

iPSC: induced pluripotent stem cells

JNK: c-Jun N-terminal kinase

LCDs: low-complexity domains

LLPS: liquid-liquid phase separation

MALAT1: metastasis associated lung adenocarcinoma transcript 1

MDC1: mediator of DNA damage checkpoint protein 1

miRNA: micro-RNA

MMC: mitomycin C

MMEJ: microhomology-mediated end-joining

MMR: mismatch repair

MMS: methyl-methane sulfonate

MRN: MRE11–RAD50–NBS1

mRNPs: messanger ribonucleoproteins

NBS: Nijmegen breakage syndrome

NCI: neuronal cytoplasmic inclusions

ncRNAs: non-coding RNAs

NCS: neocarzinostatin

NEAT1: nuclear paraspeckle assembly transcript 1

NER: nucleotide excision repair

NES: nuclear export signal

NF: neuro filament

NHEJ: Non Homologous End Joining mechanism

NIIs: neuronal intranuclear inclusions

NLS: nuclear localization signal

NMD: nonsense mediated RNA decay

PABP-1: poly-A binding protein

PAR: poly ADP-ribose

PARP1/ PARP2: poly(ADP)ribose polymerases 1/ 2

PD: Parkinson's disease

PIKKs: phosphatidylinositol 3-kinase-like protein kinases

Pol II: RNA polymerase II

Pol IV: RNA polymerase IV

Q/N: glutamine/asparagine-rich

RAN: repeat-associated non-AUG translation.

RBP: RNA binding proteins

RNF8: ring finger protein 8

RNP: ribonucleoprotein

ROS: Reactive Oxygen Species

sALS/ fALS: sporadic/ familial ALS

SDS: sodium dodecyl sulphate

SETX: senataxin

SGs: stress granules

SHM: somatic hyper-mutation

siRNA: short interfering RNA

SMA: Spinal muscular atrophy

SR: serine/arginine

SSA: single-strand annealing

SSBR: single-strand break repair

SSBs: single-strand breaks double-strand breaks

ssDNA: single stranded DNA

TCR: T-cell receptor

TDP-43: Tar DNA binding protein 43

TDPBR: TDP-43 binding region

Tdt: Terminal deoxynucleotidyl transferase

TIAR: TIA-1 related protein

UBD: ubiquitin-binding domain

UV: ultraviolet

VCP: valosin-containing protein

XLF: XRCC4-like factor

Table of Contents

To You, Mamma

Introduction

1. DNA damage and DNA damage response (DDR)

1.1 Types of DNA damages and their implication in pathologies

Genome preservation is of crucial importance because of DNA ability to encode the information for most cellular functions and to guarantee the faithful transmission of the genetic asset, across generations. As a consequence DNA must be protected from the risk of damage spontaneously generated or arising from the environment. DNA damage includes several types of lesions that can range from very frequent chemical modifications on the nitrogenous bases like alkylation and deamination, to single-strand breaks (SSBs) or double-strand breaks (DSBs) that are the most harmful ones because both strands are simultaneously damaged increasing the risk of alterations of the encoded information (Ciccia and Elledge 2010). In fact, these lesions can have serious consequences like mutagenesis and disruption of regulatory DNA elements (Bauer, et al. 2015), thus have to be efficiently counteracted.

Cellular metabolism *per se* is a potential source of a plethora of DNA alterations such as dNTPs misincorporation during replication, DNA bases deamination, alkylation and oxidative lesions due to Reactive Oxygen Species (ROS) that are physiological byproducts of oxidative respiration (Lindahl and Barnes 2000). Moreover, the DNA chain is characterized by intrinsically labile N-glycosil bonds that render dNTPs susceptible to the loss of nitrogenous bases (primarily depurination) with the consequent risk of DNA cleavage in short times (Lindahl 1993).

As a whole, these DNA alterations can occur with a frequency of 10^5 per cell daily, with DSBs occurring with an incidence of about 50 per cell, every day (Lindahl and Barnes 2000). Physical and chemical agents that we frequently encounter in our everyday life, represent instead exogenous causes of DNA damage. Indeed, physical factors like ultraviolet (UV) rays from sunlight can promote photoproducts like pyrimidine dimers whereas ionizing radiation (IR) used in

anticancer medical treatments causes oxidative lesions, SSBs and DSBs. A well-known example of chemical damage is cigarettes smoke that concurs to hurt lungs and other tissues inducing oxidative lesions and DNA adducts. Other chemical compounds belonging to different classes of DNA damaging agents which are used in cancer chemotherapy are: alkylating agents such as methyl-methane sulfonate (MMS), crosslinking agents like mitomycin C (MMC) and cisplatin, topoisomerase inhibitors as camptothecin (CPT) and etoposide, which trapping topoisomerase-DNA covalent complexes, induce SSBs and DSBs formation (Ciccia and Elledge 2010).

Given the importance of DNA integrity, it is expected that DNA damage and the potential resulting mutations, contribute to the development of different diseases. The paradigm for this phenomena is cancer pathology in which genomic instability is continuously fed by vicious cycles of events such as aberrant cell proliferation, DNA-replication stress caused by activated oncogenes, DNA damage accumulation and defects in DNA repair. In addition, at later stages of solid cancer progression, chronic hypoxia might also contribute to genomic instability (Jackson and Bartek 2009). Accumulation of DNA lesions including strand breaks and oxidation of bases also correlates with vascular diseases such as aortic aneurysms and atherosclerosis (Uryga, et al. 2016) and it has been found that in obesity and diabetes, serum levels of 8-hydroxy 20-deoxy-guanosine (8-OHdG), a sensitive biomarker for oxidative DNA damage, is increased (Shimizu, et al. 2014). Furthermore, various neurodegenerative disorders like Alzheimer's, Huntington's, Parkinson's diseases and Amyotrophic lateral sclerosis (ALS) have been associated with the accumulation of different kinds of DNA damage (Jackson and Bartek 2009), (Coppede 2011b). The susceptibility of the nervous system to DNA damage can be justified, because neurons exhibit huge mitochondrial respiration rates that produce high levels of ROS (Weissman, et al. 2007). In addition, damaged cells are poorly replaced due to the reduced regenerative capacity of the nervous tissue (Rass, et al. 2007). Additionally, neurons are terminally differentiated post-mitotic

cells, which have to rely on the error-prone "Non Homologous End Joining repair" (NHEJ) mechanism for DSBs repair in place of the most error-free mechanism of homologous recombination (HR), rather active in S-G2 phases (Jackson and Bartek 2009) (Kulkarni and Wilson 2008). Both DSBs repair mechanisms will be discussed in details below. In line with the concept that DNA damage represents such an hazard to the health of the nervous system, it is increasingly evident that a strong contribution to the mentioned neurodegenerative disorders arises from alterations of all those mechanisms that prevent or counteract DNA damage (Duker 2002). This complex set of pathways activated in presence of DNA damage is collectively termed the DNA-damage response (DDR) and is discussed below.

1.2 A General overview of the DNA damage response pathway

DDR signalling involves hundreds of different proteins, in an ample cascade of events, which start after the detection of the DNA lesions *per se*. Some factors act as physical sensors of the altered DNA structure, other as scaffold proteins essential to sustain protein-protein interactions and increase the local density of proteins with enzymatic activity such as polyADP rybosilases, kinases, phosphatases and ubiquitin ligases which modify hundreds of targets, downstream. This results in a complex signalling cascade, which originates at sites of DNA damage into multi-protein complexes, cytologically detectable as DDR foci (Lukas, et al. 2011). From there, activated enzymes spread inside the nucleoplasm and the entire cell, inducing post translational modifications on several protein targets, thus impinging on virtually any aspect of cellular metabolism (Jackson and Bartek 2009) (fig. 1).

One of the outcomes of DDR signalling is the cell-cycle checkpoints enforcement that slows-down cell cycle progression, thus leading to DNA repair before cell division is completed. When damage cannot be efficiently repaired, some cell types enter the non-proliferative state of cellular senescence while others undergo apoptosis. These mechanisms are both useful to avoid the accumulation and

expansion of heavily damaged, unhealthy cells that in the long term could cause pathologies as cancer or neurodegenerative diseases (Zhou and Elledge 2000)

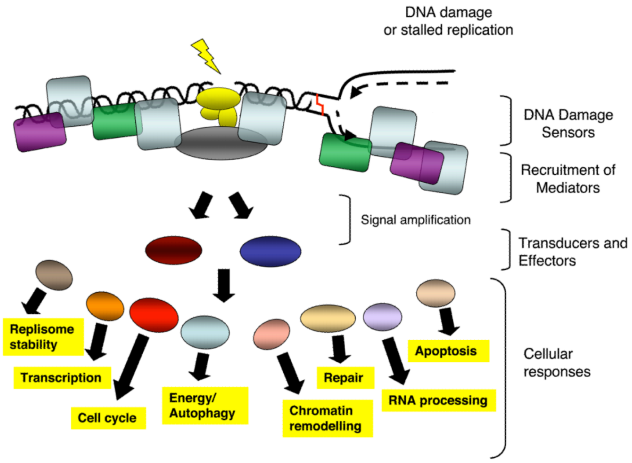


Fig.1|Model for the DDR (Jackson and Bartek 2009).

To highlight the level of complexity of DDR it should be mentioned that cells take advantage of multiple DNA repair machineries evolved to counteract specific categories of DNA injuries. Examples of big classes of such repair mechanisms are the mismatch repair (MMR) that replaces mis-incorporated bases, the base excision repair (BER) designed to remove chemically altered DNA bases and the nucleotide excision repair (NER) that resolves bulky lesions such as pyrimidine dimers and intra-strand crosslinks through the removal of 22-30 bp oligonucleotides harbouring the damaged site. Moreover, SSBs are repaired by single-strand break repair (SSBR), whereas DSBs are opportunely neutralized either by NHEJ or HR. In particular, NHEJ occurs efficiently throughout all cell cycle phases and re-ligates close DNA ends, often producing small insertions or deletions whereas HR is S-G2 phases specific since uses homologous sequences on the paired chromosome for accurate repair (Sancar, et al. 2004), (Sirbu and Cortez 2013). The

above repair mechanisms can operate independently to cope with simple lesions or cooperate on the same chromosomal site for the repair of more complex lesions such as DSB clusters and long stretches of single stranded DNA regions, which need an even more fine tuning by the DNA damage response (DDR)(Jackson and Bartek 2009; Mladenov and Iliakis 2011).

To enter into some of the molecular details of DDR activation, poly(ADP)ribose polymerases 1 and 2 (PARP1 and PARP2) of the PARP family, which catalyze the addition of poly ADP-ribose (PAR) chains on various target proteins, move to sites of DNA damage where act as molecular sensors of SSBs and DSBs and recruit other DDR factors and chromatin modifying complexes (Schreiber, et al. 2006). Targets of PARP1 and 2 at sites of DNA damage are histone tails and PARP1 itself (Schreiber, et al. 2006). Other very important DDR sensors specific for the detection of DSBs, are the MRE11-RAD50 and NBS1 (MRN) complex and KU70/KU80 (KU) ((Ciccia and Elledge 2010). These DNA damage sensor proteins bring alongside, some of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs) which include: ataxia telangiectasia-mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK). These central kinases of the DDR signalling cascade are activated upon specific DNA damage contexts. In particular, DSBs recruit both KU and the MRN complex, which activate DNA-PK and ATM respectively (Ciccia and Elledge 2010). ATM activation is required for both HR (Lavin 2008) and NHEJ (Bredemeyer, et al. 2006)) while DNA-PK is a key player in NHEJ (Harper and Elledge 2007), (Meek, et al. 2008). Instead, RPA-coated ssDNA is the primary signal for the activation of ATR, which accumulates at stalled replication forks and at resected DSBs (Cimprich and Cortez 2008). Once recruited to DNA damage sites and activated, the mentioned kinases work as the main transducers of the DDR signalling, phosphorylating hundreds of substrates (Zhou and Elledge 2000). Importantly, ATM/ATR activate the downstream checkpoint kinases CHK1 and CHK2, which in turn reduce cyclin-dependent kinases (CDKs) activity, by modulating the

function of the cell cycle phosphatase CDC25 (Branzei and Foiani 2008). Moreover, they stabilize p53 (Zhou and Elledge 2000) responsible for the transcriptional activation of the CDK inhibitor p21 (Riley, et al. 2008). Ultimately, inhibition of CDKs arrests cell-cycle progression at the G1-S, intra-S and G2-M phases to increase the time available for DNA repair before replication or mitosis. In parallel, ATM/ATR signalling promotes repair by regulating DNA-repair-proteins both at transcriptional and post-transcriptional levels as well as modifying them post-translationally (Huen and Chen 2008) and engaging several repair factors at sites of damage. If DNA repair is not efficiently carried out, DDR signalling keeps being chronically active and triggers apoptosis or cellular senescence which consists in a permanent cell-cycle arrest(Campisi and d'Adda di Fagagna 2007).

1.3 Response to DNA DSBs

Several physiological processes in vertebrates require DSBs production as an intermediate step. Examples of such processes are V(D)J recombination, class-switch recombination (CSR) and somatic hyper-mutation (SHM) that generate immunoglobulin (Ig) and T-cell receptor (TCR) diversity, in B- and T-lymphocytes, responsible for the recognition of a wide variety of antigens (Jackson and Bartek 2009). Also DNA replication leads to DNA break formation as a consequence of some replication intermediates (Branzei and Foiani 2008). Moreover DSBs are also involved in HR occurring during meiosis that is activated by the topoisomerase II-related enzyme, Spo11 (Jackson and Bartek 2009).
Despite these few physiological roles, DSBs are classified as “complex DNA damage” induced for example by exposure to ionizing radiations (IR) (Mladenov and Iliakis 2011). Particularly, double stranded breaks in the DNA helix can arise from two SSBs that are closely located on opposite DNA strands or from chemical or enzymatic conversions of minor lesions and base damages that face an existing SSB (fig. 2). Independently from their source, DSBs interrupt the continuity of the

DNA chain with the high risk of information loss. Moreover they require complex repair mechanisms due to the absence of a complementary strand that works as a template for the reconstruction of the original sequence. For these reasons, DSBs are considered the most severe lesions challenging genome integrity and cell survival and must be counteracted by highly sophisticated mechanisms of DDR signalling and DNA repair (Mladenov and Iliakis 2011).

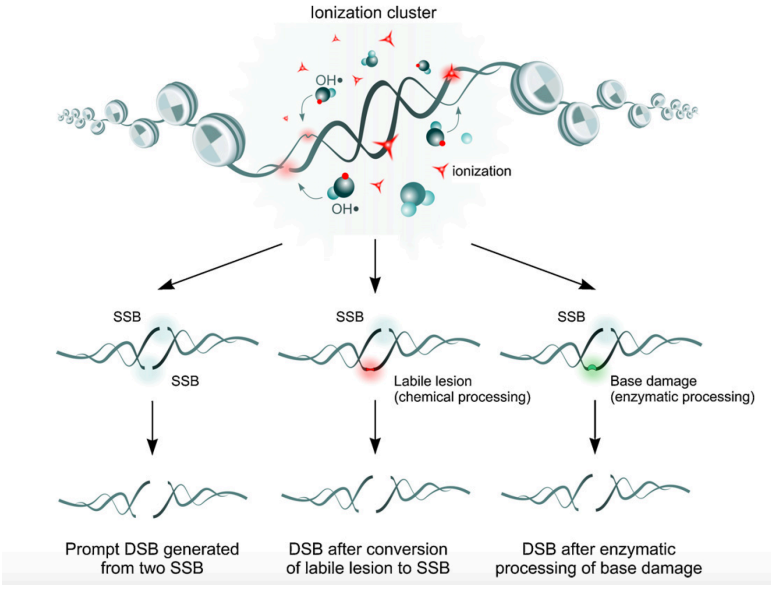


Fig. 2| IR releases its energy in the form of ionization clusters producing complex DNA damage from which DSBs can form directly, after chemical processing of sugar lesions, or after the enzymatic processing of base damage (Mladenov and Iliakis 2011).

Following DSBs generation ATM, which relocates to DSB sites (Shiloh and Ziv 2013), is believed to switch from a homodimeric inactive form to an active monomer (Dupre, et al. 2006). This conformational change is associated with the ATM autophosphorylation at Serine 1981 (Bakkenist and Kastan 2003) and other Serine residues (Kozlov, et al. 2011) together with the acetylation of Lysine 3016 (Sun, et al. 2007). As already mentioned, MRE11–RAD50–(MRN) is one of the first complexes to be recruited to DSB sites, where acts as a

damage sensor, although it is also required for both NHEJ and homology-directed repair (HDR) (Mladenov and Iliakis 2011). In particular, it is the NBS1 component of the MRN complex that recruits ATM and promotes its retention at DSBs (Difilippantonio and Nussenzweig 2007). Once activated, ATM phosphorylates the Ser 139 of the H2AX histone variant (referred to as γH2AX) which is simultaneously dephosphorylated at the Tyr 142 (an otherwise constitutively phosphorylated residue) (Lukas, et al. 2011), generating a direct binding site for the scaffold protein MDC1 (mediator of DNA damage checkpoint protein 1) (Stucki, et al. 2005) (fig. 2). MDC1 itself is phosphorylated by ATM, thus leading to the recruitment of the E3 ubiquitin ligase RNF8 (Mailand, et al. 2007) (ring finger protein 8) that promotes the histone H1 ubiquitylation in turn recognized by the ubiquitin-binding domain (UBD) of RNF168 (Doil, et al. 2009). The E3 ligase activity of RNF168 provides additional docking sites for several DSB repair factors, including breast cancer type 1 susceptibility protein (BRCA1) and p53-binding protein 1 (53BP1) that are carried to the break sites (fig. 3).

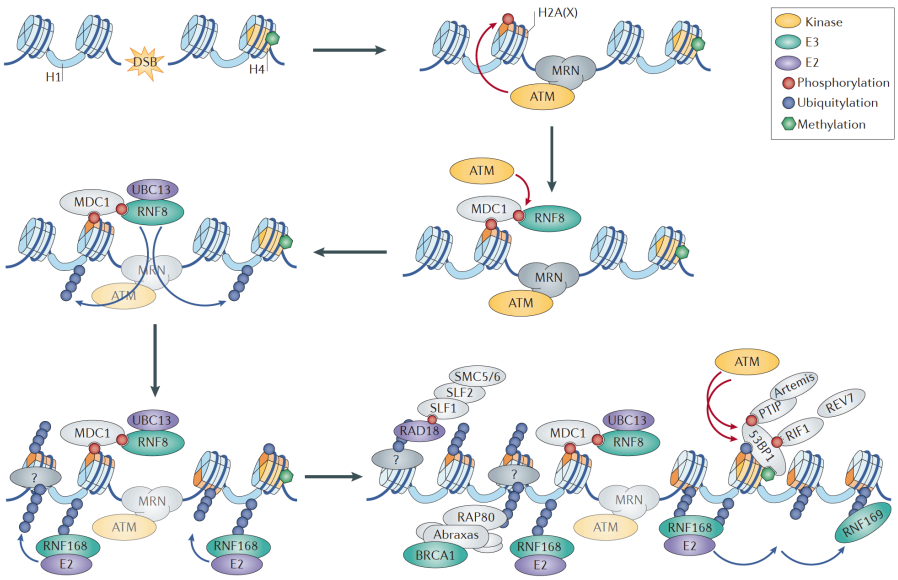


Fig.3| Ubiquitin-dependent protein assembly at double-strand break (DSB) sites by the RNF8–RNF168 pathway (Schwertman, et al. 2016). Details, within the text.

53BP1 was shown to contribute to both the intra-S-phase and G2-M checkpoints and to behave as a mediator that recruits groups of substrates to the ATM and ATR kinases, since its loss leads to a decreased phosphorylation of key checkpoint target proteins (Wang, et al. 2002). The ubiquitylation pattern involved in DDR, also promotes chromatin silencing around DSBs (Shanbhag, et al. 2010) so that transcription is repressed until DNA is fully repaired (Capozzo, et al. 2017). Therefore, the overall DNA damage response causes a massive accumulation of DDR mediators in large segments of lesion-flanking chromatin, building up nuclear foci of even few micrometers, that can be observed by light microscopy (Lukas, et al. 2011). Differently from the initial and autonomous primary recruitment of DDR sensors, DDR foci originate from the H2AX phosporylation event. Indeed, this modification causes the recruitment of MDC1 and other DDR mediator proteins 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; Francia, et al. 2016).

1.3.1 DSBs repair mechanisms

The universal importance of DSBs repair mechanisms is attested by the profound conservation of repair factors and proteins in eukaryotes from yeast to humans (Karpenshif and Bernstein 2012). Although different DSBs repair pathways exist, they can be divided in two main categories (fig. 4):

NHEJ is predominant in the G1 phase of the cell cycle and involves the direct ligation of DNA ends with little or no homology, representing for this reason an error-prone process during which nucleotides can be lost or gained at the ends prior to ligation. Differently, HR is the most accurate repair process since it requires a homologous DNA duplex as a template to recover the missing nucleotide

sequence. Given that HR relies on sequence homology to repair DSBs, it is restricted to the S and G2 phases of the cell cycle, when the presence of duplicated sister chromatids is ensured (Kadyk and Hartwell 1992).

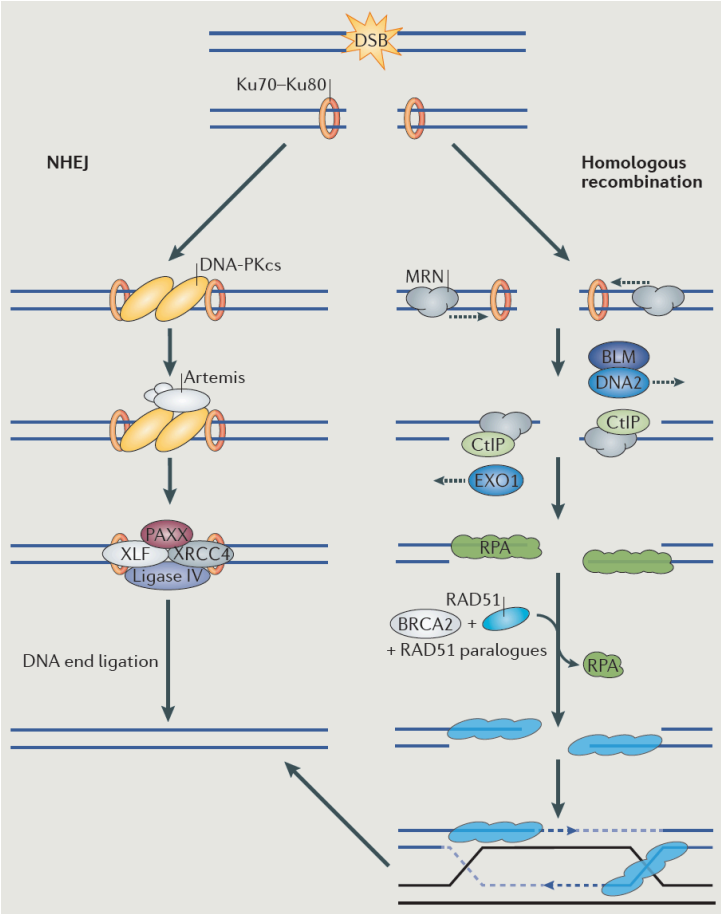


Fig.4| Scheme of NHEJ and HR repair pathways (Schwertman, et al. 2016). Details, within the text.

HR initiates with the DSBs end resection, a two-step nucleolytic degradation that produces 3′ single-stranded DNA (ssDNA) whose major regulator is the MRN complex. Resection of DSBs also involves the C-terminal binding protein (CtIP),

the helicase Sgs1 and the nucleases Dna2 or EXO1 (exonuclease 1) (Mimitou and Symington 2009) and is a key event to commit cells to the HR repair because long ssDNA overhangs are poor substrates for NHEJ (Symington 2016).

Once produced, 3′ ssDNA tails are bound by the Replication Protein A (RPA), which protects them from further damage (Wang and Haber 2004). RPA is then replaced with the conserved Rad51 recombinase through the aid of the recombination mediators BRCA2 and RAD52. The Rad51 nucleoprotein filament mediates the search for the homologous double stranded DNA and then its invasion, causing the displacement of the non-complementary strand and forming a structure termed displacement-loop (D-loop) (San Filippo, et al. 2008). The invading end of the D-loop is extended by the DNA polymerase leading to the copying of the sequence lost at the break-site. Finally, resolution of the D-loop structure can occur by two different mechanisms resulting in both crossover or non-crossover products (West, et al. 2015).

During NHEJ, broken DNA ends are rapidly bound and protected by a component of the DNA-PK oloenzyme, the Ku70–Ku80 heterodimer that has high affinity for DNA and promotes the recruitment of the catalytic subunit of DNA-PK (DNA-PKcs). The self-phosphorylation together with that of other NHEJ proteins by DNA-PKcs engages end-processing factors among which the nuclease ARTEMIS that trims the DNA ends making them compatible for ligation. Final end joining is then achieved by the activity of a ligase complex consisting of the DNA ligase IV and the associated components XRCC4 and the XRCC4-like factor (XLF) (Schwertman, et al. 2016). Moreover, a variant of the canonical NHEJ repair is the KU and DNA ligase IV independent, Alternative non-Homologous and joining mechanism (alt-NHEJ). In alt-NHEJ some short (<18 nt) or longer (>200 bp) homologous sequences around the break can be revealed by end resection, leading to the microhomology-mediated end-joining (MMEJ) (Sfeir and Symington 2015) or the single-strand annealing (SSA) (Undrill and Smith 1978) respectively.

1.4 Non-coding RNAs contribution to DDR

Different evidences exist about the involvement of various types of non-coding RNAs (ncRNAs) in DDR (d'Adda di Fagagna 2014). One contribution of such ncRNAs is relative to micro-RNA (miRNA) that were discovered to regulate transcripts levels of many DDR factors such as ATM (Hu, et al. 2010), DNA-PKcs (Hu, et al. 2010), BRCA1 (Moskwa, et al. 2011) and RAD51 (Wang, et al. 2012). Another category is represented by long ncRNAs that control the expression of downstream genes of DDR (Hung, et al. 2011). As example, PANDA is a DNA-damage induced antisense transcript generated from the Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) gene. It has been demonstrated that both CDKN1A and PANDA transcripts are promoted by p53 and ultimately mediate G1 arrest and cell survival respectively, thus regulating the cell cycle checkpoint (Hung, et al. 2011). Other studies indicate that some DDR factors bind to different kinds of ncRNAs. Indeed it was observed that the structural region of 53BP1 responsible for foci targeting, includes a Tudor domain (typical of proteins with a role in RNA metabolism) and that 53BP1 foci are sensitive to RNase A, suggesting that an RNA component is involved in the accumulation of this DDR mediator near the damaged site (Pryde, et al. 2005). The Ku80 subunit of the KU eterodimer also revealed to bind telomerase RNA in a mutually exclusive manner with respect to DNA, in Saccharomyces cerevisiae (Pfingsten, et al. 2012). Roles of ncRNAs within the DSBs repair process were also attested. There are evidences that RNA can serve as a template for DNA synthesis in different model systems like Saccharomyces cerevisiae (Storici, et al. 2007), E. Coli and human cell lines (Shen, et al. 2011).

Moreover, as anticipated above, in last years it was established that certain species of site-specific small ncRNAs have a direct role in DDR signalling. A study conducted in Arabidopsis thaliana using an inducible DSB site demonstrated that after break was achieved, small RNAs of 21-24 nts termed DSB-induced RNAs

(diRNAs) were produced. It was found that diRNAs generation and processing requires ATR, the RNA polymerase IV (Pol IV), and Dicer-like proteins, whereas Ago2 was found to be the effector protein recruiting diRNAs (Wei, et al. 2012). In the same study diRNAs biogenesis was also observed to occur in human cells. Subsequently it was demonstrated that the complex formed by Ago2 and diRNAs acts during HR by favouring Rad51 recruitment and retention at DSBs (Gao, et al. 2014). Moreover a role for diRNA in NHEJ repair was also proved in Arabidopsis thaliana (Qi, et al. 2016). Another study in Schizosaccharomyces pombe took advantage of a site-specific DSB system, to show that RNA polymerase II (Pol II) is recruited at the broken site, leading to the formation of RNA-DNA hybrids that are involved in the HR repair process (Qi, et al. 2016).

1.4.1 DDRNAs and dlincRNA: role in DDR signaling and DNA repair

Consistently with the above studies our group discovered a novel class of ncRNAs that since actively involved in DDR, have been defined DNA damage response RNAs (DDRNAs) (Francia, et al. 2012). DDRNAs processing resulted to be dependent on the double-stranded RNA-specific endoribonucleases type III DICER and DROSHA. Indeed, depletion of both RNases impaired the build up of IR induced DDR foci containing active DDR factors like pATM and 53BP1, in both human cells and zebrafish larvae. On the contrary, whether γH2AX containing foci still decreased after DICER and DROSHA depletion in the zebrafish model, which lack the DNA-PK kinase, they remained unaltered in human cells, likely because of their redundant phosphorylation by DNA-PKcs (Francia, et al. 2012). Accordingly, irradiated cells treated with RNase A and transcription inhibitors showed the same impairment in DDR foci accumulation, suggesting that RNAs themselves are essential for foci production. Indeed the specific addition of small (20–35 nt) RNAs was sufficient to support DDR foci reformation, in the absence of messenger RNAs. To determine the origin site of DDRNAs, it was used a cell line

harbouring a target site for an inducible endonuclease, placed in between some bacterial repeats. It was observed that, in cut cells upon RNase A treatment, the DDR focus reformed only after addition of RNA molecules extracted from the same cell line but not when parental cells, devoid of the integrated construct, were used as RNAs source (Francia, et al. 2012). These results suggest that DDRNAs are generated at the damage site. Next-generation sequencing of small RNAs from the inducible cell system ultimately confirmed the existence of several short RNAs mapping to the cut locus, some of which having the potential to form double-stranded species (Francia, et al. 2012).

A more recent study carried out by our group, further dissected the mechanisms of biogenesis and action of DDRNAs (Michelini, et al. 2017). Particularly it was demonstrated that DDRNAs arise from longer RNAs, newly synthesized at DSBs sites and named damage-induced long non-coding RNAs (dilncRNAs). DilncRNAs are transcribed in both convergent and divergent directions relative to DNA ends, by the RNA polymerase II which is in turn recruited through the MRN complex (Michelini, et al. 2017). An important aspect of DDRNA biogenesis and site-specific function is that dilncRNAs are DDRNA precursors undergoing DICER and DROSHA processing and also represent the chromatin bound landing platform for DDRNAs association to the damaged site by complementary base pairing (fig.4). A similar paradigm is active at centromeres to silence transcription of satellite repeats (Francia 2015).

Moreover DDRNAs trigger DDR foci formation and together with dilncRNAs, physically recruit 53BP1 via its Tudor domain (Michelini, et al. 2017). This model is consistent with the impairment of DDRNA recruitment, DDR activation and DNA repair, observed following RNAPII inhibition (Michelini, et al. 2017). Importantly, the administration of sequence-specific antisense oligonucleotides (ASO) that match and block dilncRNAs and DDRNAs interactions, leads to the same inhibition of DDR. (Michelini, et al. 2017) (fig. 5).

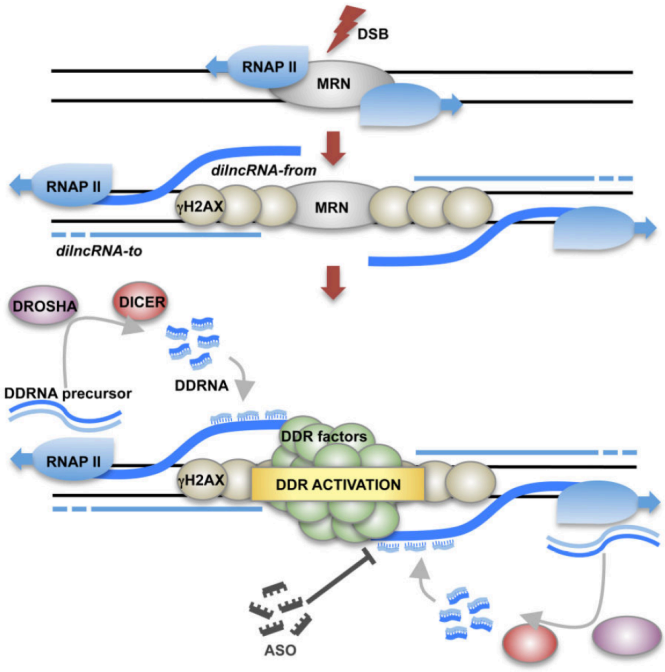


Fig.5| Model of dilncRNAs and DDRNAs biogenesis and action
MRN recruits RNAPII to the DSB and induces the bidirectional synthesis of dilncRNA-from (blue) and dilncRNA-to (light blue). DROSHA and DICER process the resultant long double-stranded RNA, generating DDRNAs, which pair with nascent unprocessed single-stranded dilncRNAs; together they bind to 53BP1 and fuel DDR focus formation. ASOs promotes site-specific inhibition of DDR, interfering with dilncRNA:DDRNA pairment (Michelini, et al. 2017).

All the results listed here can be reproduced also at de-protected telomeres, which are well known activators of DDR, highlighting the physiological relevance of this phenomena in different pathological contexts associated with telomere dysfunction, among others chronological aging and progeria syndromes (Rossiello, et al. 2017).
The discovery of DDRNAs and dilncRNAs as upstream players of the DDR cascade introduces a new layer of knowledge related to the field of RNAs that as a whole is made up by RNAs themselves as well as RNA binding proteins (RBP) and factors related to the RNA metabolism. Therefore, the intriguing direct contribution of RNAs to DDR, suggests that other RNA binding factors could have an important role in the intricate mechanism of DDR activation, thus paving the

way to further investigations related to RNA-protein interactions at sites of DNA damage.

2. Neurodegenerative pathologies: role of DNA damage in neurodegeneration and aging

Cell types with different life length, must cope with DNA lesions for time periods of different duration. Indeed, highly proliferative cells such as epithelial intestinal cells can compensate for DNA repair failures and subsequent cell death because of the high rates of tissue turnover, whereas post-mitotic cells such as neurons cannot be replaced and must be able to efficiently solve DNA damage to guarantee the health status of the nervous system. Moreover, the high rate of oxygen consumption of neuronal cells contributes to its high sensitivity to ROS accumulation (Madabhushi, et al. 2014). The resulting ROS-induced DNA alterations are counteracted by BER and NER mechanisms that share a general scheme of repair consisting of damage sensing, excision, gap-filling DNA synthesis and ligation (Madabhushi, et al. 2014). ROS-mediated attack on the DNA backbone can also produce SSBs (Caldecott 2008) that in addition to be *per se* detrimental may further predispose to DSBs formation (fig. 3) (Mladenov and Iliakis 2011).

Human neural development starts with the specification of neural progenitors during gastrulation (Madabhushi, et al. 2014). The neural progenitors pool is expanded through symmetric divisions, whereas neurons start to be generated with the asymmetric divisions of the progenitor cells. Then, new born neurons reach their final destinations in the appropriate regions of the central nervous system (CNS), where undergo further differentiation (Madabhushi, et al. 2014). It is extremely important that the proper DNA repair mechanism is effective in the right stage of development, otherwise the functionality of the nascent nervous system could be compromised (McKinnon 2013). Therefore the HR-mediated DSB repair acts during progenitor proliferation to preserve as much as possible the genetic

information. On the contrary, HR is unlikely to operate in post-mitotic differentiated neurons, which primarily rely on the NHEJ repair (Madabhushi, et al. 2014).

It is noteworthy that the aging process has been shown to be caused by accumulation of unrepaired DNA damage in various ways (d'Adda di Fagagna 2008). Indeed there are different evidences that unrepaired DNA damage accumulates with age (Madabhushi, et al. 2014) (fig. 6).

For instance it was observed that oxidative lesions enrich in the promoter regions of genes implicated in critical neuronal functions and this correlates with dramatic changes in the expression profiles of such genes contributing to the physiological age-related cognitive decline (Lu, et al. 2004). Furthermore, in addition to directly alter the DNA helix, DNA damage also impacts on chromatin organization and some studies suggest that the chromatin conformation might not be faithfully restored to its original state, following DNA repair {Oberdoerffer, 2007 #78}. As a consequence, wide chromatin changes due to chronic DNA damage can occur with age as well as in neurodegeneration. Indeed, DNA damage also influences the chromatin state globally, since it was demonstrated that exposure of cells to DNA-damaging agents leads to the relocation of the histone deacetylase SIRT1 to damaged sites (Oberdoerffer, et al. 2008). Thus, chronic genotoxic stress causes a persistent redistribution of SIRT 1 and a consequent large-scale transcriptional de-repression of its canonical target genes. Interestingly the pattern of deregulated genes due to SIRT 1 delocalization matches that observed in aging mice brain (Oberdoerffer, et al. 2008).

Another heavy contribution to aging, linked to DNA damage could arise from the conversion of DNA lesions into mutations falling into genes encoding for DDR factors, especially DNA repair factors (fig. 6) (Vijg and Suh 2013).

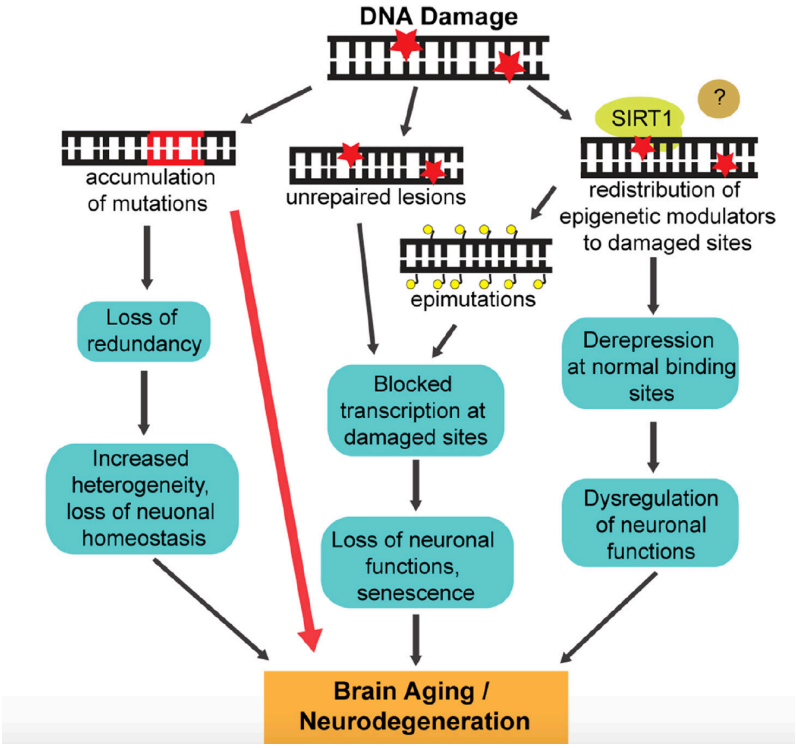


Fig. 6| Effects of DNA Damage in Aging and Neurodegeneration. Left: Defective repair of DNA damage can induce mutations perturbing the homeostasis of the nervous system. Particularly mutations of DNA repair factors can promote neurodegeneration (red arrow). **Middle:** the accumulation of unrepaired lesions can block the transcription of critical neural genes, leading to cognitive decline. **Right**: DNA damage dependent redistribution of epigenetic modulators like SIRT1, can trigger global changes on chromatin inducing wide transcriptional deregulation of otherwise repressed targets (Madabhushi, et al. 2014).

Importantly, the existence of many congenital diseases with neurological phenotypes caused by mutations in DNA repair factors highlights the necessity of preserving genomic stability in the nervous system (Madabhushi, et al. 2014). As an example ataxia telangiectasia (A-T) is caused by null mutations of ATM, the central kinase in DDR, relevant for different DNA repair mechanisms, and is a multisystem disease primarily characterized by neurological defects as ataxia and cerebellar atrophy, overall representative of a progressive neurodegenerative

phenotype (Biton, et al. 2008). Another related neurodegenerative disorder sharing similar defects although slower in its progression, is the A-T-like disease (A-TLD) caused by hypomorphic mutations in MRE-11, a subunit of the MRN complex, the key sensor of DSBs (Taylor, et al. 2004). On the contrary, the Nijmegen breakage syndrome (NBS) due to mutations in the NBS1 component of the same complex is characterized by microcephaly and mental deficiency as neurological hallmarks, rather than neurodegeneration (Digweed and Sperling 2004). Different research efforts aimed to elucidate the mechanisms underlying such divergent neurological defects in pathologies that all share defective ATM activation and it was found that the level of functional ATM is determinant to drive the opposite neurological outcomes (Shull, et al. 2009). Particularly, it has been established that during neurogenesis ATM promotes the elimination of excessively damaged post-mitotic neural cells by triggering their apoptosis (Lee, et al. 2001). Accordingly, the complete loss of function of ATM and mutations in MRE11, responsible for DNA damage accumulation in A-T and A-TLD respectively, also abrogate the ATM-induced apoptosis of damaged cells (Lee, et al. 2001), (Shull, et al. 2009). Therefore, the survival of such damaged neurons, that will die progressively over longer times, is supposed to contribute to neurodegeneration (Madabhushi, et al. 2014). Conversely, in NBS the defective MRN complex allows for the retention of enough ATM activity to induce DNA damage-associated apoptosis of neuronal cells, thus promoting microcephaly (Shull, et al. 2009).

Defects in the DNA repair mechanisms have also been characterized in age-associated neurodegenerative pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Adamec, et al. 1999), (Mullaart, et al. 1990), (Martin 2001), (Bender, et al. 2006). Since the most reported damage in neuronal cells is oxidative stress, it is not surprising that the expression of BER factors was found reduced in aging and disorders like AD (Canugovi, et al. 2013), (Borgesius, et al. 2011). In addition it has been widely demonstrated that also DNA strand breaks accumulate in pathologies as AD and

ALS (Adamec, et al. 1999), (Mullaart, et al. 1990), (Martin 2001) and an increase in DNA DSBs has been reported in several mouse models of neurodegeneration (Suberbielle, et al. 2013). At this regard, it is noteworthy the inducible p25/CDK5 mouse model (CK-p25 mice) of AD (Cruz, et al. 2003). Indeed, the Cyclin-dependent kinase 5 (Cdk5) is a brain-specific serine/threonine kinase that requires the cyclin-like partner p35, to act catalytically (Lew, et al. 1994). Remarkably, analysis of pre-symptomatic CK-p25 mice expressing p25, a cleavage product of p35 that has been associated to AD (Su and Tsai 2011), revealed the presence of many DNA DSBs in the forebrain prior to the onset of all other pathological signs, suggesting that DSBs could be the initiating pathogenic stimulus (Kim, et al. 2008). However it is still unclear which are the sources of DSBs in neurodegenerative pathologies, since DSB formation is relatively more rare than other types of DNA lesions, even in proliferating cells that are subjected to DNA-replication dependent damage. It has been speculated that the mere physiological neuronal activity can promote DSBs within neurons and AD mice models have revealed higher basal levels of DSBs as a consequence of synaptic dysfunctions due to beta amyloids accumulation (Dobbin, et al. 2013).

Another source of genome instability that has been linked to neurological diseases is represented by the accumulation of RNA:DNA hybrids known as R-loops. These structures likely arise from the hybridization of nascent RNAs protruding from the transcribing RNA polymerase, to the DNA template strand (thread-back model) and are composed by the RNA–DNA duplex together with the remaining displaced ssDNA (Aguilera and Garcia-Muse 2012) (fig 7).

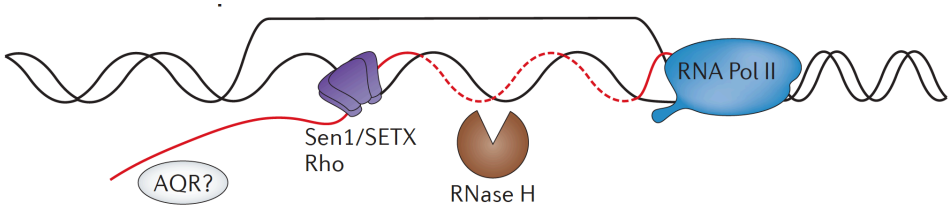


Fig.7| R-loops structure and methods for their resolution. R-loops are formed by DNA-RNA hybrids and a displaced ssDNA, when a transcribed mRNA binds to its DNA template. R-loops can be removed by the RNase-H activity or by helicases like Rho (bacteria), Sen1 (yeast) and human senataxin (SETX) that unwound the DNA-RNA hybrids. Other helicases as aquarius (AQR), may also play a part. (Santos-Pereira and Aguilera 2015).

R-loops have been found to mediate physiological processes like DNA replication of mitochondrial and plasmidic DNA and the Ig class-switch recombination (CSR), but they have been also implicated in transcription activation and termination and in the regulation of the chromatin structure (Santos-Pereira and Aguilera 2015). However, R-loops may also represent a potential source of genome instability so that once formed they should ideally persist only temporary. Indeed, R-loops resolution is physiologically ensured by the activity of RNase H enzymes, which degrade their RNA moiety or by the RNA–DNA helicases, which unwind the RNA–DNA hybrids (Wahba, et al. 2011), (Hong, et al. 1995), (Skourti-Stathaki, et al. 2011) (fig. 5). Specific RNA-binding proteins (RBPs) may also prevent the generation of R-loops by interacting with the nascent mRNA and impeding its association with DNA (Santos-Pereira and Aguilera 2015). Despite these scavenger mechanisms, R-loops are able to threaten cell genome in various ways, being a source of DNA damage and replication stress (Santos-Pereira and Aguilera 2015). First of all, due to the intrinsic structure of such RNA-DNA hybrids, the displaced ssDNA is more exposed to various DNA-damaging agents and mutagenic enzymes. Additionally, working in place of RNA primers, R-loops were found to

drive non-canonical and origin-independent DNA replication events, which might be mutagenic, in E.coli (Kogoma 1997) and in yeast cells (Stuckey, et al. 2015). Finally it has been demonstrated in various organisms, that R-loops can interfere with the replication-forks progression, promoting DNA breaks formation (Santos-Pereira and Aguilera 2015), (Brambati, et al. 2015). In addition to their role in genome instability R-loops can be detrimental for cells in other ways and are implicated in various neurological disorders. As an example the Fragile X syndrome is a neurodevelopmental pathology caused by a decreased expression level of the *FMR1* gene due to the expansion of a CGG repeat in its 5′ untranslated region (5′ _UTR). Particularly, the *FMR1* mRNA itself generates R-loops on the CGG repeats promoting its down-regulation (Loomis, et al. 2014).

Importantly, R-loops production has been also associated with ALS cases characterized by the expansion of the GGGGCC hexanucleotide repeat in the chromosome 9 open-reading frame 72 (*C9orf72*) gene. Indeed in this ALS subtype, R-loops contribute to the generation of aborted transcripts harbouring the repeats, which impair the nucleolar function of patients cells (Haeusler, et al. 2014). Importantly it has been also demonstrated that C9orf72 repeat expansions were causative of R-loops-induced DSBs and of defective ATM activation, which results in downstream alterations of both DDR and DNA repair (Walker, et al. 2017). All these effects were also recapitulated in mice nervous systems, after C9orf72 RNA or dipeptide repeats expression and were strictly correlated with neurodegenerative phenotypes, supporting the idea that genome instability is a direct promoter of neuronal degeneration (Walker, et al. 2017).

Consistently with the model that R-loops accumulation is a pathological mechanism behind neurodegeneration, defective activities of enzymes responsible for R-loops resolution like SETX, have been associated with neurodegenerative disorders such as ataxia-ocular apraxia type 2 (AOA2) (Moreira, et al. 2004) and ALS type 4 (ALS4) (Chen, et al. 2004).

Overall these studies indicate that DNA damage is widely implicated in the pathogenesis of different congenital and degenerative diseases of the nervous system, underlying the importance to further investigate the extent of the causal link between DNA damage and neuronal pathologies.

3. The spectrum of TDP-43 proteinopathies

3.1 Amyotrophic lateral sclerosis (ALS): clinical, istological, genetic features and DNA damage role

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that affects upper and lower motor neurons. Upper motor neurons (or cortico-spinal motor neurons) innervate the motor cortex and make synapses with lower motor neurons departing either from the brainstem to innerve the tongue muscle (bulbar motor neurons) or from the spinal cord to innerve skeletal muscles of limbs (spinal motor neurons) (Taylor, et al. 2016) (fig. 8).

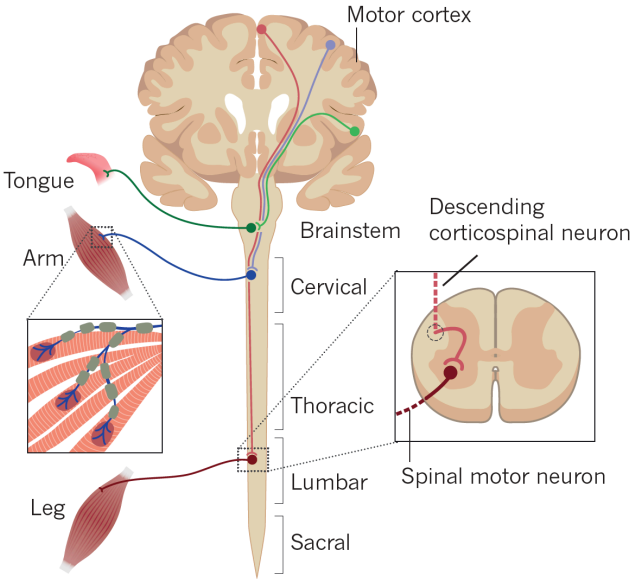


Fig.8| Routes of cortico-spinal, bulbar and spinal motor neurons, that are affected in ALS. (Taylor, et al. 2016)

ALS starts focally and progressively spreads (Ravits and La Spada 2009), ultimately leading to muscular paralysis and death due to respiratory failure, generally in 3-5 years from the diagnosis (Taylor, et al. 2016). The average age of ALS onset is of about 60 years and its incidence is 2-3 individuals per one hundred thousands each year in Europe, where most related epidemiological studies were conducted (Al-Chalabi and Hardiman 2013). There are various ALS phenotypes classified on the base of the initial region of onset and the relative ratio of affected lower and upper motor neurons, which imply different clinical manifestations (Ravits and La Spada 2009). For example, *progressive muscular atrophy* primarily affects lower motor neurons causing muscular decay, *primary lateral sclerosis* hits cortico-spinal motor neurons and is characterized by spasticity and *bulbar ALS* affects brainstem motor neurons that innervate the tongue muscle, leading to its atrophy and causing defects in speech and swallowing. About 20% of all ALS cases can also manifest cognitive, behavioural and language disturbances associated with frontotemporal dementia (FTD), another neurodegenerative disorder caused by the loss of fronto-temporal cortical neurons (Seelaar, et al. 2011), (Taylor, et al. 2016), (Conlon, et al. 2018). The coexistence of both conditions in a certain fraction of cases, suggests their belonging to the same spectrum of neurodegenerative diseases. The idea that ALS and FTD are overlapping syndromes rather than separate pathologies is also widely supported by their huge similarity with regard to the pathological markers (Ling, et al. 2013), discussed beyond. Both sporadic and familial ALS forms exist (sALS and fALS), the latter representing only about 10% of all ALS cases and being dominantly transmitted in families (Taylor, et al. 2016).

Many genetic variants have been found to be associated with fALS and to a less percentage, also with sALS. Most of genes whose mutations have been linked to ALS regulate various aspects of RNA metabolism, protein homeostasis, protein clearance processes and cytoskeletal structure, in motor neurons (Taylor, et al. 2016). The first genetic variants to be associated with ALS are those of the SOD-1

gene, which were found in about 20% of fALS and 2% of sALS events (Rosen, et al. 1993). The protein encoded by SOD1 is a Cu-Zn superoxide dismutase enzyme that acts as antioxidant by producing O_2 and H_2O_2 from reactive superoxide (Rosen, et al. 1993). About 170 SOD-1 mutations have been related to ALS, all of which inducing toxic effects without compromising or by slightly affecting, the dismutase activity (Taylor, et al. 2016), (Bruijn, et al. 1998). Thus, it has been hypothesized that at least a fraction of such mutations cause dismutase misfolding, in turn responsible for the toxic cytoplasmic accumulation of the protein in motor neurons and astrocytes (Bruijn, et al. 1997). Another mutation that has been associated to a substantial fraction of fALS and sALS cases is the amplification of the GGGGCC repeats in C9orf72 (DeJesus-Hernandez, et al. 2011), (Renton, et al. 2011). These repeats range from 2 to 23 copies in healthy subjects and are expanded to various hundreds of copies in ALS-FTD patients. This expansion also showed to increase from 10% to 50% the probability that FTD could develop in parallel with ALS (Bruijn, et al. 1997).

Three major mechanisms have been proposed about how the hexanucleotide expansion could promote toxicity in C9orf72 ALS-FTD (C9 ALS-FTD) individuals (fig. 9). Although the role of the product of C9orf72 is still unclear, it has ben speculated that the protein works as a guanine–nucleotide exchange factor (GEF) for several Rab GTPases (Webster, et al. 2016). Thus, since C9orf72 was weakly expressed in C9 ALS–FTD affected individuals, its pathogenicity was supposed to be linked with C9orf72 loss of function (Waite, et al. 2014). Nonetheless, it was found that the complete depletion of C9orf72 led to several phenotypes among which splenomegaly, lymphadenopathy and to alterations of macrophages and microglial cells in mice models, without compromising motor neurons (O'Rourke, et al. 2016), (Burberry, et al. 2016). Overall, the experimental data were not sufficient to corroborate an exclusive pathogenic role of C9orf72 loss of function and different evidences rather point to the gain of toxic functions associated with the expanded repeats.

Hexanucleotide repeats of C9orf72 are transcribed in both directions leading to RNA foci enriched in sense and antisense transcripts (Gendron, et al. 2013a), (Mori, et al. 2013a). These RNA accruals can exert toxic roles, working as baits for different RBPs that are sequestered and impaired in their physiological functions (fig. 9) (Lee, et al. 2013), (Conlon, et al. 2016). Moreover, these expanded repeats are prone to form secondary structures like G-quadruplets that can contribute to toxicity by favouring the abortive transcription of the locus and by boosting R-loops production (discussed bottom) in addition to stabilize the interaction with various RBPs (Taylor 2014). A third toxic mechanism postulated for C9 ALS–FTD involves dipeptide repeats proteins (DPR) that are translated from the hexanucleotide repeats in an AUG independent manner, through the exploitation of the secondary structure formed by the C9orf72 repeats (fig. 9) (Zu, et al. 2011), (Zu, et al. 2013). Different DPR proteins composed by glycine-alanine (GA), glycine-arginine (GR), proline-arginine (PR), proline-alanine (PA) and glycine-proline (GP) are produced by the translation of sense and antisense repeat-associated RNAs in all the reading frames, and accumulate in cytoplasmic and nuclear inclusions typical of C9 ALS–FTD (Zu, et al. 2013). It was shown that among all the dipeptides, those containing arginine are the most harmful. Indeed GR and PR hamper nucleolar RNA processing, promoting cell death (Kwon, et al. 2014) and can interfere with the biophysical features of proteins with low-complexity domains (LCDs), ultimately altering the dynamic properties of membraneless organelles like stress granules (SGs) (Lee, et al. 2016).

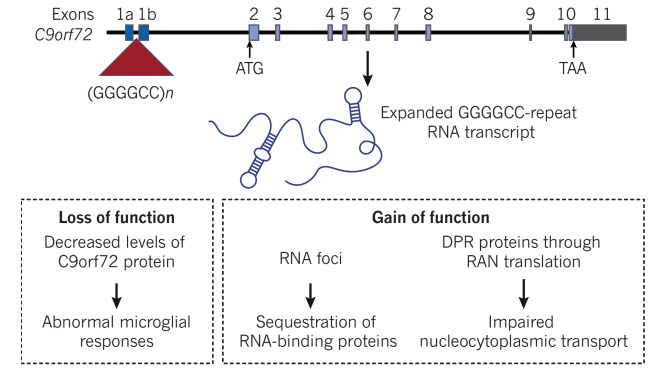


Fig.9| Proposed pathogenic mechanisms for C9 ALS–FTD. (Taylor, et al. 2016). RAN: repeat-associated non-AUG translation.

In addition to the most frequent SOD-1 and C9orf72 mutations, other less represented mutations of genes like *TARDBP*, fused in sarcoma (*FUS)*, *HNRNPA1*, *SQSTM1*, *VCP*, *OPTN, PFN1*, alsin (*ALS2) and senataxin (SETX)* have been associated to the ALS pathogenesis (Taylor, et al. 2016), (Coppede 2011a). Finally, additional genetic variants have been proposed to affect the disease predisposition and progression as well as the pathological clinical signs, such as those related to the ATXN2 (Elden, et al. 2010) and EPHA4 (Van Hoecke, et al. 2012) genes. However, independently from the presence or not of causative or predisposing mutations, there are some characteristic pathological signatures that distinguish ALS related disorders both familial and sporadic, from other neurodegenerative diseases. The main pathological hallmarks of ALS cases and of FTD subtypes characterized by frontotemporal lobar degeneration (FTLD-U) are neuronal cytoplasmic inclusions (NCI). Such inclusions have been found to be negative for the tau protein (whose modified forms are characteristic of AD and PD) and positive for phosphorylated and ubiquitinated proteins (Forman, et al. 2004), (Wightman, et al. 1992), (Okamoto, et al. 1992). Extensive studies focused on the identification of the main proteins composing such inclusions and converged on the discovery of TDP-43, encoded by the TARDBP gene, as specific core component

(Arai, et al. 2006), (Neumann, et al. 2006). Particularly, TDP-43 was found within cytoplasmic, rounded or skein-like shaped inclusions in motor neurons of the spinal cord and in neurons of the frontal cortex and hippocampus in both ALS and FTLD-U, although at different extents (Arai, et al. 2006), (Neumann, et al. 2006). The presence of TDP-43 within cytosolic ubiquitinated inclusions was often accompanied by its nuclear clearance, suggesting that in ALS affected neurons its nucleus-cytoplasm redistribution might be responsible for its loss of function, causative for the disease (Neumann, et al. 2006). Moreover, additional neuronal intranuclear inclusions (NIIs) in the spinal cord, and cytoplasmic tau-positive inclusions in glial cells, all negative for ubiquitin and positive for TDP-43, have been reported in both pathologies (Arai, et al. 2006) (fig. 10).

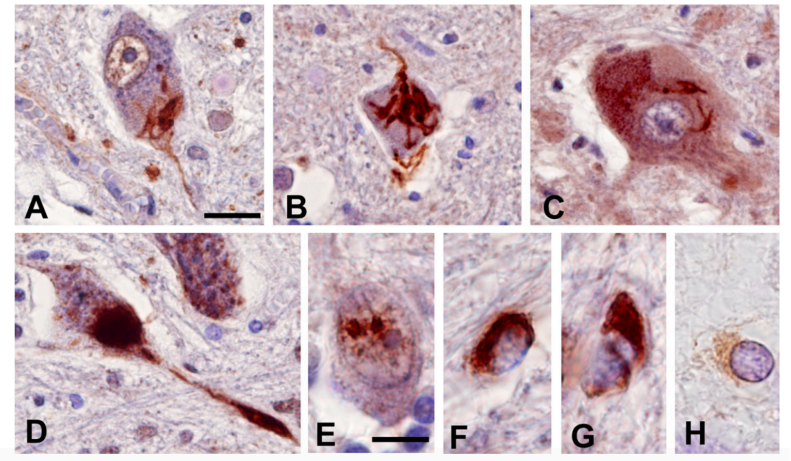


Fig.10| Immunostaining of the spinal cord in ALS. Spinal motor neurons with skein-like cytoplasmic inclusions, that are positive for ubiquitin (A) and TDP-43 (B-C). Neurons with cytoplasmic compact rounded inclusions and dendritic neurite-like structures, both positive for TDP-43 (D); neuronal intranuclear inclusions (E) and glial inclusions (F-G), positive for TDP-43; tau-positive glial inclusions (H). The sections are counterstained with hematoxylin. (Arai, et al. 2006)

Importantly, immunoblots of urea extracted, insoluble fractions from spinal cords and frontotemporal cortices of ALS and FTLD cases probed for TDP-43, reveal bands at 45 kDa and 25 kDa as well as a higher smears in addition to the bands of

the endogenous protein. The highest bands are representative of phosphorylated and widely modified forms of TDP-43 whereas the lowest bands have been identified as C-terminal cleavage products of the full-length protein (Arai, et al. 2006), (Neumann, et al. 2006). These additional bands are in line with the presence of consensus motifs for caspase 3 within the TDP-43 structure (Zhang, et al. 2007), which once cleaved, give rise to C-ter fragments (CTFs) of 35 and 25 kDa, able to aggregate in cultured cells (Zhang, et al. 2009). The 35 kDa CTF is also translated from an alternative translational start site of TDP-43 (Xiao, et al. 2015). The presence of characteristic cytoplasmic inclusions harbouring TDP-43 prompts to the re-definition of ALS and FTDL-U as TDP-43 proteinopathies. Importantly, almost all ALS cases are marked by the presence of TDP-43 inclusions except for those caused by mutations in the SOD-1 gene, which are rather marked by SOD-1 positive inclusions (Mackenzie, et al. 2007). However other types of pathological accruals, like RNA foci (DeJesus-Hernandez, et al. 2011) and inclusions containing both p62 (Al-Sarraj, et al. 2011) and dipeptide repeat proteins (Mori, et al. 2013b) are present in parallel to those containing TDP-43, in C9orf72 ALS-FTD, whereas FUS positive inclusions often immune-reactive for also TDP-43, are detected in sALS and fALS, non SOD-1 cases (Deng, et al. 2010).

In the last years of investigations, a lot of mechanisms have been suggested to contribute to ALS pathogenesis (fig.11).

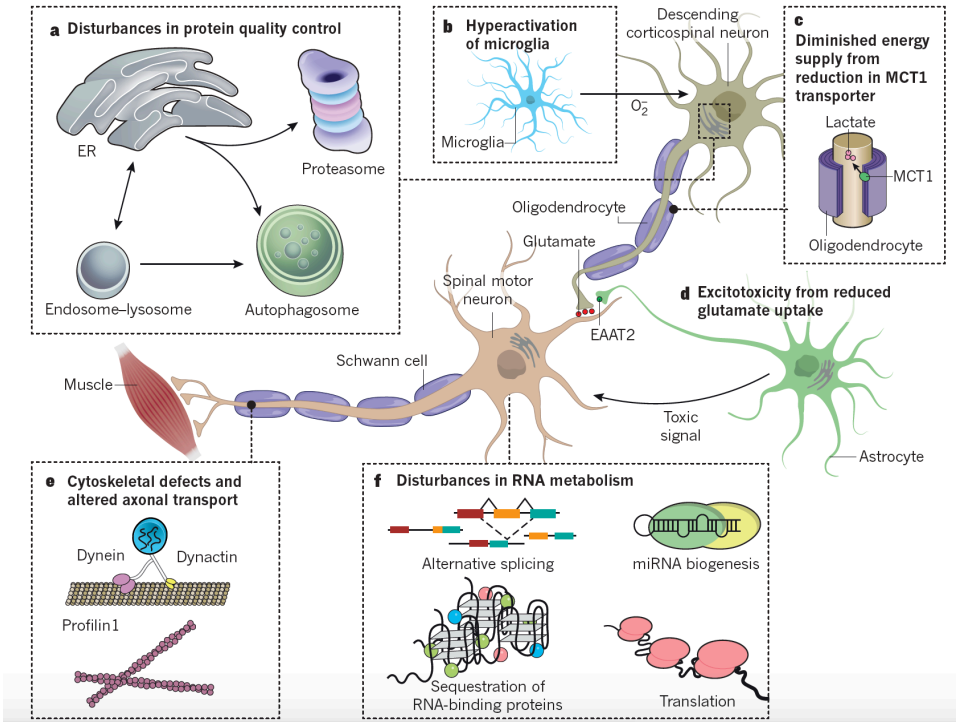


Fig.11| Mechanisms of disease implicated in ALS. (Taylor, et al. 2016).

First of all, it must be considered that ALS linked mutations do not exclusively affect the activity of motor neurons but also of glial cells, making ALS a disorder subjected also to non-cell-autonomous mechanisms. In particular microglia, oligodendrocytes and astrocytes have been all implicated in defective processes linked to SOD-1 and C9orf72 ALS cases (Taylor, et al. 2016). As an example, mutant SOD-1 revealed to counter-intuitively boost microglia production of superoxide through a fine mechanism involving the activation of the GTPase RAC1, finally promoting neuronal inflammation (Harraz, et al. 2008). Moreover SOD-1 mutations demonstrated to impair the energy supply provided by oligodendrocytes to motor neurons (Lee, et al. 2012).

Additionally, astrocytes have the important role to 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).

Many ALS related variants occur in genes involved in autophagy and proteasomal pathways, two branches of the protein quality control system. Examples of such genes are ubiquilin-2, sequestosome-1, optineurin and the valosin-containing protein (VCP) (Taylor, et al. 2016). The finding of such mutations supports the idea that altered clearance of defective and misfolded proteins concurs to ALS toxicity. Additionally, motor neurons are strongly dependent on the axonal transport to ensure the correct supplying of proteins and RNAs in neuritis and synaptic sites that are distant from the cell bodies, where these molecules are produced (Taylor, et al. 2016). Mutations of several RBPs among which FUS, TDP-43 and hnRNPA1 that regulate the delivery of RNA containing granules along the entire axon, can compromise this transport, thus damaging neuronal cells and function (Taylor, et al. 2016). Also mutations in cytoskeletal motor proteins that carry various cellular cargos hamper both anterograde and retrograde axonal transport, favouring neurodegeneration (Williamson and Cleveland 1999), (Williamson and Cleveland 1999), (Puls, et al. 2003).

Another important aspect that can drive ALS pathogenesis is related to the events underlying the generation of inclusions that are typical of ALS-FTD (Taylor, et al. 2016). Most of such inclusions are composed of proteins with LCDs, which are prone to aggregate (Lin, et al. 2015). Indeed, LCDs allow the liquid-liquid phase separation (LLPS) process that consists in the splitting of a mixed and homogenous phase in two different phases, a liquid one and a droplets-like one (Hyman, et al. 2014) (fig. 12).

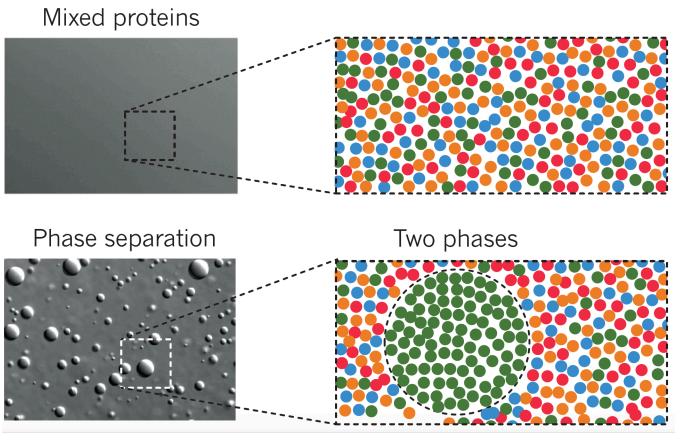


Fig. 12| Scheme of the phase separation process (Taylor, et al. 2016).

This process serves to generate physiological relevant membraneless organelles like nucleoli and SGs, involved in different aspects of RNA metabolism (Molliex, et al. 2015). Within droplets, RBPs are in close proximity and can undergo to aggregation with production of fibrils, if membraneless organelles are stabilized or their resolution is impeded (Molliex, et al. 2015) (fig. 13).

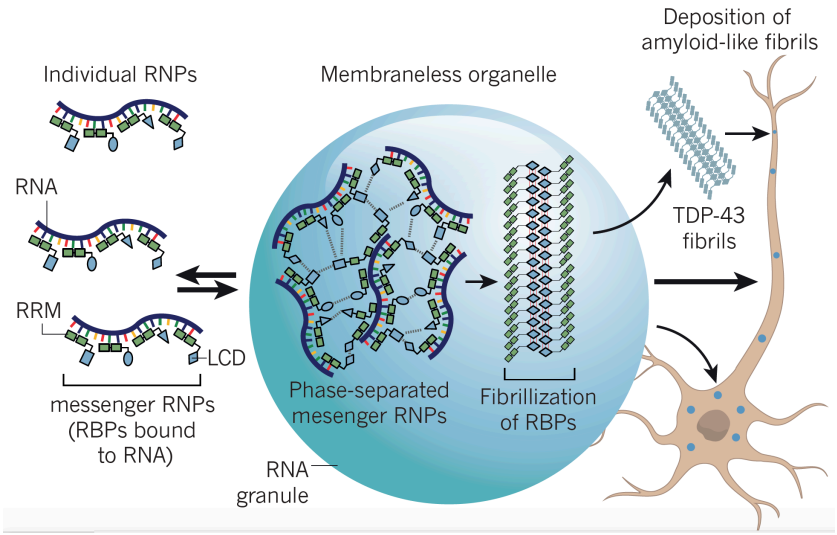


Fig. 13| Phase separation contributes to the production of membraneless organelles, which can promote fibrillization of RBPs through their LCDs (Taylor, et al. 2016).

Consistently, many ALS mutations fall within the LCD domains of various RBPs (Lagier-Tourenne, et al. 2010), (Kim, et al. 2013). One consequence of the accumulation of RBPs in cytoplasmic inclusions is their depletion from nuclei, which causes the impairment of their nuclear functions. Since FUS, TDP-43 and hnRNPA1 are important splicing regulators, their nuclear loss reflects the alteration of various splicing events that might contribute to motor neurons defects (Taylor, et al. 2016).

There are many studies attesting how also increased DNA damage correlates with ALS pathology. As previously said, the oxidative damage is one of the most encountered, in disorders affecting the nervous system. At this regard, oxidative DNA lesions in nuclear and mitochondrial genomes have been widely reported in ALS affected subjects (Ferrante, et al. 1997), (Bogdanov, et al. 2000), (Murata, et al. 2008) and have been associated with mutations in ALS-linked genes like ALS2

(Cai, et al. 2005) and SETX (Suraweera, et al. 2007). Also DNA breaks have been found to characterize ALS. Consistently, SOD-1 mutant mice were reported to accumulate both nuclear and mitochondrial SSBs and DSBs, the latter increasing with the age progression (Martin, et al. 2007). In addition, as already said ALS expanded repeats of C9orf72 have been associated with increased DSBs and DDR activation (Farg, et al. 2017), as well as R-loops accumulation due to defective ATM driven repair (Walker, et al. 2017). A direct connection between DNA damage and ALS pathology also arises from studies regarding the ALS protein, FUS (Madabhushi, et al. 2014). Indeed, FUS was found to have roles in the genome stability maintenance (Madabhushi, et al. 2014) and in DSBs repair through HR and NHEJ (Penndorf, et al. 2018). Importantly, it was shown that FUS localizes to DNA damage sites in a PARP-1 dependent manner (Rulten, et al. 2014) and its role in DNA repair seems to rely on the interaction with HDAC1, through its G-rich C-terminal domain, which is often mutated in FUS-related ALS cases (Wang, et al. 2013). Accordingly, motor cortices from ALS patients with such FUS mutations were enriched of DNA damage relative to healthy brain samples (Wang, et al. 2013). It was also showed that both FUS and TDP-43 have a role in the prevention or repair of the UV induced, transcription-linked DNA damage (Hill, et al. 2016). Additionally, another study comparing the response to DSBs of hMSCs derived from sALS patients and control hMSCs, show that the former are more sensitive to the induced damage, since they activate autophagy mediated cell death (Wald-Altman, et al. 2017). All these studies suggest that in addition to all the other proposed pathogenic mechanisms, DNA damage accumulation and or its defective repair is another important mark of ALS that might underlie motor neurons cell death in ALS.

3.2 The DNA/RNA binding protein TDP-43: structure and functions

Tar DNA binding protein 43 (TDP-43) is a ubiquitously expressed DNA/RNA binding protein of 414 amino acids, evolutionary conserved in invertebrates and mammals, mainly located in the cell nucleus (Smethurst, et al. 2015). Its name derives from its ability to bind and inhibit the TAR regulatory element implicated in the gene expression of the HIV-1 virus (Ou, et al. 1995). Specifically, TDP-43 belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) family and is composed of a N-terminal domain, a nuclear localization signal (NLS), two RNA binding domains (RRM1 and RRM2), a nuclear export signal (NES), a glutamine/asparagine-rich (Q/N) site and a glycine-rich domain (Smethurst, et al. 2015) (fig. 14).

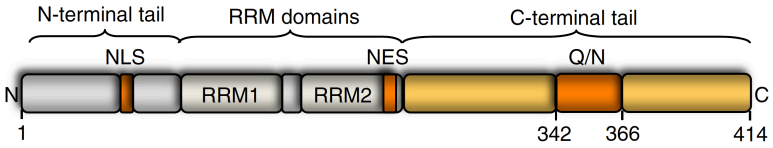


Fig. 14| Protein structure of TDP-43. (Smethurst, et al. 2015)

RRM domains have affinity with RNA sequences enriched in UG. Particularly, it has been shown that the binding occurs with single or double stranded repeats of at least six UG or TG, in a proportional fashion respect to increasing numbers of repeats (Buratti and Baralle 2001). RRM1 was found to be necessary and sufficient for the nucleic acid binding activity through phenylalanine residues 147 and 149, whereas RRM2 seems to contribute mostly to the correct conformation of the protein-RNA complex (Buratti and Baralle 2001). Additionally, TDP-43 has an unstructured region at its C terminus that is rich in glycine/serine and glycine/asparagine (Lim, et al. 2016), is subjected to post-translational modifications (Gsponer and Babu 2009) and to the strict regulation of its expression (Gsponer, et al. 2008), features that are typical of unstructured proteins.

Within the unstructured region, a stretch of 23 amminoacids rich in Q/N residues, has been defined as prion like domain since it is responsible for the TDP-43 tendency to aggregate and is similar to domains of other proteins, which are able to assume amyloid conformations that propagate from cell to cell (Udan and Baloh 2011). Importantly a lot of ALS/FTD mutations fall within this region of TDP-43 and are believed to increase the intermolecular aggregation of the protein (Udan and Baloh 2011). It has been established that TDP-43 is able to intrinsically modulate the amount of its transcripts, leading to the auto-regulation of the protein level present in the cell. Indeed, a study demonstrates that the ectopic expression of an inducible TDP-43 led to a decreased level of the endogenous protein in HEK293 cells (Ayala, et al. 2011). This kind of auto-regulation requires that TDP-43 binds through the RRM1 domain to its transcripts in a region inside the 3'UTR that has been defined TDPBR (TDP-43 binding region). This regulation has been proposed to rely on a pathway of mRNA degradation involving exosomes (Ayala, et al. 2011). Another proposed regulatory mechanism involves instead the splicing activity of TDP-43 on its own transcripts (Polymenidou, et al. 2011). Indeed, the protein is able to bind an intron in the 3' UTR of the mRNA, driving an alternative splicing event that generates mRNAs with a premature stop codon ultimately leading to transcripts degradation by the nonsense mediated RNA decay (NMD) process (Polymenidou, et al. 2011).

TDP-43 has been shown to have a plethora of roles related to the RNA metabolism (fig. 15), thus alterations in such processes due to its loss or dysfunction, can favour ALS related toxicity in different ways (Ratti and Buratti 2016).

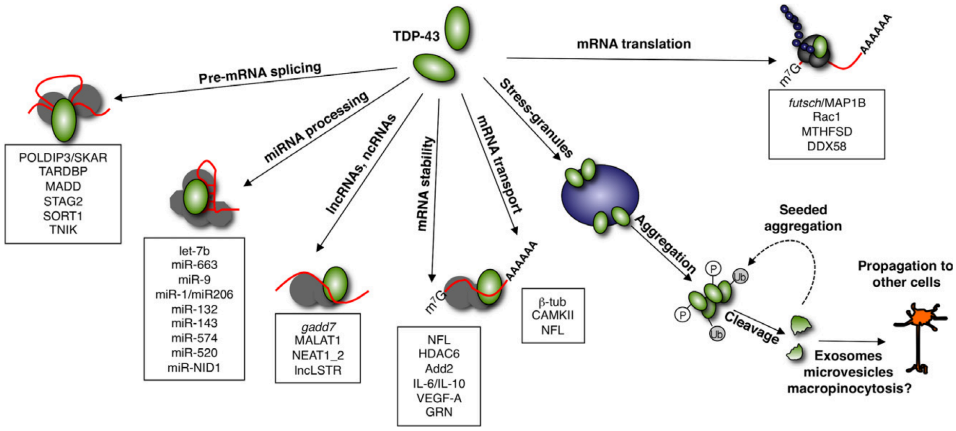


Fig. 15| Scheme depicting the functions of TDP-43. (Ratti and Buratti 2016)

In addition to regulate the splicing of its own mRNA, TDP-43 was shown to modulate splicing events of other transcripts (Ratti and Buratti 2016). The first discovered target of the splicing regulatory activity of TDP-43 was the cystic fibrosis transmembrane conductance regulator (CFTR) gene, whose mutations are causative of the related pathology (Buratti, et al. 2001). In particular TDP-43 was found to induce the skipping of the 9th exon by binding the polymorphic TG repeats near the upstream 3' splice site (Buratti, et al. 2001). Other splicing events found to be directly headed by TDP-43 are those affecting transcripts of genes as POLDIP3/SKAR, which regulates cell growth and favours global protein translation (Fiesel, et al. 2012), the key receptor for neuron viability SORT1 (Prudencio, et al. 2012) and STAG2 and MADD, respectively involved in chromatids segregation during cell division and neuronal survival (De Conti, et al. 2015).

An additional relevant role of TDP-43 is the regulation of mRNA stability. One of the RNA target stabilized by the interaction of its 3' UTR with TDP-43 is that of the human low molecular weight neuro filament protein (hNFL), whose altered

stoichiometry, has been linked with the formation of neurofilament (NF) aggregates in ALS pathology (Strong, et al. 2007). Another important mRNA substrate reduced in its expression and probably also in its stability after TDP-43 knockdown, is GRBP (McDonald, et al. 2011) a core marker of SGs, which are widely implicated as intermediates in the mechanism of TDP-43 aggregation (fig. 13). TDP-43 also acts as a translational regulator since it was demonstrated that in dendrites of rat hippocampal neurons it is organised in RNA-containing granules and following opportune neuronal stimuli, is able to regulate local protein translation (Wang, et al. 2008). The role of TDP-43 in translational regulation has been straightened by the discovery of other targets with important roles in neuronal functions, like Rac1 implicated in mammalian spinogenesis (Majumder, et al. 2012).

Another important function ascribed to TDP-43 is the transport of mRNA over axons and dendrites (Alami, et al. 2014). In fact RNA granules containing TDP-43 together with other RBPs, are subjected to both anterograde and retrograde transport (Fallini, et al. 2012) and TDP-43 mutations affecting transport efficiency, damage neurons contributing to their degeneration (Alami, et al. 2014). There are also some studies attesting that TDP-43 regulates the expression of some ncRNA and lncRNA whose alterations have been implicated in neurodegerative disorders (Ratti and Buratti 2016). Particularly, it has been observed that two lncRNAs, the nuclear paraspeckle assembly transcript 1 (NEAT1) and the metastasis associated lung adenocarcinoma transcript 1 (MALAT1), are bound by TDP-43 at higher rates in FTLD than in healthy brain samples. NEAT1 has a role in paraspeckle formation and MALAT1 regulates serine/arginine (SR) splicing factors distribution, in nuclear speckles as well as their phosphorylated levels (Tollervey, et al. 2011).

It is noteworthy that TDP-43 has also crucial roles in RNA silencing driven by miRNA (Ratti and Buratti 2016). Particularly, it directly contributes to the processing of many miRNAs like let-7b, miR-663, miR-132, mir-574, mir- 143 and mir-558 (Buratti, et al. 2010), (Kawahara and Mieda-Sato 2012) whose biogenesis

is altered following TDP-43 depletion. A more global involvement of TDP-43 in miRNA regulation comes up from different studies demonstrating its contribution to the function of the miRNA biogenesis machineries involving the RNases DROSHA and DICER (Kawahara and Mieda-Sato 2012), (Di Carlo, et al. 2013). DROSHA is part of two complexes differing in protein composition and molecular mass, the smallest of which is known as microprocessor, contains DGCR8 and is strictly responsible for pri-miRNA processing into pre-miRNA (Gregory, et al. 2004). Importantly, TDP-43 was shown to be an accessory component of the larger complex formed by DROSHA and other RNA-associated proteins such as DEAD-box and DEAH-box RNA helicases and hnRNPs (Gregory, et al. 2004). In addition to directly bind some pre-miRNA and pri-mRNA and to interact with the DROSHA complex in the nucleus, TDP-43 was also found to bind the DICER complex in the cytoplasm (Kawahara and Mieda-Sato 2012). Through all these protein-protein and protein-RNA interactions, TDP-43 contributes to the maturation of some miRNA species in both growing cells and cells committed to a neuronal fate (Kawahara and Mieda-Sato 2012). Moreover TDP-43 was found to regulate the amount of the microprocessor complex, stabilizing DROSHA at the protein level in cells undergoing neural differentiation and this occurs also for DGCR8, although to a less extent (Di Carlo, et al. 2013). The role of TDP-43 in miRNA biogenesis and regulation is in line with the hypothesis that miRNA deregulation is another kind of alteration contributing to ALS pathology (Taylor, et al. 2016). Indeed, it was observed that DICER depletion in glial cells and spinal motor neurons induced neurodegeneration and typical ALS pathological signs (Volonte, et al. 2015). Consistently, also alterations of the ALS-related FUS protein affect several sets of miRNAs implicated in neural differentiation and motor neurons survival (Morlando, et al. 2012), (De Santis, et al. 2017).

Neurons differentiated from induced pluripotent stem cells (iPSCs) derived from ALS patients with different mutations in TARDBP revealed also a reduction in the levels of both the unprocessed and the mature forms of mir-9, after the treatment

with the stress inducing agent staurosporine (Zhang, et al. 2013). Particularly, neurons derived from cells with the most pathogenic M337V mutation on TARDBP, showed diminished mir-9 levels, even in the absence of any stress stimulus (Zhang, et al. 2013). The low expression of this miRNA was found to be associated to TDP-43 mutations also in Drosophila melanogaster (Li, et al. 2013b), and might be pathogenic since mir-9 plays relevant roles in neuronal specification (Volonte, et al. 2015). Indeed, miR-9 reduction contributes to the de-regulation of the neuro filament (NF) transcript, leading to an altered NF stoichiometry at the protein level, which as said, is a reported feature of ALS. Additionally, miRNA profiles reflecting a pro-inflammatory status have also been found in leukocytes of ALS patients or included in vesicles as exosomes that are present in the plasma of the patients and are useful as diagnostic biomarkers (Volonte, et al. 2015). Another relevant study revealed a global down-regulation of miRNA in motor neurons of both sALS and fALS patients (Emde, et al. 2015). In this study the decrease of motor neuronal miRNAs resulting from DICER dysfunction, correlated with SGs formation due to the overexpression of wild type and ALS-linked mutant forms of TDP-43 and FUS. At this regard, it was hypothesized that changes in the interactions carried out by DICER with its cofactors and with SGs proteins are responsible for the decreased DICER activity in terms of miRNA biogenesis. Moreover, treatments of two ALS mice models with enoxacin, an antibiotic able to reinforce the interaction between the TRBP component of the DICER complex and the pre-miRNA substrates ultimately boosting their processing, ameliorated ALS clinical signs (Emde, et al. 2015).

3.3 Stress Granules and ALS

3.3.1 Insights in SGs composition, assembly, disassembly and function

Stress Granules (SGs) are cytoplasmic membrane-less organelles belonging to the wider class of ribonucleoprotein (RNP) granules that are enriched in RNA and proteins (Protter and Parker 2016). Other nuclear and cytoplasmic assemblies belonging to the same class are nucleoli, paraspeckles, processing bodies (P-bodies), neuronal granules and germ cell granules (Protter and Parker 2016). SGs and P-bodies are strictly correlated since both organelles contain untranslated mRNAs and have in common some protein components (Kedersha and Anderson 2007). Moreover they are often positioned nearby in the cytoplasm and they also have dynamic exchanges of messanger ribonucleoproteins (mRNPs) (Kedersha, et al. 2005). Nevertheless, SGs exclusively form in presence of stresses of different origin and result in the inhibition of the initial steps of protein synthesis (Protter and Parker 2016), whereas P-bodies are constitutive and are thought to serve for mRNA degradation (Aizer, et al. 2014). Particularly, stresses that can trigger SGs formation are: viral infections, exposure to oxidative agents, heat shock, chemical components as puromycin, increased osmolarity and serum deprivation. All of them converge on the phosphorylation of the translation initiation factor eIF2a, thus preventing the formation of the ternary complex eIF2-GTP-tRNA (Met) that normally binds the 48S pre-initiation complex to start protein translation (Kedersha and Anderson 2002). The 48S complex is then bound by the RBP TIA-1 and the TIA-1 related protein (TIAR), which block translation through polysomes decay (Kedersha and Anderson 2002). An eIF2a-independent pathway also resulting in stalled translation, involves instead the inhibition of the eIF4F complex (Frydryskova, et al. 2016). The ultimate effect of this global translation stall is supposed to be the reduction of energy consumption during the time the stress persists (Dewey, et al. 2012). Once stress is removed, mRNAs can be reassembled into polysomes to resume translation or can be transferred to P-bodies for

degradation (Dewey, et al. 2012). In addition to mRNAs and the stalled translation preinitiation complex, SGs are also composed of many other proteins with various functions in signalling cascades like those mediated by TOR and RACK1, in post-translation modifications and mRNPs remodelling (Protter and Parker 2016). SGs are organised in two moieties, an internal core more compact and enriched in RNA and proteins and a surrounding shell where these factors are more dispersed (Jain, et al. 2016) (fig. 14). Moreover it was observed that SGs are very dynamic structures: they move, they can fuse or split in two and they can exchange factors with the surrounding cytoplasm (Kedersha, et al. 2005). These exchanges were shown to occur extremely fast, on average in half a minute, for most of SGs constituents (Buchan and Parker 2009).

Importantly, SGs composition strongly depends on the cell types and on the specific kind of stress the cell receives (Dewey, et al. 2012), (Markmiller, et al. 2018). However some SGs protein markers characterize these organelles constitutively, independently from the context in which they form. Such constitutive SGs markers include the proteins TIA-1 and TIAR, the Ras GTPase-activating protein-binding protein 1 (G3BP), the poly-A binding protein (PABP-1) and the eIF3 and eIF4G factors forming the translation initiation complex (Dewey, et al. 2012), (Buchan and Parker 2009). All these proteins work as SGs nucleating factors, which start the formation of SGs seeds that later on can incorporate additional components as RBPs like hnRNP A1, FUS and TDP-43 and helicases as DDX4 and DDX6 (Protter and Parker 2016). TDP-43 itself figures as a facultative component of SGs but is specific since it does not incorporate into PBs (Aulas and Vande Velde 2015). A comparison of different studies shows that TDP-43 recruitment into SGs depends more on the cell type than on the type of stress inflicted (Dewey, et al. 2012). Indeed, HeLA cells were shown to be prone to form TDP-43 positive SGs in various stress conditions while the neural cell lines SH-SY5Y and Neuro2a have proven to be more refractory to TDP-43 localization into SGs (Dewey, et al. 2012). It has been observed that the TDP-43 transfer within

SGs requires its RRM1 domain and a portion of the glycine rich domain (Colombrita, et al. 2009). Moreover, a study showed how inhibition of the c-Jun N-terminal kinase (JNK) signalling inhibited TDP-43 localization in SGs upon different treatments inducing oxidative stress (Meyerowitz, et al. 2011). Since TDP-43 has not obvious consensus sites for JNK, the researchers speculated that TDP-43 localization into SGs might be a consequence of its interaction with other SGs components and TDP-43 interactors like hnRNPs, which are target of JNK. Additionally, a recent study has further extended the list of proteins found within SGs, using G3BP as bait and has highlighted how their composition varies in different cell lines (Markmiller, et al. 2018). As previously mentioned, SGs assembly is supposed to be promoted by the LLPS of RBPs, which can weakly and dynamically interact through their LCDs. In the context of membrane-less organelles formation like SGs, LLPS is a process leading some components to be concentrated in some districts within the cell (Hyman, et al. 2014). This results in the production of two distinct liquid phases that are not surrounded by defined boundaries but that stay apart because although in each phase the composition of the molecular species changes, the lack of a difference in the chemical potential between the two phases impedes their mixing (Hyman, et al. 2014). The hypothesis that SGs could arise from LLPS involving RBPs is in line with the ability of LCDs to initiate LLPS in vitro (Molliex, et al. 2015). Importantly, LLPS is a relevant process also connected to nuclear responses as the DDR (Altmeyer, et al. 2015). Indeed, it has been demonstrated that FUS as well as other LCDs containing proteins of the FET family, phase separate and assemble at sites of DNA damage following the formation of PAR chains that are sensed by the IDRs of such proteins (Altmeyer, et al. 2015).

To explain how LLPS could favour SGs formation despite these organelles also contain cores characterized by more stable interactions, a multistep model of assembly has been postulated (Protter and Parker 2016). According to such model, specific and strong interactions between mRNPs, first ensure the nucleation of SGs

cores and then the close proximity of LCDs contained in RBPs leads to LLPS that in turn favours the constitution of the more dynamic SGs shells. Particularly, after nucleation SGs grow incorporating additional mRNPs and forming cores that fuse and are ultimately surrounded by shells, giving rise to larger and cytologically visible SGs. The assembly, dynamicity and remodeling of SGs are ascribed to the activity of ATPases as DEAD-box, MCM and RVB helicases and chaperones (Jain, et al. 2016), (Cherkasov, et al. 2013). Indeed, in the presence of stress, such ATPases guarantee that SGs components are promptly exchanged between core and shell and with the surrounding environment, by temporarily cutting off SGs internal interactions. Differently, after stress removal, ATPases activity is required for triggering SGs disassembly (Protter and Parker 2016). The complete clearance of SGs finally involves the autophagy pathway triggered by other ATPases such as the VCP/Cdc48 ubiquitin segregases (Buchan, et al. 2013).

3.3.2 SGs in ALS pathogenesis

Different evidences correlate oxidative stress, an established promoter of SGs, with neurodegenerative diseases like ALS and FTLD (Shaw, et al. 1995), (Abe, et al. 1995). Additionally, ALS and FTLD pathologies have been extensively linked with mutations within genes whose products can contribute to SGs formation or to their impaired clearance. As discussed above, examples of these genes are RBPs like TIA-1, hnRNPA1, FUS, TDP-43 and genes related to autophagy such as optineurin and ubiquilin-2 (Protter and Parker 2016), (Dewey, et al. 2012). Strikingly, although the co-localization of SGs markers with TDP-43 pathological inclusions in ALS/FTLD is still object of debate, different groups observed that in spinal cord and frontal cortex tissues of ALS and FTLD patients, TDP-43 positive aggregates overlapped with the SGs markers TIA-1, eIF3, PABP-1 (Liu-Yesucevitz, et al. 2010), (Bentmann, et al. 2012). Moreover, according with some in vitro

experiments (Molliex, et al. 2015), (Lin, et al. 2015), an interesting hypothesis now considered valid in the scientific community is that persistent SGs can progressively degenerate into more stable and pathological structures. As demonstrated in in vitro studies, the high concentration of prion-like domains present in LCDs of SGs marker proteins could lead to the production of amyloid-like structures, which exert pathological effects on cells (Li, et al. 2013a). Indeed, these stabilized structures might sequester important players of various cell pathways and overall seem to negatively impact on all those processes that were shown to be altered in ALS pathogenesis, such as RNA metabolism and the axonal transport of RNA granules (Protter and Parker 2016).

It has also been proposed that TDP-43 accumulation into SGs contributes to their transition into more persistent, aggregate-like forms that, differently from TDP-43 negative SGs, cannot be reverted after treatment with cycloheximide, a chemical compound whose action is to block the elongation phase of translation and to dissolve canonical SGs organelles (Parker, et al. 2012). In light of all these evidences, chronic environmental stresses such as the oxidative one and genetic mutations affecting RBPs with relevant functions in RNA and protein homeostasis, as well as in axonal transport of neuronal cells, might be causative of ALS/FTD pathologies. In particular, such factors could concur individually or in synergy, to produce altered and persistent SGs that may act as precursors of more stable TDP-43 positive aggregates, which definitively characterize the spectrum of TDP-43 proteinopathies.

Aims of the research

As widely reported, the hallmark of ALS/FTLD is the presence of neuronal and glial TDP-43 positive inclusions (Arai, et al. 2006), (Neumann, et al. 2006), (Zhang, et al. 2008). At present, how such TDP-43 inclusions result pathogenic is under debate. On one side it is thought that TDP-43 aggregation could sequester the endogenous protein, hampering its canonical nuclear functions (loss of function hypothesis) (Winton, et al. 2008), on the other hand, TDP-43 aggregates *per se* could exert some toxic functions, detrimental for cell viability (gain of function hypothesis) (Johnson, et al. 2009), (Zhang, et al. 2009). These pathogenic mechanisms aren't mutually exclusive and a third cumulative hypothesis contemplates their coexistence.

A relevant feature of ALS is the enrichment in oxidative DNA damage (Ferrante, et al. 1997), SSBs and DSBs (Farg, et al. 2017), (Martin, et al. 2007) within affected neurons. In line with this notion, FUS, another ALS-linked protein sharing structural and functional similarities with TDP-43, has been already implicated in DSBs repair (Penndorf, et al. 2018). Moreover, TDP-43 is an established physical and functional interactor of DICER and DROSHA (Kawahara and Mieda-Sato 2012), (Di Carlo, et al. 2013), whose role in DDRNAs biogenesis has been demonstrated to be essential to trigger an effective DDR (Francia, et al. 2012). It was recently shown that the overexpression of WT or ALS-linked TDP-43 and FUS mutants could trigger SGs generation, resulting in dampened DICER activity and reduced miRNAs processing (Emde, et al. 2015).

In light of this combined knowledge, in my PhD thesis it was hypothesized that TDP-43 could have a role in the DDR process and that its ALS-related mutations, its aggregation or both processes could hinder the efficacy of DDR. The putative TDP-43 contribution to DDR might be elicited directly or indirectly, by affecting DICER/DROSHA activities, as an example. The resulting DDR impairment could in turn be one pathogenic aspect that fuels TDP-43 proteinopathies. In this perspective, my project was aimed to understand whether and how TDP-43 is involved in DDR, by using two different approaches that evoke the loss of function

or the gain of function theories, proposed to explain the disease mechanisms of TDP-43 proteinopathies. One approach consisted into overexpress TDP-43 to mimic the gain of function hypothesis in conventional cell systems and was useful to investigate the impact of the inclusions themselves on DDR. The other strategy used to mimic the loss of function theory was to deplete TDP-43 by means of its knock down and allowed us to investigate whether TDP-43 plays a physiological role in DDR. In both cases, the DDR signaling was studied by analyzing how DDR markers distributed within manipulated cells and how efficiently DDR foci accumulated in both basal conditions and after drug-induced DSBs generation. Importantly, trying to transpose part of the results in ALS patient's cells, I'm also preliminarily investigating TDP-43 distribution and the responsiveness to DDR of fALS fibroblasts with a specific mutation in the TARDBP gene.

Materials and methods

Cell culture

HeLA and U2OS cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L glutamine and 1% penicillin/streptomycin. SHSY5Y cells were cultured in a medium composed of DMEM:F-12 in a 1:1 ratio, with added 15% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were grown at 37°C under a humidified atmosphere with 5% CO2. When indicated DNA damage was induced by treating cells with Neocarzinostatin (NCS) at a final concentration of 50 ng/ml, for 20 minutes. ALS and healthy fibroblasts were kindly provided by Dr. Cristina Cereda from the Mondino Institute.

For all the experiments for which quantification is provided, we analyzed an average number of cells comprised between 50 and 180 per each biological replicate. More precise indications of the number of cells analyzed in each experiment are reported in the figure legends.

Plasmid transfection

Cells 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 Lipofectamine 2000 Reagent (Life Technologies). The two solutions were then mixed together and incubated for 10 minutes at 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 24 h of transfection, cells were collected for subsequent analysis.

Plasmid used as control in this study is pcDNA3.1+ (Addgene). The Myc tagged plasmid expressing TDP-43 WT was a kind gift of Dr. Emanuele Buratti on concession of Dr. Petrucelli Leonard (Mayo Clinic, Jacksonville, Florida) whereas

the Flag tagged plasmid expressing TDP-43 M337V was kindly provided by Prof. Eran Hornstein on concession of Prof. Dr. Markus Landthaler (Max Delbrück Center for Molecular Medicine-MDC, Berlin, Germany).

Chemical treatments

Treatment with DNA-PK and ATM inhibitors: Cells transfected with Lipo2000 with a Myc tagged TDP-43 WT expressing vector or an empty vector, or incubated with the transfecting agent only, where treated at 20 h post transfection with the DNA-PK inhibitor KU-60019 (Sigma Aldrich) or the ATM inhibitor NU7441 (Tocris Bioscience) at the final concentration of 5 uM, for 3 hours. Untreated cells were added with the same volume of DMSO. NCS treatment to induce DNA damage was done after 160 minutes, where indicated.

Treatment with the proteasome inhibitor: Cells transfected with Myc TDP-43 WT, the empty vector or added with the transfecting agent only, where treated in parallel with the proteasome inhibitor MG-132 (SIGMA) at a final concentration of 5 μM, for 24 hours. An equal volume of DMSO was added to untreated cells. When indicated, NCS treatment was made 20 minutes before fixation.

Treatment with Sodium (meta)arsenite: Sodium (meta)arsenite powder (SIGMA) was solubilized in H_2O and used at a final concentration of 500 μM for 45 minutes. Untreated cells were added with equal volumes of clean medium. To induce DNA damage where indicated, NCS treatment was made 20 minutes before fixation, meaning 25 minutes after arsenite addition.

UV-C irradiation

Cells were plated to have a 80-90% of confluence into 30 mm^2 dishes, washed once with PBS, dried and subjected to UV-C irradiation at both 40 J/m^2s^2 and 100 J/m^2s^2 (Tomas, et al. 2017), without plate lid. Not irradiated cells where treated in the same way. Both control and irradiated cells were then left to recover for 30 minutes

in serum free medium at 37°C, before fixation.

RNA interference (RNAi)

Short interfering RNAs (siRNAs) are commonly used to knockdown a specific gene of interest. For siRNA transfection, cells were plated in 6 multi-well plates so that they were at around 40-50% confluence, the day of the transfection. For each transfected well 250 µl of serum-free medium (Opti-MEM) were mixed with siRNA oligo (usually 10 nM as final concentration) 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 substituted with 1.5 ml of fresh culture medium. The mix was then added to the cells that were left in the incubator until the analysis. Knockdown by siRNA transfection is transient and usually biological effects are studied within 72-96 hours post transfection. The sequences of ON-TARGET plus SMARTpool siRNA oligonucleotides (Dharmacon) for human TDP-43 as well as nontargeting control siRNAs are reported in the table below.

Target mRNA	Sequences
TDP-43	GCUCAAGCAUGGAUUCUAA
	CAAUCAAGGUAGUAAUAUG
	GGGCUUCGCUACAGGAAUC
	CAGGGUGGAUUUGGUAAUA
Non-targeting CONTROL	UGGUUUACAUGUCGACUAA
	UGGUUUACAUGUUGUGUGA
	UGGUUUACAUGUUUUCUGA
	UGGUUUACAUGUUUUCCUA

Indirect immunofluorescence (IF)

Cells were grown on glass coverslips, washed twice with ice-cold PBS, fixed with 4% PFA for 10 minutes at RT and permeabilized with 0.2% Triton X-100 for 10 minutes at RT, for most of antibodies used. Some antibodies could require a different fixation method to work effectively and in such cases cells were fixed with a 1:1 mix of cold Methanol-Acetone for 3/ 4 minutes.

In any cases, cells were then washed twice in PBS, incubated for 1 hour in blocking solution (PBG, 0.5% BSA, 0.2% gelatin from cold water fish skin in PBS) 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 and incubated with 4'-6-Diamidino-2-phenylindole (DAPI, 0,2 µg/ml, Sigma-Aldrich) for 2 minutes at RT. Cells were briefly washed with PBS and water and coverslips were then mounted with Aqua Poly/Mount (Polysciences) mounting medium and let dry overnight at room temperature. Coverslips were air dried before microscope analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (tunel) assay

This assay was developed to detect the apoptosis-induced DNA fragmentation of apoptotic cells by imaging. To perform the Tunel assay I used the *In Situ Cell Death Detection Kit, TMR red* (Roche). In particular, the kit uses the Terminal deoxynucleotidyl transferase (Tdt) that adds TMR red-labeled nucleotides to free 3'OH DNA ends, if present. Cells were grown on coverslips and transfected for 24 h with Myc-TDP-43 WT, flag-TDP-43 M337V or the empty vector, fixed with 4% PFA for 10 minutes at RT, washed twice with PBS and treated with a mixed solution formed by Tdt and TMR nucleotides. Tunel reaction was protracted for 60 minutes in a humid and dark chamber, at 37°C. Cells used as negative control were treated with the TMR nucleotides only. Cells used as positive control were pre-

treated with DNase (Ambion, Life technologies) at a final concentration of 300 U/ml in an appropriated buffer with added PBG (0.5% BSA and 0.2% gelatin from cold water fish skin in PBS), for 30 minutes in a humid chamber, washed three times with PBS and subjected to the tunel reaction. After that cells were washed three times with PBS and incubated with 4'-6-Diamidino-2-phenylindole (DAPI, 0,2 µg/ml, Sigma-Aldrich) for 2 minutes at RT. Cells were briefly washed with PBS and water and coverslips were then mounted with Aqua Poly/Mount (Polysciences) mounting medium and let dry overnight at room temperature. Coverslips were air dried before microscope analysis.

Image capture and analysis

Immunofluorescence images were acquired using 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). Comparative immunofluorescence analyses were performed in parallel with identical acquisition parameters and exposure times using CellProfiler (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. All data for imaging analyses were plotted with the GraphPad Prism software.

For all the analysis in which it was required, the distinction between cells with and without TDP-43 positive inclusions was made through a comparison of the original images and the images in output from CellProfiler where nuclei are numbered in an automated manner. All the analysis regarding measures of DROSHA intensity and counts of 53BP1 foci have been restricted to cells overexpressing TDP-43 WT (either with and without TDP-43 positive granules), to ascertain that the effects were specifically linked to TDP-43 granules. To this aim a threshold intensity of

the Myc staining was chosen to distinguish Myc-TDP-43 overexpressing cells from not overexpressing cells. For Cyclin A intensity measurements, cells with pan-nuclear γH2AX, due to their strong signals, were clearly distinguished from the other cells, by eyes.

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® Turbo™ transfer pack) using the Trans-Blot® Turbo™ 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 Luminata™ Classico or Crescendo (Millipore). Proteins were visualized by autoradiography on ECL films (Amersham), using various exposure times and manually developed.

Antibodies

Antibody	Company	Host	Applications	
			IF	WB
TDP-43	Proteintech	Rabbit	1:500	1:1000
TDP-43	Abcam	Mouse	1:100	
c-Myc	Santa Cruz	Mouse	1:250	
G3BP	BD transduction laboratoires	Mouse	1:100	
TIA-1	Saanta Cruz	Goat	1:50	
Vinculin	Millipore	Mouse		1:1000
Vimentin	Sigma	Mouse		1:1000
γH2AX	Millipore	Mouse	1:800	1:1000
53BP1	Bethyl	Goat	1:800	
pATM	Rockland	Mouse	1:400	
pATM	Abcam	Rabbit		
pDNA-PK	Abcam	Rabbit		1:3000
Cyclin A	Santa cruz	Rabbit	1:200	
cleaved Caspase 3	Cell signaling	Rabbit	1:200	
DROSHA	Abcam	Rabbit	1:250	
R-loops (S9.6)	Prof. Marco Foiani's group (IFOM)	Mouse	1:20	
p-p38	Cell signaling	Rabbit		1:1000
FK2	ENZO	Mouse		1:500
Alexa 488/647	Life-Tech	mouse/ rabbit/ goat	1:300	
Invitrogen 555	Abcam	mouse/ rabbit/ goat	1:300	

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.

Results

1. Cytoplasmic TDP-43 inclusions alter DDR signaling

1.1 Increased levels of TDP-43 lead to the generation of cytoplasmic TDP-43 positive inclusions in HeLA cells

As mentioned above, TDP-43 cytoplasmic granules are hallmark of the ALS/ FTLD pathological spectrum, independently from the presence or not of TDP-43 genetic mutations that instead account for a relatively minor fraction of ALS cases. Importantly, in line with the gain of function hypothesis, such inclusions could be pathogenic in virtue of some toxic properties not fully characterized yet. Since TDP-43 also contains a LCD, which is intrinsically prone to aggregate, we tested if high concentrations of the protein might promote its homotypic interactions and trigger its self-aggregation (Li, et al. 2013a). Thus, I firstly evaluated if the overexpression of the TDP-43 protein in its WT form could give rise to aggregation. To this aim, I used three common cell lines originated from tumors of different organs, HeLA, U2OS and SHSY5Y that are particularly suitable for plasmid transfection and immunofluorescence (IF) analysis, an approach which we used to visualize TDP-43 localization (fig. 16 A). As shown in the figure, overexpression of a Myc-tagged version of TDP-43 WT resulted in the formation of cytoplasmic granules in a certain fraction of HeLA cells. Granules accumulation didn't occur in U2OS and SHSY5Y, indicating that different cell types might have different tendency to accumulate TDP-43 cytosolic inclusions. Indeed, this could be due to different levels of expression of TDP-43 *per se* or to a different rate of clearance of cytoplasmic granules, once formed. For this reason, we performed most of the experiments investigating the impact of TDP-43 aggregation on nuclear DNA stability, in HeLA cells.

Then, to test if ALS-linked TDP-43 mutations might increase granules formation, I overexpressed TDP-43 M337V (a mutation associated to fALS cases (Rutherford, et al. 2008), (Sreedharan, et al. 2008)) in parallel to the TDP-43 WT protein in HeLA cells (fig.16 B, D). Consistently I observed that TDP-43 WT overexpression

leads to the formation of clear cytoplasmic inclusions in about 10-15% of cells whereas the overexpression of the mutant TDP-43 M337V induces formation of cytoplasmic inclusions less frequently respect to the WT protein (fig. 16 B-C). Importantly, the different propensity to aggregate of the two TDP-43 isoforms was not related to the level of expression of the two proteins since mutant TDP-43 was even more expressed than the WT protein in HeLA, as assessed by WB (fig. 16 D) suggesting that the M337V mutation does not increase the propensity of TDP-43 to form cytoplasmic inclusions.

Of note, TDP-43 accumulation in cytoplasmic inclusions does not always result in its nuclear depletion (fig.16 B). This suggests that the phenotype observed upon TDP-43 overexpression might not be associated with its nuclear loss of function. It should be mentioned that cytoplasmic inclusions containing endogenous TDP-43 were also visible in a smaller fraction of control HeLA cells transfected with an empty vector (e.v), suggesting that plasmid transfection might contribute to a kind of stress that *per se* induces the formation of granules containing the endogenous protein. Thus, in line with the present literature (Aulas and Vande Velde 2015), (Dewey, et al. 2012), we observed that TDP-43 has an intrinsic tendency to be incorporated into cytoplasmic inclusions. Nevertheless, the formation of these structures is not promoted by the ALS linked mutation M337V.

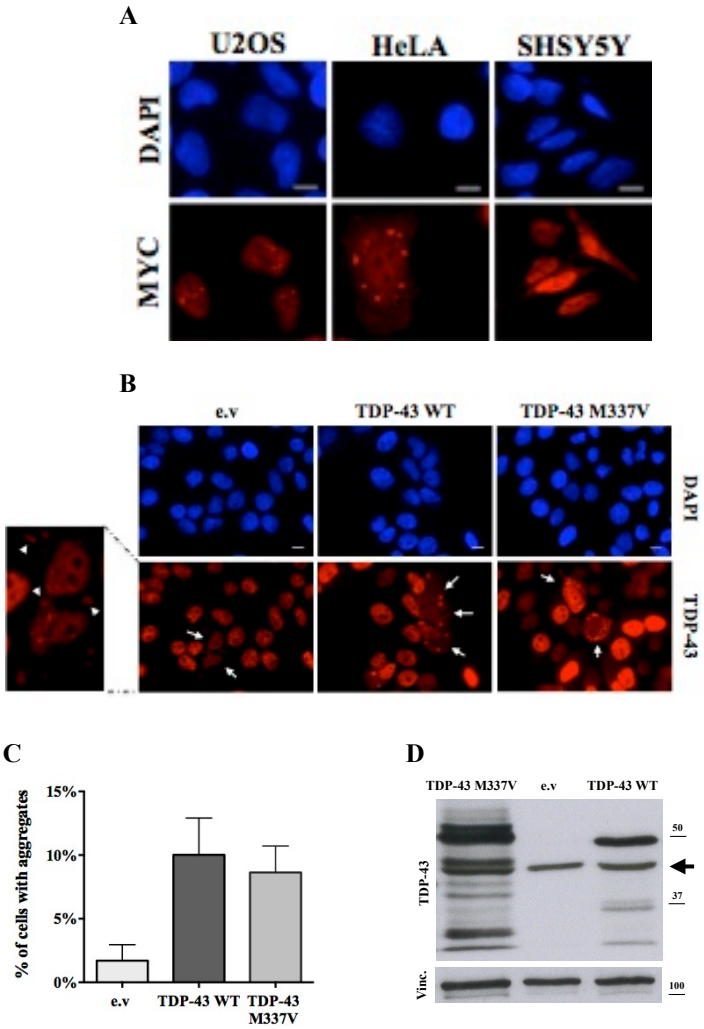


Fig. 16| Overexpression of TDP-43 WT and TDP-43 M337V produces cytoplasmic TDP-43 positive granules in HeLA cells.
A. Imaging of U2OS, HeLA and SHSY5Y cell lines overexpressing Myc-TDP-43 WT stained for Myc and counterstained with DAPI. Scale bar: 10 um. **B.** Imaging of HeLA cells overexpressing Myc-TDP-43 WT, TDP-43 M337V or transfected with an e.v as control, stained for TDP-43. Arrows indicate cells with cytoplasmic granules. A few control cells are able to form TDP-43 positive granules and are magnified to the right (faint granules are indicated by arrowheads). Nuclei were counterstained with DAPI. **C.** Percentages of cells forming TDP-43 granules in each indicated condition. Error bars represent SEM from three independent experiments. For this analysis, we analyzed from 90 to 260 cells for each condition, per biological replicate. **D.** Western blotting images showing the expression of tagged and endogenous TDP-43 (black arrow) in each condition, in HeLA.

1.2 Cytoplasmic TDP-43 positive granules *per se* cause ATM and DNA-PK dependent, nuclear wide phosphorylation on Ser 139 of H2AX

1.2.1 TDP-43 containing inclusions correlate with pan-nuclear γH2AX signals

Since my project has the aim of studying DDR defects in cells presenting TDP-43 dysfunction, we investigated the activation of well-known DDR markers in cells overexpressing TDP-43 isoforms. Thus, we stained HeLA cells overexpressing TDP-43 WT and the fALS-linked mutant TDP-43 M337V, with an antibody against γH2AX, the histone modification that decorates sites of DNA damage. Intriguingly, the fraction of cells positive for TDP-43 inclusions presented a very peculiar and strong pan-nuclear γH2AX signal even in the absence of exogenous DNA damage, indicative of the generation of a strong genotoxic stress (fig. 17 A-B). Indeed, TDP-43 overexpression *per se* is not sufficient to induce genotoxicity since cells equally transfected and overexpressing TDP-43, which do not form TDP-43 inclusions are negative for γH2AX pan nuclear signals (fig. 17 A). This intriguing observation indicates that cells forming cytoplasmic foci of TDP-43 gain a toxic function that has negative impacts on genome stability.

In light of this result, we also evaluated which was the cellular response to exogenously provided DNA damage in these cells. Thus, we performed the same experiment in parallel with cells treated with neocarzinostatin (NCS) (fig. 17), a radiomimetic drug able to exogenously induce DSBs, similarly to IR (Kuo, et al. 1984). As expected, cells devoid of TDP-43 inclusions, properly mounted canonical γH2AX positive DDR foci upon NCS treatment (FIG. 17 A-B). Instead, cells with TDP-43 inclusions accumulated γH2AX with a pan-nuclear distribution and with a higher mean nuclear signal respect to cells negative for TDP-43 inclusions (fig. 17 A), thus impeding the distinction of discrete γH2AX foci. Once γH2AX signals are very strong in cells with TDP-43 containing granules, such increased signals are also appreciable in cells overexpressing both isoforms of TDP-43, by blotting protein lysates with an antibody against γH2AX (fig. 17 C).

From the above results appeared that the fraction of cells forming cytoplasmic granules upon TDP-43 M337V expression is lower than that relative to TDP-43 WT expression (fig.16 C) and that the effects in terms of γH2AX distribution depend on TDP-43 granules formation rather than on TDP-43 overexpression *per se*.

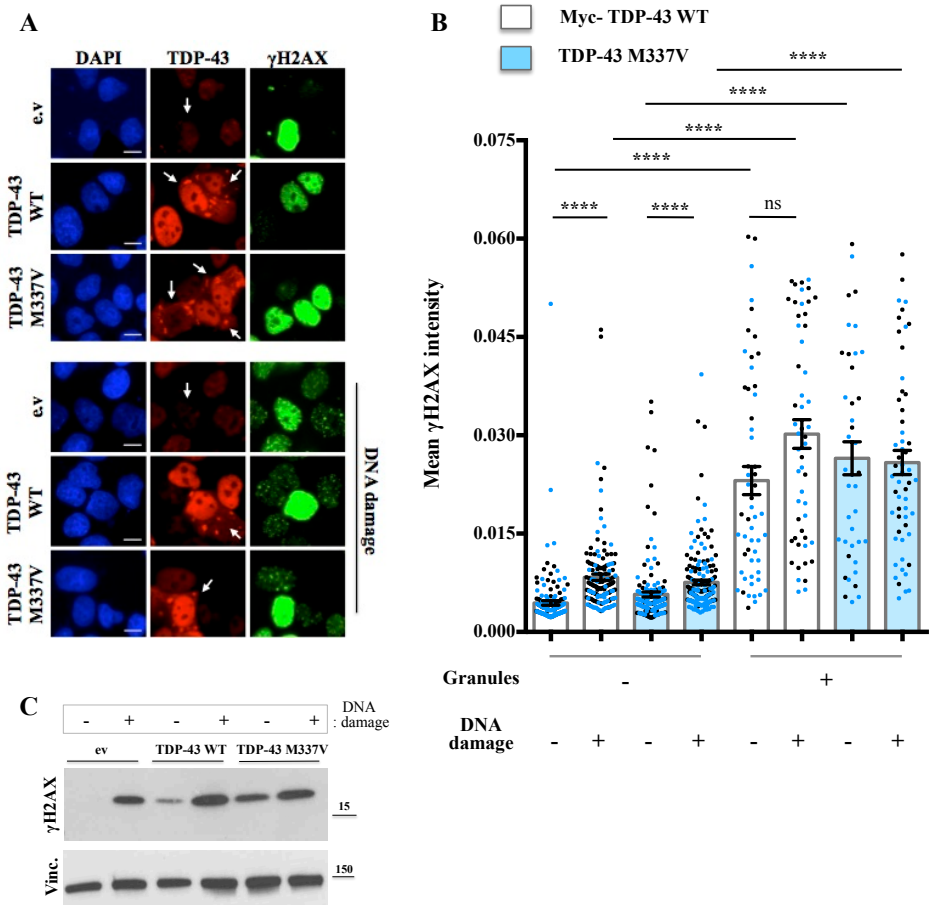


Fig. 17| Cytoplasmic TDP-43 positive granules lead to nuclear γH2AX accumulation;
A. Imaging of HeLA cells expressing TDP43 WT, TDP43-M337V and control cells, untreated or treated with NCS to induce DNA damage and immunostained to detect TDP-43 and γH2AX; arrows indicate cells with cytoplasmic granules; nuclei were counter-stained with DAPI. Scale bar: 10 um. **B.** Mean γH2AX intensity was measured in cells overexpressing TDP-43 WT (white bars) and TDP-43 M337V (light blue bars), by separating cells with TDP-43 granules from cells without TDP-43 granules, in each condition. Error bars represent SEM from two independent experiments, discernable by the different color of spots. P-value ≤ 0.05. **C.** For the quantification shown, between 80 and 140 cells have been analyzed for each condition, per biological replicate. Lysates of cells from the

indicated experimental conditions were blotted for γH2AX.

Thus, we conclude that high level of TDP-43 expression leads to its incorporation in cytoplasmic inclusions in a fraction of cells, an event that occurs also in the absence of specific ALS-linked mutations. Therefore subsequent analysis have been performed by taking advantage mainly of TDP-43 WT overexpression. To investigate the impact of TDP-43 inclusions on genome instability we compared cells forming TDP-43 granules with the surrounding cells also overexpressing TDP-43 WT but lacking granules, which we considered as the more accurate negative control.

1.2.2 Pan-nuclear γH2AX in cells with TDP-43 positive granules does not represent an apoptotic signal

A proposed mechanism for motor neurons degeneration in ALS pathology is the activation of the apoptosis program in affected cells (Martin 1999).
Noteworthy, in addition to represent a marker of DSBs by forming typical nuclear foci, phosphorylation on serine 139 of γH2AX, especially in its pan nuclear distribution, is reminiscent of the induction of chromosomes fragmentation during the apoptotic process (Rogakou, et al. 2000). In particular, pan-nuclear γH2AX could mark nuclei of cells undergoing intermediate steps of apoptosis (Solier and Pommier 2014). Thus, I tested if the nuclear-wide γH2AX accumulation observed in cells with TDP-43 inclusions coincides with apoptotic signals, by performing the terminal deoxynucleotidyl transferase dUTP nick end labeling (tunel) assay in cells overexpressing WT and M337V TDP-43, in parallel to control cells. The Tunel technic detects DNA ends generated during the genome fragmentation induced by the activation of apoptosis, ultimately leading to the formation of rounded apoptotic bodies. Intriguingly, adherent cells, which frequently showed

pan-nuclear γH2AX associated with TDP-43 aggregation in previous analysis (fig. 17 A), were all negative to tunel staining (fig. 18 A) whereas tunel signals were clearly detected in the same sample within rounded apoptotic bodies and in positive control cells intentionally treated with nucleases, confirming the efficacy of the assay into detecting apoptotic cells (fig. 18 A-B). Importantly, the percentage of apoptotic bodies positive to tunel signals was identical in cells transfected with EV or TDP-43 expressing vectors, indicating that the low level of apoptosis detected, was typical of the cell population, occurring regardless to the overexpression of TDP-43 or the formation of its cytoplasmic inclusions (18 B).

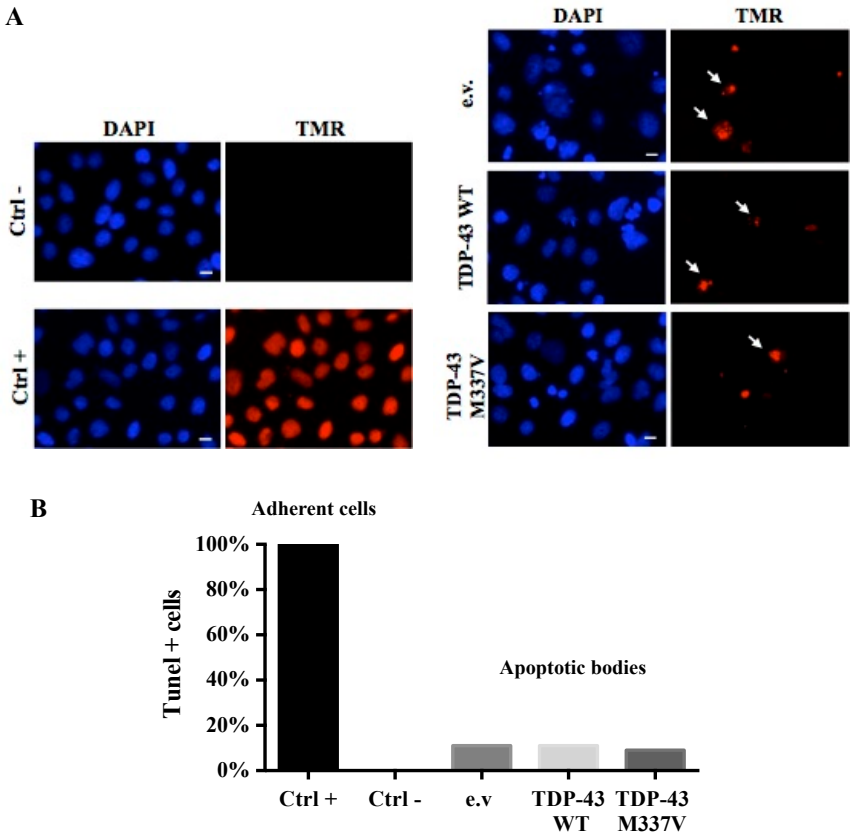


Fig. 18| TDP-43 overexpression doesn't trigger apoptosis.
A. Imaging of HeLA cells overexpressing TDP-43 WT, TDP-43 M337V or control cells, subjected to tunel assay to detect apoptosis. Cells pretreated with DNase and untreated cells were used as positive

and negative technical controls, respectively. TMR-nucleotides bound to free 3'OH DNA ends reveal apoptotic signals in red, also indicated by arrows; nuclei were counterstained with DAPI. Scale bar: 10 um. **B.** Quantification of apoptotic cells by tunel assay in each indicated condition. For the quantification shown, between 30 and 70 cells have been analyzed for each condition.

To strengthen this observation, we performed an immunostaining with the well-known apoptotic marker cleaved caspase 3 (Wolf, et al. 1999). Consistently with previous results, among cells overexpressing TDP-43 neither cells with TDP-43 inclusions nor cells with pan-nuclear γH2AX were positive to the staining for this apoptotic marker, while apoptotic bodies present in the cell populations regardless to TDP-43 overexpression, were positive for both cleaved caspase-3 and γH2AX (fig. 19). This indicates that cells enriched in nuclear γH2AX, associated with TDP-43 aggregation, are not undergoing apoptosis.

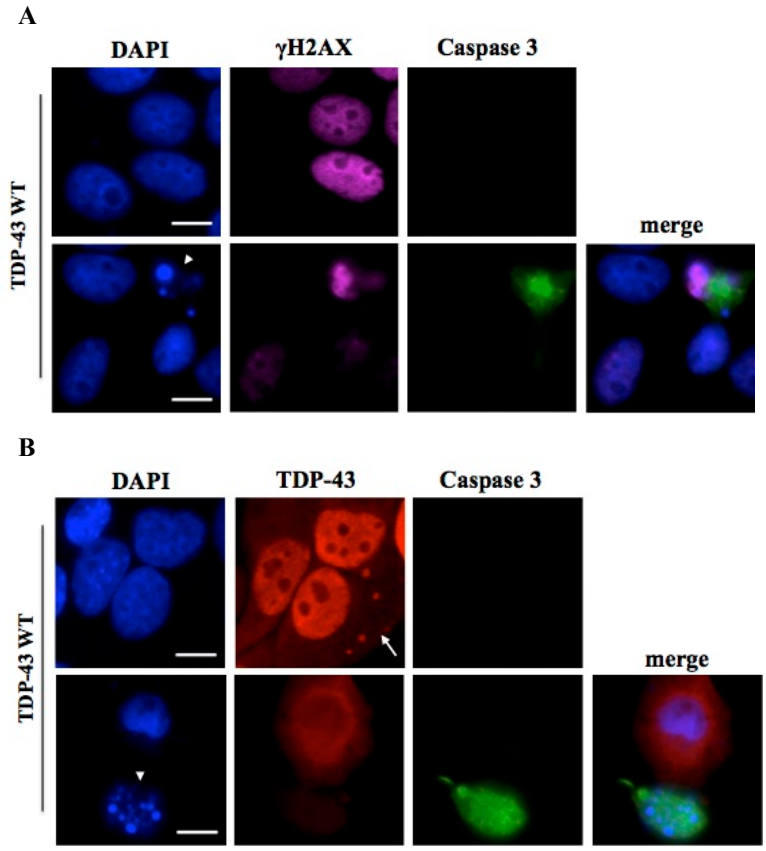


Fig.19| Pan-nuclear γH2AX in cells with TDP-43 granules is not associated with the activation of caspase 3. Representative images of HeLA cells overexpressing TDP-43 WT, stained for γH2AX and cleaved caspase 3 **(A)** or for TDP-43 and cleaved caspase 3 **(B).** Nuclei were counterstained with DAPI. Scale bar: 10 um. The arrow indicates cells with cytoplasmic granules. **A-B** bottom panels show DAPI positive apoptotic bodies (arrowheads), also positive for cleaved caspase 3 and γH2AX **(A)**.

1.2.3 Pan-nuclear γH2AX is not associated to R-loops formation

As mentioned above, R-loops could cause replicative stresses and genomic instability (Santos-Pereira and Aguilera 2015). Moreover they have been associated to some ALS subtypes (Farg, et al. 2017), (Chen, et al. 2004) and importantly, defects in RBPs could shift the equilibrium toward the exacerbated production of such DNA:RNA hybrids (Santos-Pereira and Aguilera 2015). In light

of these notions, I investigated if formation of TDP-43 granules and consequent pan-nuclear γH2AX, could correlate with R-loops generation, by performing an immunofluorescence with a monoclonal antibody raised against DNA:RNA hybrids, the clone S9.6 (Boguslawski, et al. 1986). The obtained staining was mostly cytoplasmic due to the signal coming from mitochondria, as previously observed (Sollier and Cimprich 2015). However, the staining with the S9.6 antibody was negative in the nuclei of cells overexpressing TDP-43 WT, as well as in those of cells forming TDP-43 positive inclusions (fig. 20). This data indicate that TDP-43 inclusions and pan-nuclear γH2AX do not correlate with accumulation of R-loops and consequent replication stress and DNA damage generation (Santos-Pereira and Aguilera 2015), (Walker, et al. 2017).

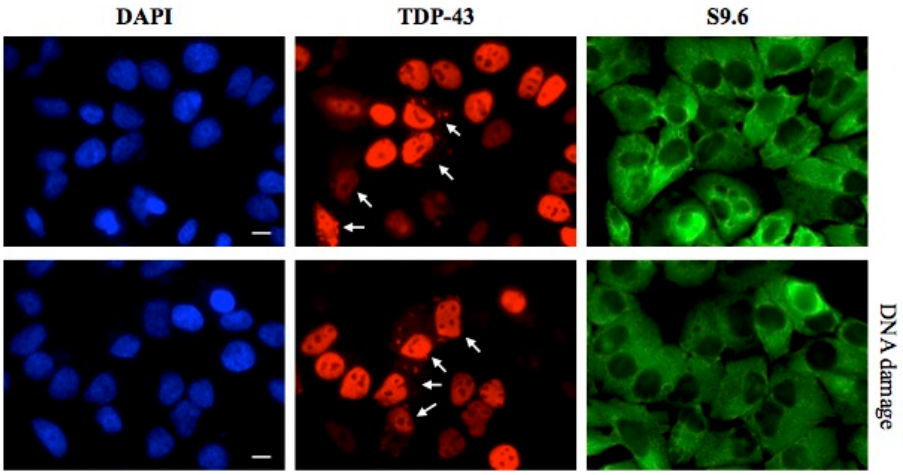


Fig. 20| TDP-43 granules are not associated to the formation of R-loops. Representative images of HeLA cells overexpressing TDP-43 WT, stained to detect TDP-43 and R-loops (S9.6). Arrows indicates cells with cytoplasmic granules. Nuclei were counterstained with DAPI. Scale bar: 10 um.

1.2.4 Pan-nuclear γH2AX does not correlate with replication stress

Having failed in identifying a causative role for apoptosis and R-loops accumulations in the formation of a pan-nuclear γH2AX signal, we wondered which other mechanisms could be responsible for such genotoxic effect. At this

regard, a study showed that pan-nuclear accumulation of γH2AX marked a high fraction of cells in S-phase upon UV-irradiation (de Feraudy, et al. 2010). Thus, I tested if the γH2AX enrichment observed in cells with TDP-43 granules was characteristic of cells in S phase, which could be subjected to replication stress. To this aim, I evaluated whether the pan nuclear γH2AX signal of cells experiencing TDP-43 inclusions was in fact associated with the passage into S-G2 phases of the cell cycle, by staining for Cyclin A, which is highly expressed in these two phases (Henglein, et al. 1994), (Pagano, et al. 1992) (fig.20). As shown in the figures, all cells with strong γH2AX signals had very low or null levels of Cyclin A expression (fig. 21) suggesting that such cells are preferentially in the G1 phase and are neither replicating nor in the G2 phase. Differently, cells devoid of pan nuclear γH2AX showed the expected Cyclin A positivity with different intensities (fig. 21 B) representative of the different levels of its expression in the various phases of the whole cell cycle. Of note, mutually exclusion between γH2AX and Cyclin A signals occurs with identical frequency both in damaged (+ NCS) and undamaged conditions, demonstrating that the cell cycle arrest of cells with TDP-43 inclusions occurs before exposure to DNA damage inflicted for 20 minutes for experimental purposes (fig. 21 C).

The evidence that formation of TDP-43 inclusions causes a G1/S cell cycle arrest is coherent with the idea that pan-nuclear γH2AX could reflect the accumulation of physical DNA damage such as DSBs that are responsible for the induction of cell cycle checkpoints (Jackson and Bartek 2009), (Branzei and Foiani 2008).

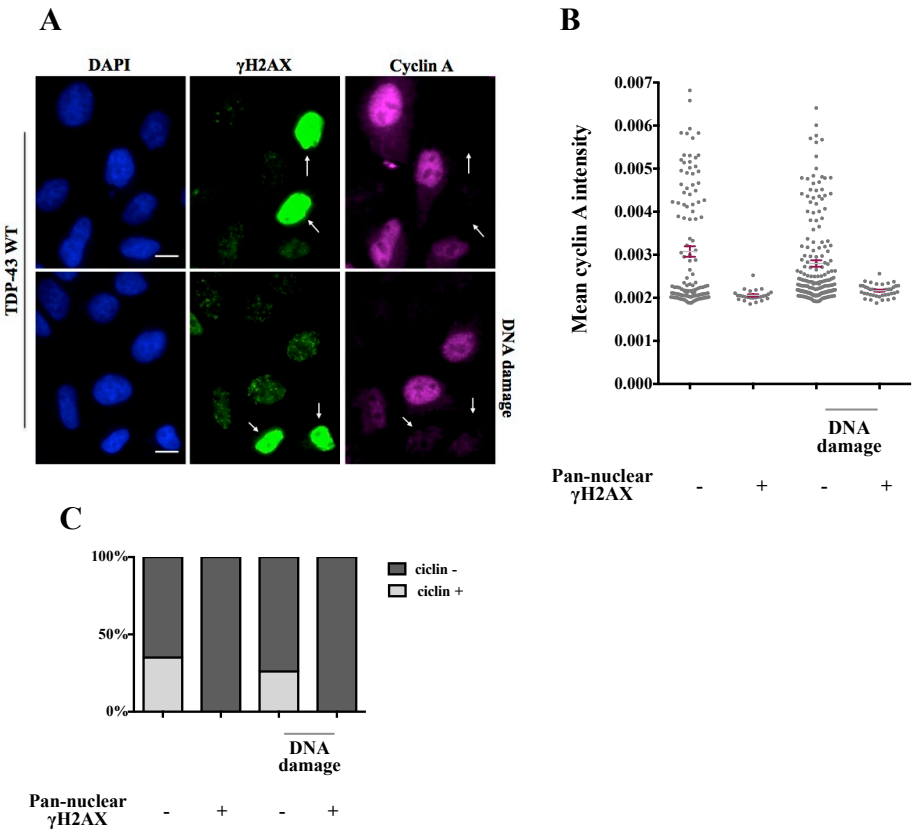
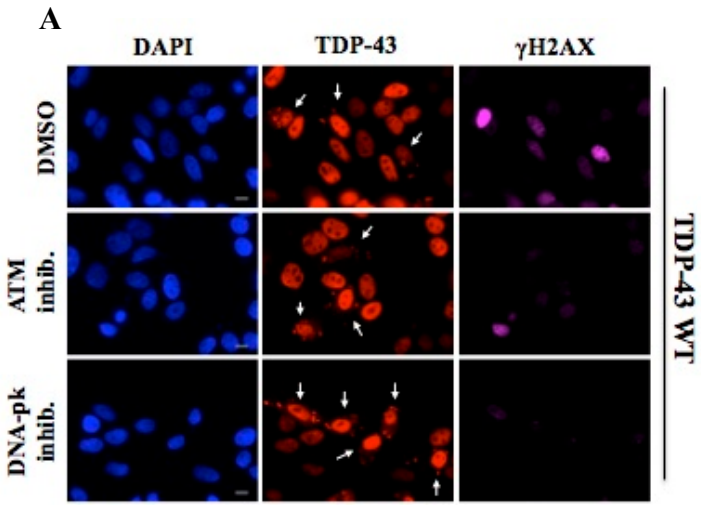


Fig. 21| Pan-nuclear γH2AX is typical of cells in the G1 phase.
A. Representative images of HeLA cells overexpressing TDP-43 WT, in basal conditions or after DNA damage induction, stained to detect γH2AX and Cyclin A. Arrows indicate cells with pan-nuclear γH2AX. Nuclei were counterstained with DAPI. Scale bar: 10 um **B.** Measure of mean Cyclin A intensity in each indicated conditions; Error bars represent SEM (n=1). **C.** Quantification of the percentages of cells positive and negative for Cyclin A in each indicated condition. The threshold Cyclin A intensity to differentiate positive and negative cells (0.003) was chosen on the base of the cyclin intensity distribution of cells without pan-nuclear γH2AX in absence of damage (B). For the quantification shown, 150 untreated and 220 NCS-treated cells have been analyzed, respectively.

1.2.5 ATM and DNA-PK kinases are responsible for the nuclear wide phosphorylation on H2AX

The two main kinases responding to DSBs are ATM and DNA-PK, and a recent study showed that pan-nuclear γH2AX accumulates also in response to the strong

activation of both kinases in different contexts of chromatin structure alterations (Meyer, et al. 2013). Thus, I investigated if the same kinases were responsible for γH2AX accumulation in cells with TDP-43 inclusions. To this aim, TDP-43 WT overexpressing cells have been treated with the ATM and the DNA-PKcs inhibitors (KU60019 and NU7441) separately for three hours and the amount of γH2AX accumulation was evaluated by IF (fig. 22) both in basal conditions and upon DNA damaging treatments in order to have a positive control to verify that both inhibitors worked (fig. 22 C). Treatment with ATM and DNA-PK inhibitors significantly decreased both the frequency (fig. 22 A) and the intensity (fig. 22 B) of pan-nuclear γH2AX in cells with TDP-43 cytoplasmic inclusions, suggesting a key role of ATM and DNA-PK kinases in this phenomenon. The activation of ATM and DNA-PK in damaged samples, and their inhibition by KU60019 and NU7441 treatment, was confirmed by western blotting (fig. 22 C).



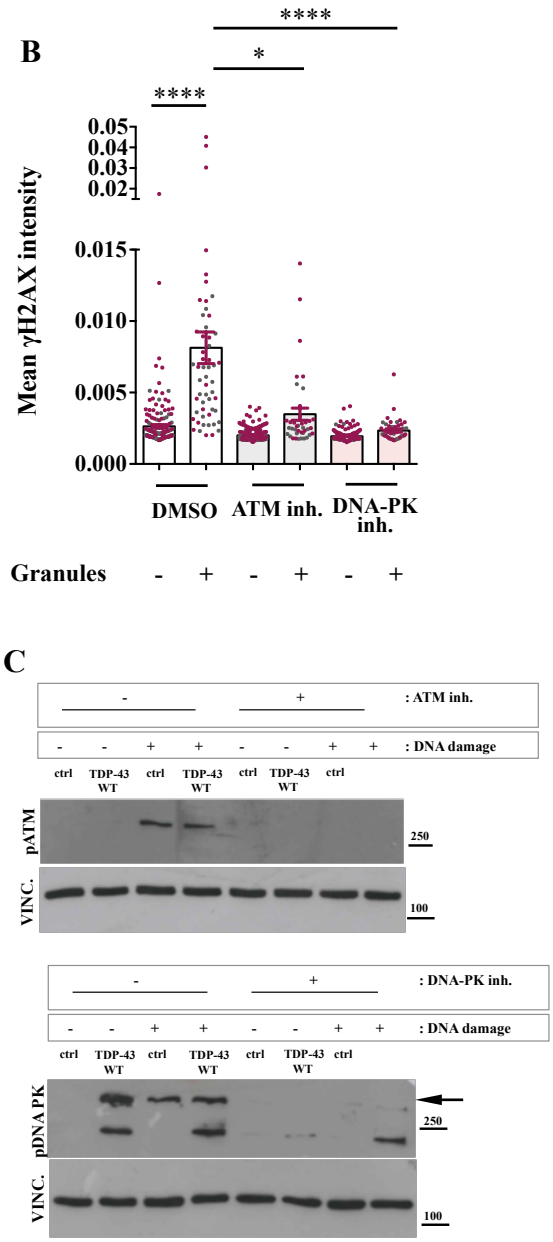


Fig. 22| Pan-nuclear γH2AX in cells with TDP-43 granules, is dependent on ATM and DNA-PK kinases activation

A. Imaging of HeLA cells overexpressing TDP-43 WT and immunostained to detect TDP-43 and γH2AX, after treatment with the ATM inhibitor KU60019, the DNA-pk inhibitor NU7441 or with

DMSO as control. Cells with TDP-43 positive granules are indicated by arrows; Nuclei were counterstained with DAPI. Scale bar: 10 um **B.** Mean γH2AX intensity measured in cells expressing TDP-43 WT, by separating cells with TDP-43 granules from cells without TDP-43 granules, in each indicated condition. Error bars represent SEM from two independent experiments, discernable by the different color of spots. P-value ≤ 0.05. For the quantification shown, between 90 and 160 cells have been analyzed for each experimental condition, per biological replicate. **C.** Lysates of cells overexpressing TDP-43 WT treated with KU60019, NU7441 or control cells blotted to detect pATM or pDNA-PK. Ctrl cells were additioned with the transfection mix only.

Overall, these results indicate that cytoplasmic TDP-43 aggregation induces a nuclear wide activation of both DNA-PK and ATM, which are in turn responsible for the phosphorylation of H2AX.

1.3 Cells with TDP-43 positive granules do not properly respond to DNA damage

In light of the unveiled contribution of ATM activation in cells experiencing TDP-43 inclusions, I also analyzed the auto-phosphorylation of ATM on S1981 (pATM), in basal conditions and upon NCS-induced DNA damage (fig. 23). Intriguingly, in undamaged conditions pATM staining appeared increased in cells with TDP-43 granules relative to control cells (fig. 23 A, C). However, differently from control cells treated with DSBs inducing agents, which normally present bright and discrete pATM foci, cells experiencing TDP-43 inclusions present a diffuse and pan nuclear staining for pATM in basal conditions, suggesting that its activation is not associated with its accumulation at sites of DNA damage. More importantly, upon exogenously induced DNA damage by NCS treatment, most of cells with TDP-43 inclusions were unable to accumulate discrete pATM foci rather they showed a homogenous and diffuse nuclear ATM activation (fig. 23 A-C). Differently, surrounding cells lacking TDP-43 inclusions formed bright pATM foci as expected (fig. 23 A). These observations suggest that ATM is chronically active in cells experiencing TDP-43 inclusions. Nevertheless these cells are defective in mounting pATM positive DDR foci upon exogenous induction of DSBs by acute treatment with NCS. Thus, cells with TDP-43 granules loose the ability to effectively respond to acute DNA damage, possibly leading to chronic DNA

damage accumulation and consequent ATM activation. This defect might confer to these cells a particular sensitivity to DNA damaging stimuli.

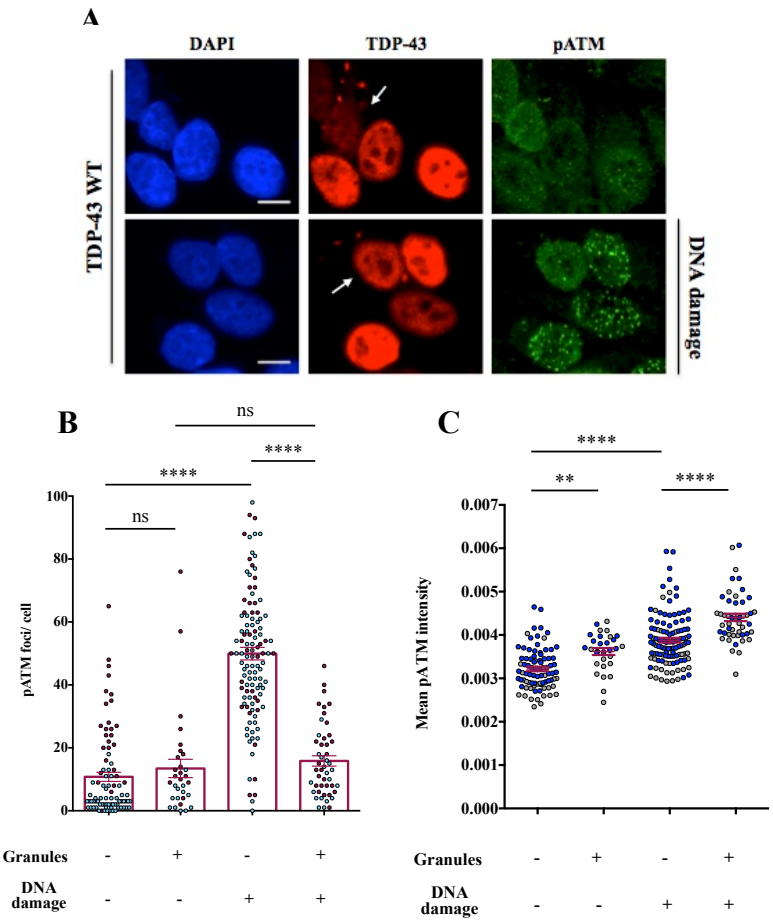


Fig. 23| Cytoplasmic TDP-43 positive granules lead to wide-nuclear pATM activation and impair pATM foci production in presence of DNA damage:
A. Imaging of HeLA cells expressing TDP-43 WT, immunostained to detect TDP-43 and pATM in basal conditions or upon DNA damage induction; cells with TDP-43 positive granules are indicated by arrows; nuclei were counter-stained with DAPI. Scale bar: 10 um **B.** Counts of pATM foci per cell and **C.** mean pATM intensity, measured in cells expressing TDP-43 WT, by separating cells with TDP-43 granules from cells without TDP-43 granules, in each indicated condition. Error bars represent SEM from two independent experiments, discernable by the different color of spots. P-value $\leq$ 0.05. For the quantification shown, between 60 and 90 cells have been analyzed for both untreated and NCS-treated cells, per each biological replicate.

Since cells with TDP-43 inclusions were unable to correctly mount pATM foci in response to exogenously inflicted DNA damage, we tested whether in the same cells treated with NCS also the accumulation of the DDR mediator 53BP1 into well-defined foci was normal or defective. Moreover, to investigate putative effects on DDR due to the mere TDP-43 overexpression, I stained cells for the Myc-tag that marked the construct expressing TDP-43 WT. In such way I was able to evaluate if DDR impairment was strictly related to the generation of TDP-43 granules rather than to the general overexpression of the protein (fig. 24).

Similarly to what observed for pATM, cells having TDP-43 inclusions did not respond properly to DNA damage generation, showing a strong, significant defect in 53BP1 foci formation (fig. 24 A-B). Differently, adjacent cells that still overexpress TDP-43 WT but lacking granules, presented bright and discrete 53BP1 positive DDR foci (fig. 24 A-B). Noteworthy, the impairment in mounting 53BP1 foci after exogenous DNA damage induction is not due to the appearance of pan nuclear γH2AX accumulation into the same cells. In fact, a fraction of cells with TDP-43 inclusions are still able to accumulate canonical γH2AX foci upon treatment with NCS, nevertheless the same cells are completely defective for the recruitment of 53BP1 into γH2AX discrete foci (fig. 24 D). Overall, these results suggest that in addition to promote a huge genotoxic stress, cytoplasmic TDP-43 inclusions strongly interfere with the nuclear response to DNA damage and impede DDR foci formation.

1.4 TDP-43 aggregation is associated with nuclear DROSHA decrease

As described above, my laboratory contributed in unveil a role for DICER and DROSHA RNases in DDRNAs generation, important to trigger a full DDR activation (Francia, et al. 2012). Importantly, it has been established that both enzymes form complexes with TDP-43 whose contribution is considered relevant to promote miRNA biogenesis (Kawahara and Mieda-Sato 2012), (Di Carlo, et al. 2013). Moreover, the overexpression of WT TDP-43 and other ALS-linked mutant

proteins has been already associated with cytoplasmic stress granules formation, which in turn correlates with reduced DICER activity, finally resulting in decreased levels of mature miRNAs (Emde, et al. 2015). In light of the above, a possibility is that TDP-43 inclusions lead to alterations in the localization and activity of DICER and the DDRNAs biogenesis machineries, which might contribute to the anomalous DDR response associated with TDP-43 aggregation, observed in this study. Moreover, DROSHA was already found to localize into cytoplasmic stress granules, in neuritis of motor neurons (Markmiller, et al. 2018). In light of this, I investigated if DROSHA was a component of TDP-43 positive granules induced by TDP-43 overexpression also in our cellular system (fig. 24). Strikingly, DROSHA was not found within TDP-43 containing granules, but its protein levels were strongly decreased in the nucleus of cells marked by TDP-43 aggregation (fig. 24 A, C). This data provide a possible explanation of the mechanism behind the defective DDR observed in cells with TDP-43 inclusions. Indeed such DDR defects might arise from apical impairments of DDRNAs biogenesis, a process that requires a proficient DROSHA activity.

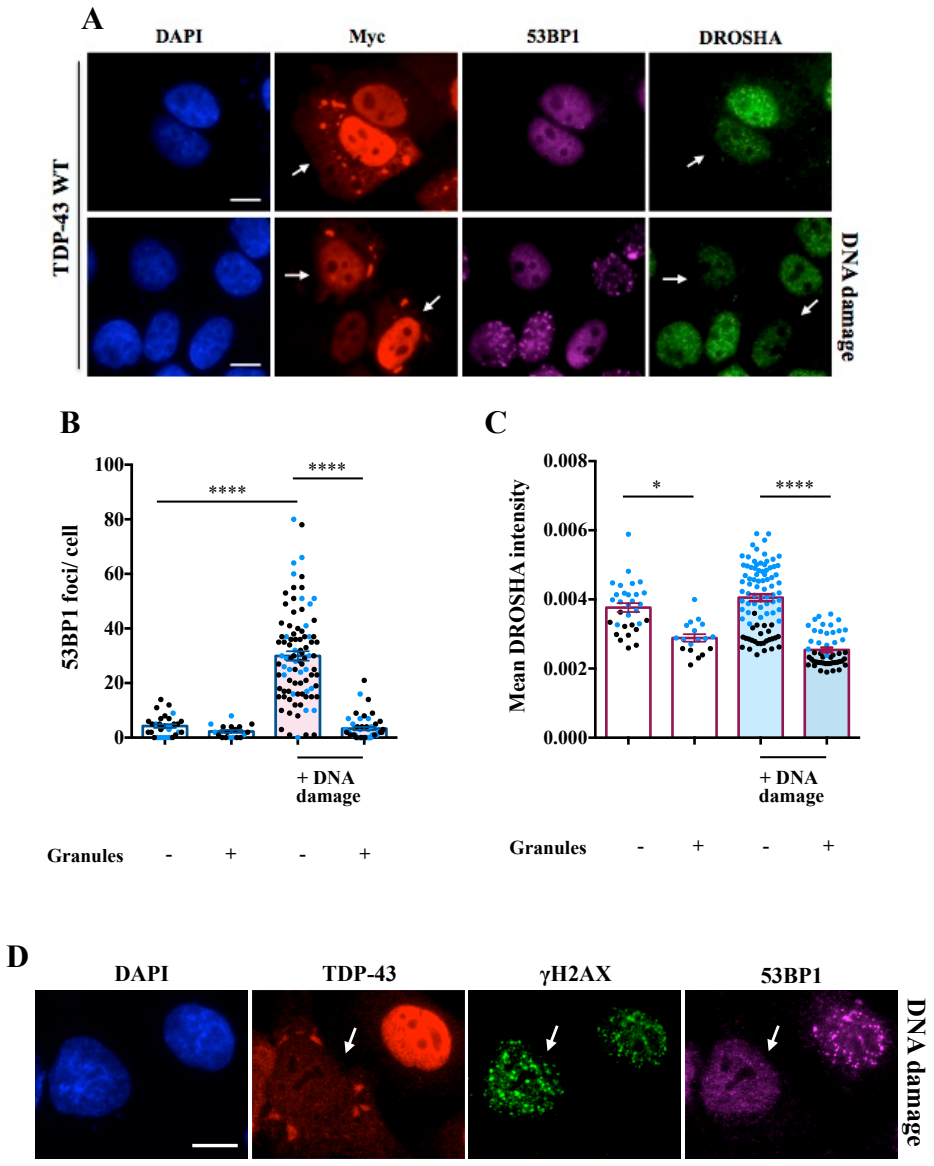


Fig. 24| Cytoplasmic TDP-43 positive granules impair 53BP1 foci formation in presence of DNA damage and correlate with nuclear DROSHA decrease.
A. Imaging of HeLA cells expressing Myc-TDP-43 WT immunostained for Myc, 53BP1 and DROSHA in basal conditions or upon DNA damage induction; cells with Myc TDP-43 positive granules are indicated by arrows; nuclei were counter-stained with DAPI. Scale bar: 10 um **B**. Counts of 53BP1 foci per cell and **C.** Mean DROSHA intensity, measured in damaged cells overexpressing Myc TDP-43 WT, by separating cells with Myc TDP-43 granules from cells without Myc TDP-43 granules, in each indicated condition. Error bars represent SEM from two independent experiments,

discernable by the different color of spots. P-value ≤ 0.05. For the quantification shown, between 20 and 80 cells have been analyzed for both untreated and NCS-treated cells per each biological replicate. **D.** Imaging of HeLA cells expressing Myc-TDP-43 WT immunostained for TDP-43, γH2AX and 53BP1 upon DNA damage induction. Cells with TDP-43 positive granules are indicated by arrows; nuclei were counter-stained with DAPI. Scale bar: 10 um

2. TDP-43 incorporation into SGs is necessary to reduce DDR efficiency and DROSHA levels

2.1 Cytoplasmic TDP-43 containing inclusions are a type of SGs

One type of cytoplasmic granules that form in response to stress stimuli is represented by SGs, which are composed of both constitutive and facultative proteins, among which TDP-43 itself (Aulas and Vande Velde 2015). Many studies attested how the overexpression of ALS-linked mutant proteins is sufficient to induce SGs, part of which also incorporate endogenous TDP-43 (Emde, et al. 2015), (Liu-Yesucevitz, et al. 2010). The overexpression of the wild type protein seems to be less efficient to induce SGs and is often showed as the control condition. Nevertheless, it induces the formation of such cytoplasmic structures at a certain extent, too (Liu-Yesucevitz, et al. 2010).

In addition to the overexpression of ALS-linked TDP-43 mutants, various stress conditions such as heat shock and oxidative stress were reported to induce SGs that also contain TDP-43 in specific cell types such as BE-M17, HeLA, Hek293T and NSC34 (Colombrita, et al. 2009), (Dewey, et al. 2012), (Markmiller, et al. 2018). Finally, in different studies the fraction of cells forming TDP-43 positive SGs was increased by combining the overexpression of TDP-43 both WT or some of its ALS-linked mutants, together with treatments inducing cellular stresses such as heat shock and administration of oxidative drugs (Liu-Yesucevitz, et al. 2010), (McGurk, et al. 2018). In light of this knowledge I considered the possibility that TDP-43 positive cytoplasmic inclusions that I observed upon TDP-43 overexpression, were in fact SGs. To test this possibility, I immunostained TDP-43 WT overexpressing cells for two well-known SGs markers: TIA-1 and G3BP

(Dewey, et al. 2012). In parallel, I treated HeLA cells with sodium Arsenite (ARS), a well-known inducer of stress granules formation by oxidative stress (Flora 2011). In fact, cytoplasmic inclusions containing TDP-43 in Myc-TDP-43 overexpressing cells, stained positive for both SG markers TIA-1 and G3BP (fig. 25). As expected, ARS treatment induced evident formation of SGs that stained positive for both TIA-1 and G3BP, in all the cells. However, none of ARS treated cells, generated granules incorporating TDP-43. Intriguingly instead, control cells transfected with an empty vector occasionally showed cytosolic inclusions containing TDP-43, which were also positive for SGs markers, indicating that cellular stress due to plasmids transfection itself, induces stress granules incorporating the endogenous protein. This might indicate that also SGs under study in our condition of TDP-43 overexpression, mimics the physiological ones.

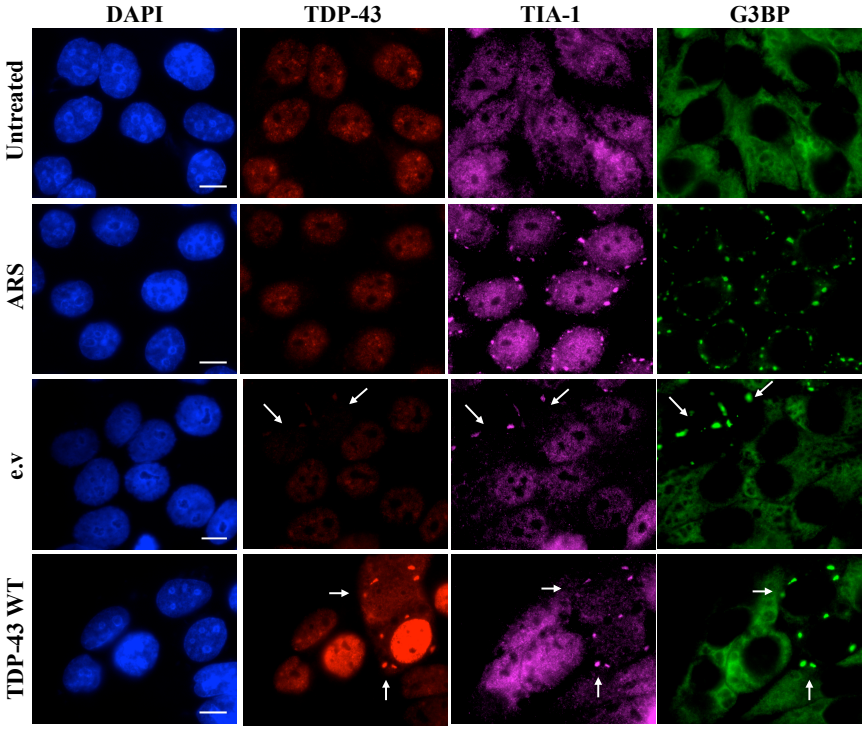


Fig. 25| Cytoplasmic TDP-43 positive granules in TDP-43 overexpressing cells are positive for SGs markers. Imaging of HeLA cells, untreated, treated with 0.5 uM ARS (positive control of SGs formation), transfected with an empty vector and overexpressing TDP-43 WT, immunostained for TDP-43, TIA-1 and G3BP. Cells with TDP-43 positive granules are indicated by arrows. Nuclei were counterstained with DAPI. Scale bars: 10 um.

2.2 ARS induced SGs don't incorporate TDP-43 in HeLA cells

As described above, our cellular system allows investigating the impact of TDP-43 positive SGs on genome instability mainly by imaging approaches, at single cell levels, due to the low fraction of cells experiencing the formation of such cytoplasmic inclusions. This aspect limits the use of technics that look at changes in the entire cell population such as western blotting or chromatin immuno-precipitation. Thus I spent some time trying to identify an experimental approach, which could induce the formation of TDP-43 positive SGs in a wider portion of the cell population. ARS is one of the most commonly used oxidative agents to induce SGs formation and different research reports show that it can induce SGs incorporating TDP-43, in various cell types like Hek293T and NSC34 (Dewey, et al. 2012). In particular, ARS-induced generation of TDP-43 positive SGs has been reported also in HeLA cells (McDonald, et al. 2011). Thus, I tested different concentrations and timing of ARS treatments with the aim of identifying a condition leading to SGs incorporating TDP-43 at a wider extent in the population respect to TDP-43 overexpression. I observed that, treatment with ARS performed in the same manner as in the study of McDonald et al. (McDonald, et al. 2011) led to the formation of typical SGs in 100% of treated HeLA cells. Nevertheless, I was not able to detect any TDP-43 into TIA-1 and G3BP positive SGs, using two different antibodies specific for the protein (fig.25, 26 A-B). This observation is not supporting our aim of having a homogeneous population of cells experiencing TDP-43 positive stress granules and is only in line with the notion that TDP-43 is a facultative SGs component, which is not frequently incorporated in SGs.

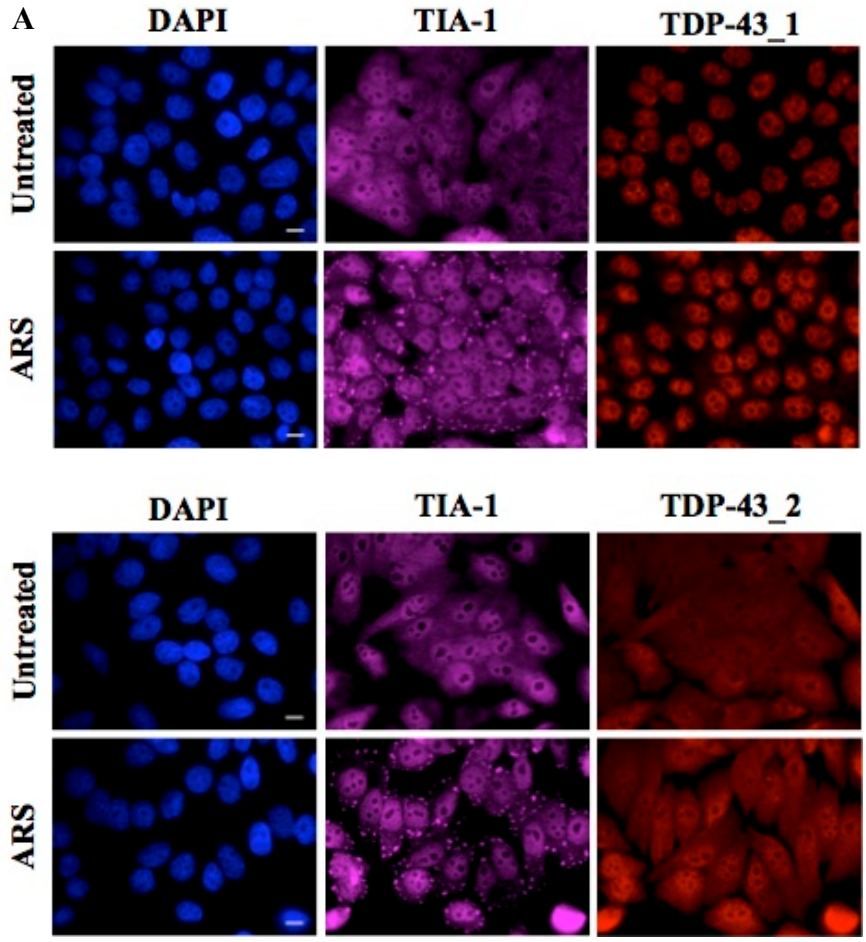


Fig.26| ARS induced SGs in HeLA don't incorporate endogenous TDP-43. Imaging of HeLA cells untreated or treated with 0.5 uM ARS, immunostained for TIA-1 and a policlonal **A.** or monoclonal **B.** antibody for TDP-43. Nuclei were counterstained with DAPI. Scale bars: 10 um.

2.3 TDP-43 negative ARS induced SGs are not able to reduce DDR and DROSHA levels

The characterization of TDP-43 positive inclusions as SGs raised the question whether DDR defects specifically observed in TDP-43 overexpressing cells, were due to SGs formation *per se* or to the incorporation of TDP-43 into such cytosolic

membrane-less organelles. Thus, I investigated if also ARS-induced SGs were able to elicit the effects of pan-nuclear γH2AX accumulation and under DNA damage conditions, of impaired DDR foci mounting. Thus I treated HeLA cells with 500 uM of ARS for 45 minutes and after fixation I stained for γH2AX and 53BP1. As shown in the figure (fig. 27) after induction of DNA damage by NCS addition to the medium of ARS treated cells, we observed that these cells are perfectly able to mount canonical γH2AX foci to a similar extent respect to untreated cells. A slight but significant increase in γH2AX foci was detected after ARS treatment in basal conditions (fig. 27 B), suggesting that ARS on its own might be partially genotoxic. Anyway, in all cells with ARS induced SGs, γH2AX distribution remained confined within discrete foci and didn't assume the pan-nuclear distribution observed in cells with TDP-43 positive SGs (fig. 27 A). Moreover, 53BP1 foci accumulation was similar in untreated and ARS-treated cells indicating that the activation of the DDR cascade was not compromised by induction of SGs *per se*. Then, we investigated if DROSHA protein level was reduced by formation of SGs induced by ARS, as revealed in cells experiencing TDP-43 positive stress granules. In line with the idea that the observed effects on DDR is not due to SGs formation *per se*, also DROSHA protein levels were unchanged after ARS-induced SGs formation (fig. 28).

Altogether these observations suggest that genotoxic stress, DDR impairment and decreased DROSHA levels, observed in cells marked by TDP-43 positive SGs, specifically depend on TDP-43 recruitment within these structures, rather than on stress response or stress granules formation *per se.*

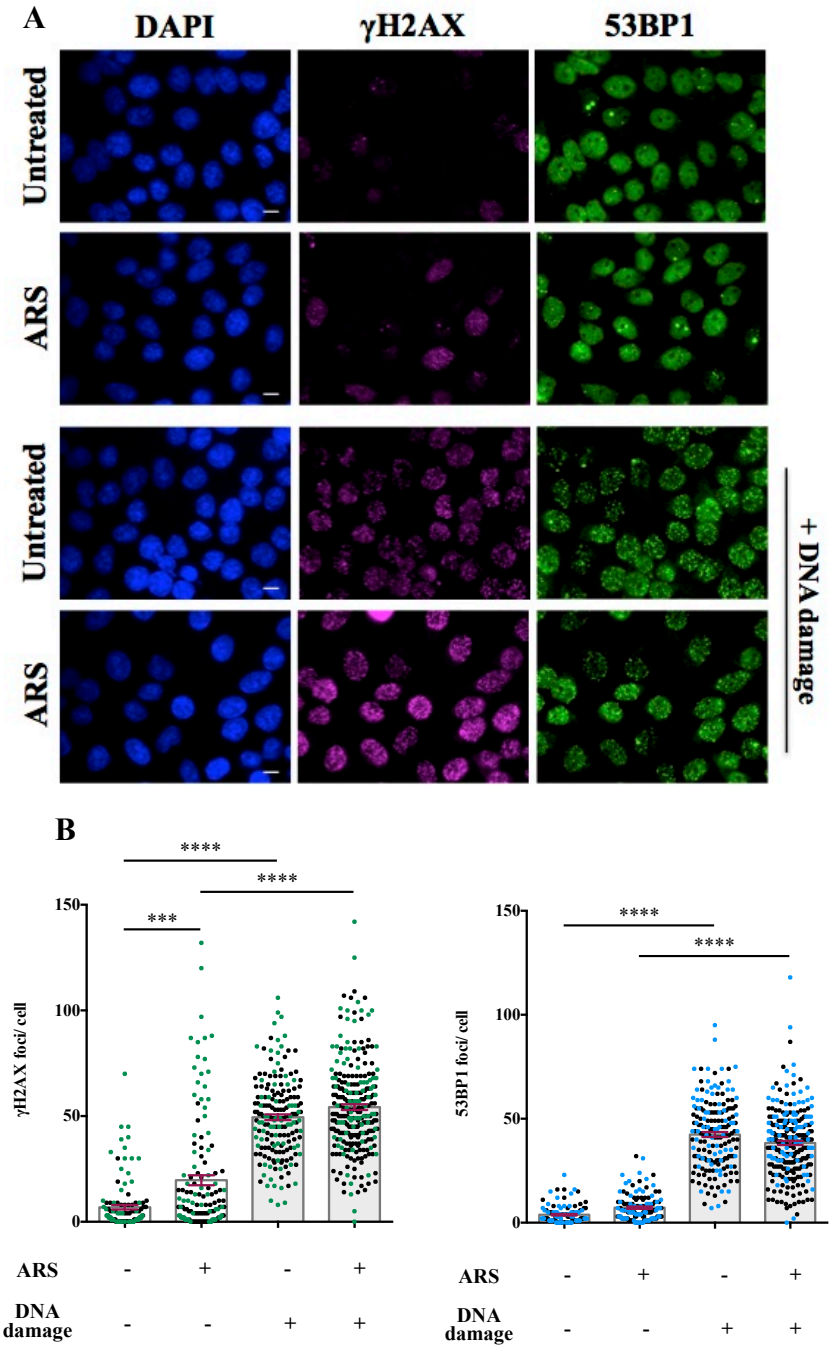
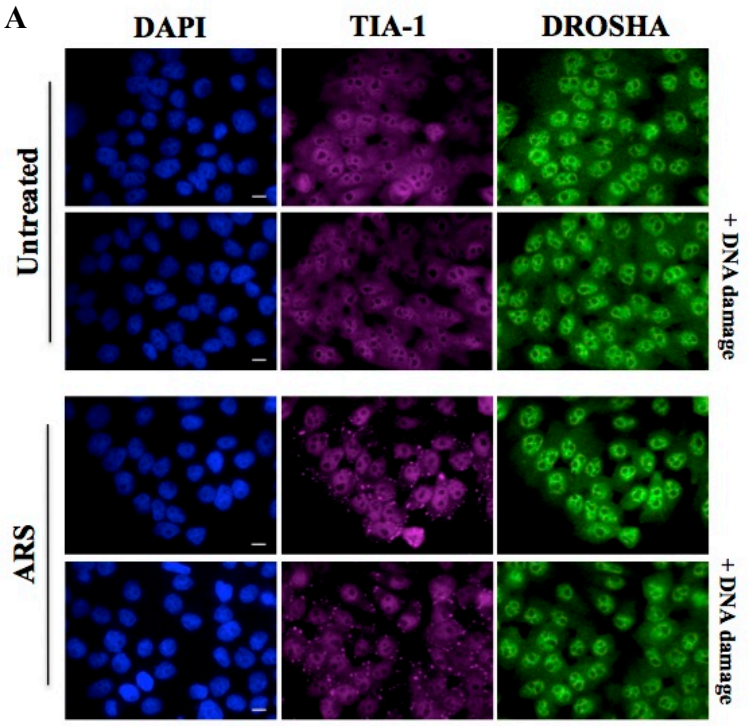


Fig. 27| ARS induced SGs are not able to impair DDR foci formation, per sé.
A. Imaging of HeLA cells, untreated or treated with 0.5 uM ARS, either in basal conditions or in presence of DNA damage, immunostained to detect γH2AX and 53BP1. Nuclei were counterstained

with DAPI. Scale bars: 10 um. **B.** Counts of γH2AX and 53BP1 foci in the indicated conditions. Error bars represent SEM from two independent experiments, discernable by the different color of spots. P-value ≤ 0.05. For the quantification shown between 60 and 180 cells have been analyzed for each experimental condition, per replicate.



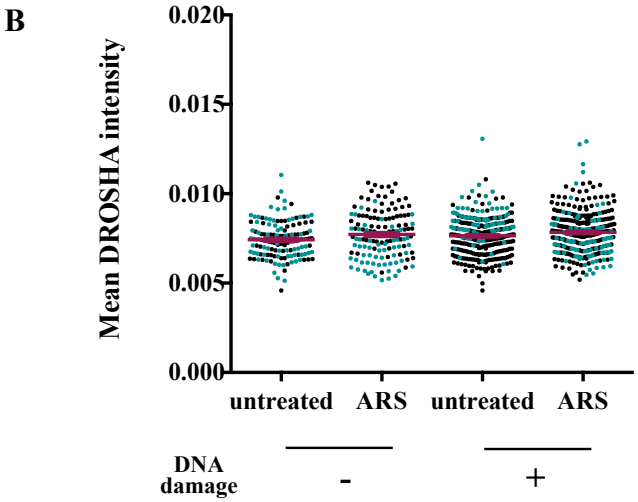


Fig. 28| ARS induced SGs don't impact on DROSHA signal. A. Imaging of HeLA cells untreated or treated with 0.5 uM ARS, either in basal conditions or in presence of DNA damage, immunostained to detect TIA-1 and DROSHA. Nuclei were counterstained with DAPI. Scale bars: 10 um. **B.** Mean DROSHA intensity, measured in untreated and ARS treated cells, upon DNA damage induction. Error bars represent SEM from two independent experiments, discernable by the different color of spots . P-value $\leq$ 0.05. For the quantification shown, between 60 and 200 cells have been analyzed for each experimental condition, per biological replicate.

3. Nuclear DROSHA clearance is not due to p38-MAPK activation or proteasome mediated degradation

3.1 DROSHA reduction is not due to the stress-induced activation of the p38 kinase

Decreased DROSHA levels might be responsible for the altered DDR response induced by TDP-43 positive SGs. This might explain the loss of 53BP1 and pATM recruitment into discrete DDR foci, since DROSHA inactivation by siRNA does not lead to strong pan nuclear γH2AX (Francia, et al. 2012). Thus I devoted some efforts to investigate the mechanism behind such nuclear DROSHA reduction. At this regard, it has been demonstrated that some kinds of stresses like heat-shock and H_2O_2 administration are able to cause DROSHA degradation following its phosphorylation by the p38 kinase, which causes its prompt export to the cytoplasm and its calpain-mediated degradation (Yang, et al. 2015). Therefore, I investigated whether SGs containing TDP-43 led to p38 phosphorylation and activation by staining cells with an antibody against the activated kinase p-p38 (fig. 29). As positive control for p38 activation we used UV-C irradiation (Tomas, et al. 2017). As shown in the figures (fig. 29 A-B), p38 was not activated in cells with TDP-43 positive SGs, differently from cells irradiated with two distinct doses of UV-C, 40 and 100 J/m^2, which showed clear p38 activation. Thus, we concluded that differently from other types of cellular stresses, the mechanism driving DROSHA down-regulation in cells experiencing TDP-43 positive SGs is not mediated by p38 activation and is not based on its calpain-mediated degradation.

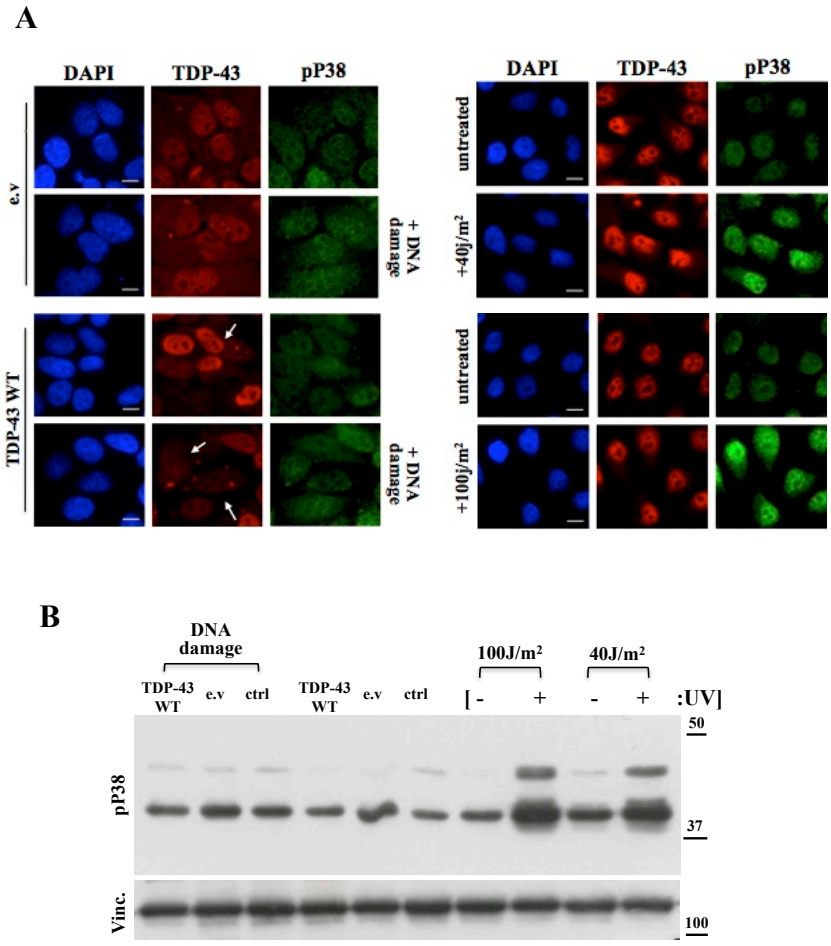


Fig. 29| Cytoplasmic TDP-43 positive SGs do not correlate with the activation of the p38 kinase. **A.** Imaging of HeLA cells expressing TDP-43 WT and control cells, untreated or treated with NCS to induce DNA damage, immunostained to detect TDP-43 and p-p38. Cells with TDP-43+ granules are indicated by arrows; Cells irradiated with 40 or 100 J/m^2 of UV-C were used as positive control of p38 activation. Nuclei were counterstained with DAPI. Scale bars: 10 um **B.** Lysates for each indicated condition were also immunoblotted for p-p38.

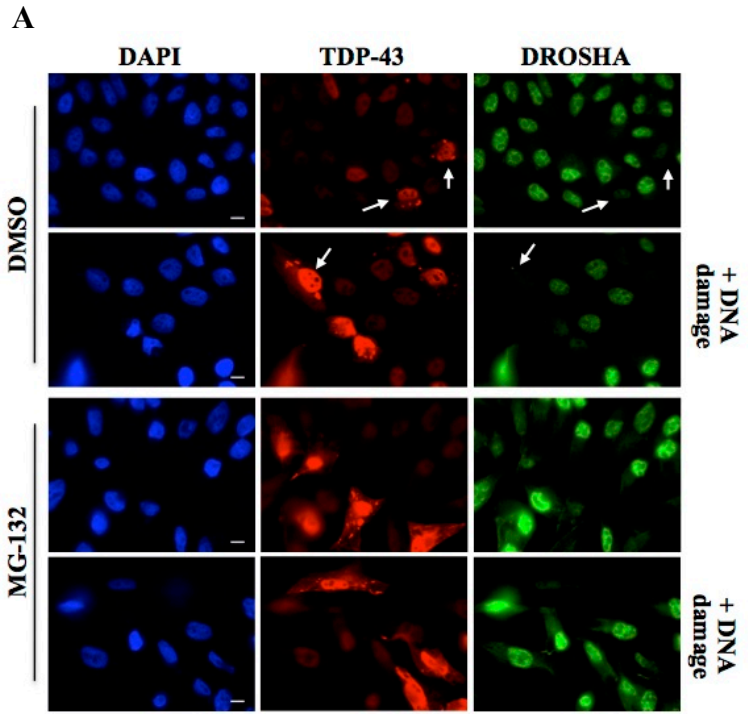
3.2 DROSHA reduction is not due to proteasome degradation

Another possibility is that DROSHA reduction in cells with TDP-43 positive SGs is due to its direct degradation by the proteasome (Ye, et al. 2015). To address this point, I treated cells with the proteasome inhibitor MG-132 for 24 hours at the dose

of 5uM, concomitantly with the overexpression of myc-TDP-43 WT by transfection, to evaluate if a proteasome block could prevent DROSHA degradation in cells with TDP-43 positive SGs. I observed that, proteasome inhibition did not impact on DROSHA protein levels and was not sufficient to recover nuclear DROSHA signals in cells with TDP-43 positive SGs (fig. 30 A).

To verify the inhibition of proteasomal function upon MG132 treatment, a WB assay using an antibody that detects ubiquitinylated proteins, which accumulate when proteasome function is inhibited, was performed and confirmed an increase in signal upon proteasomal inhibition (fig. 30 B).

This indicates that the reduction of DROSHA levels is not due to its proteasome-mediated degradation.



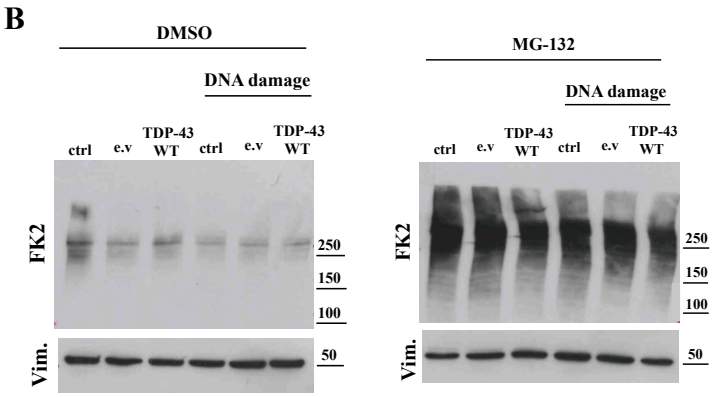


Fig. 30| Cytoplasmic TDP-43 positive SGs do not induce proteasomal degradation of DROSHA. A. Imaging of HeLA cells expressing TDP-43 WT treated with the proteasome inhibitor MG-132 in parallel to transfection (24 h), either in basal conditions or in presence of DNA damage, immunostained to detect TDP-43 and DROSHA. DMSO treatment was used as control. Cells with TDP-43 positive granules are indicated by arrows. Nuclei were counterstained with DAPI. Scale bars:10 um **B.** Lysates for each indicated condition were immunoblotted for FK2 to assess MG-132 activity. Ctrl cells were additionated with the transfection mix only. Vimentin was used as loading control.

4. Role of endogenous TDP-43 in DDR

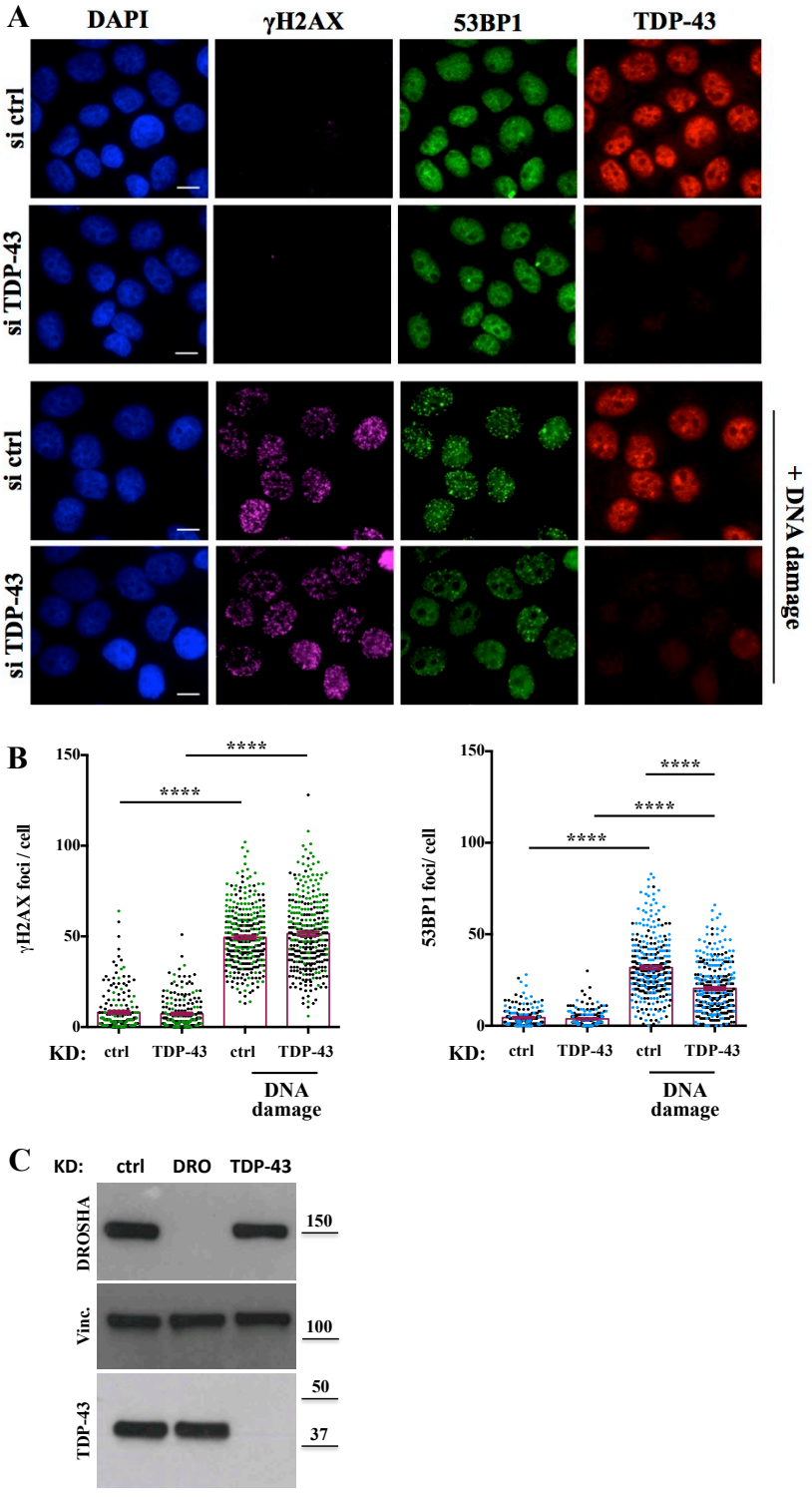
4.1 TDP-43 is necessary to ensure a proficient DDR activation

The results reported above are consistent with a model in which TDP-43 cytoplasmic inclusions confer a gain of function that interferes with genomic stability, and indicate that TDP-43 containing SGs, even in the absence of TDP-43 nuclear clearance, can contribute to threaten cells viability, by causing genotoxic stress, diminishing the efficacy of DDR and altering DROSHA levels.

Nevertheless these outcomes, don't rule out the possibility that sequestration of TDP-43 into SGs could also diminish its nuclear activities concurring to hinder cell survival. Importantly, TDP-43 shares many structural domains and functions with the ALS-linked FUS protein (Kapeli, et al. 2016), which has been discovered to play a role in DDR (Madabhushi, et al. 2014). Additionally, TDP-43 depletion has

already been associated to the generation of transcription-dependent DNA damage (Hill, et al. 2016). Thus, a possibility is that TDP-43 itself could have some roles in DDR, in addition to all the other established functions. To address the putative role of TDP-43 in DDR activation also in our cellular systems, I knocked down TDP-43 by siRNA transfection and evaluated the efficiency of DDR foci formation upon exposure of HeLA cells to NCS-induced DNA damage (fig. 31). The obtained results indicate that 53BP1 foci accumulation was hampered in cells lacking TDP-43 without altering γH2AX foci formation (fig. 31 A-B). The unaffected number of γH2AX foci in the two conditions, also confirms that the amount of damage was generated at similar extents in control cells and cells knocked down for TDP-43 (fig. 31 B). This result is consistent with the idea that also endogenous TDP-43 similarly to FUS, plays a positive role in triggering a full and proficient DDR signalling.

Moreover to evaluate whether TDP-43 depletion in addition to have impacts on DDR, might concomitantly affect nuclear DROSHA signals as in case of TDP-43 granules generation, we evaluated DROSHA protein level upon TDP-43 KD by western blotting. As shown in figure 31 C, TDP-43 silencing does not result in a decreased DROSHA protein level.


A
DAPI
γH2AX
53BP1
TDP-43
si ctrl
si TDP-43
si ctrl
si TDP-43
+ DNA damage
B
γH2AX foci / cell
150
100
50
0
****
****
KD:
ctrl
TDP-43
ctrl
TDP-43
DNA damage
53BP1 foci/ cell
150
100
50
0
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****
KD:
ctrl
TDP-43
ctrl
TDP-43
DNA damage
C
KD:
ctrl
DRO
TDP-43
DROSHA
150
Vinc.
100
TDP-43
50
37

Fig.31| TDP-43 depletion by siRNA reduces 53BP1 foci formation after DNA damage induction **A.** Imaging of HeLA cells after 72h of transfection with siRNA directed against TDP-43 or ctrl siRNA, untreated or NCS treated to induce DNA damage and stained for TDP-43, γH2AX and 53BP1. Nuclei were counterstained with DAPI. Scale bars: 10 um. **B.** Counts of γH2AX and 53BP1 foci in the indicated conditions. Error bars represent SEM from two independent experiments, discernable by the different color of spots. P-value ≤ 0.05**.** For the quantification shown, between 60 and 200 cells have been analyzed for each experimental condition, per biological replicate. **C.** Lysates for each indicated condition were immunoblotted for TDP-43 and DROSHA to assess their levels in each indicated condition.

4.2 The ALS-linked TDP-43 mutation G294V in fALS fibroblasts correlates with DDR defects when DNA damage is induced

An important problem related to studies on ALS/FTLD pathology is that motor neurons harboring pathological signs of degeneration are clinically inaccessible. This often delays the diagnosis. For this reason it would be relevant to find other cell types that can be easily obtained from patients by biopsies, to identify some disease markers that might be shared with neurons from the same person. Fibroblasts can be attractive in this sense, because they are isolated from live patients through a punch skin biopsy. In an attempt to take advantage from this cell type to gain insights into the ALS pathology, a group already evaluated how TDP-43 was distributed in sALS and fALS fibroblasts (Sabatelli, et al. 2015). Particularly, it was found that in fALS fibroblasts with different mutations on TARDBP, a consistent amount of TDP-43 shifted from the nuclear to the cytoplasmic fraction and in some cases it was distributed within very fine cytoplasmic inclusions (Sabatelli, et al. 2015). Prompted by the chance to find cytosolic TDP-43 aggregates and to study possible DDR defects associated with TDP-43 mutations in cells derived from ALS patients, in collaboration with Dr. Cristina Cereda of the Mondino Institute, I performed preliminary analysis on control fibroblasts and fALS fibroblasts with the G294V mutation on TARDBP (reported in both sALS and fALS cases (Conforti, et al. 2011)). Fibroblasts were NCS-treated to induce DNA damage and both TDP-43 localization and DDR foci accumulation were evaluated by immunofluorescence. In contrast with the

observations of Sabatelli et al. (Sabatelli, et al. 2015), in fibroblasts from the patient with the G294V mutation on TARDBP I could detect neither a nuclear depletion nor a cytoplasmic translocation of TDP-43. In accordance with my observation, a recent study did not detected any TDP-43 delocalization on sALS fibroblasts (Codron, et al. 2018). However, comparing untreated versus NCS-treated cells from the healthy donor and the fALS patient with the afore mentioned mutation, I observed that, despite γH2AX accumulation was unchanged, 53BP1 foci didn't significantly increase in fALS fibroblasts upon DNA damage induction (fig.32). Importantly, fALS fibroblasts present a higher number of endogenous 53BP1 foci in undamaged conditions, an observation consistent with our model that endogenous TDP-43 plays a positive role in DDR. Such results are preliminary and need to be confirmed by expanding the biological replicates of this analysis and the number of ALS patients from which fibroblasts derive. Nevertheless these observations suggest that TDP-43 mutations could lead to loss of function of the endogenous protein causing DDR defects, which is consistent with the outcomes I obtained upon TDP-43 depletion.

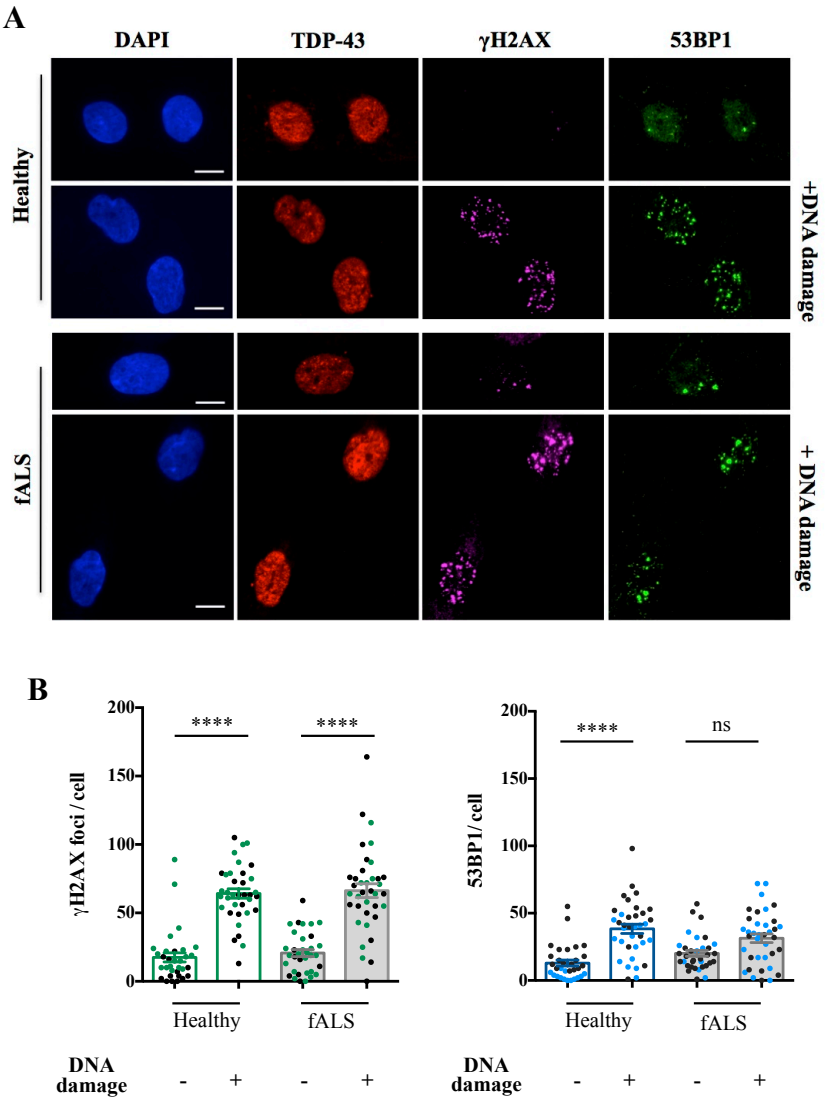

Fig. 32| fALS fibroblasts mutated on the TDP-43 gene show faint DDR activation upon DNA damage induction. A. Representative images of fibroblasts from healthy subjects and from fALS patients with the G294V mutation on TARDBP, stained for TDP-43, γH2AX and 53BP1, in basal conditions or after NCS treatment to induce DNA damage. Nuclei were counterstained with DAPI. Scale bars: 10 um. **B.** Counts of γH2AX and 53BP1 foci in the indicated conditions. Error bars represent SEM from two independent experiments, discernable by the different color of spots. P-value $\leq$ 0.05. For the quantification shown, between 10 and 25 cells have been analyzed for each experimental condition, per biological replicate.

Discussion

Aim of this project was to investigate if TDP-43 dysfunctions, typical of cells from ALS affected patients, cause DDR impairment. Thus, we evaluated how reduced levels of TDP-43 or its recruitment into cytosolic granules induces endogenous DNA damage or affects DDR activation in an in-vivo cellular system.

In particular three tumour cell lines derived from different tissues, among which the neuronal one, were chosen to test the effects of TDP-43 overexpression by IF, taking advantage from the good transfectability and flat morphology of these cell types, which facilitate concomitant imaging of nucleus and cytoplasm. We observed that cytoplasmic TDP-43 positive inclusions evoking the hallmarks of the ALS/FTLD spectrum of diseases, formed better in HeLA than in the other cell lines tested, rendering this cells particularly attractive for further analysis. Why only this cell line was able to produce TDP-43 positive inclusions is not completely clear and it is an important aspect to investigate. Indeed, we plan to continue our research trying to shed light on the cell type specific mechanisms that promote TDP-43 aggregation, contributing to elucidate why this pathological sign selectively marks cells of neural origin in ALS patients.

Importantly, it was found that TDP-43 positive inclusions are in fact SGs, membrane-less organelles that physiologically form in response to a wide range of stress conditions (Protter and Parker 2016). SGs have already been involved in ALS pathology and are believed to represent precursors of more toxic, TDP-43 containing aggregates (Dewey, et al. 2012), (Robberecht and Philips 2013), (Furukawa and Nukina 2013). At this regard TDP-43 incorporation into SGs has been proposed to drive the conversion of these highly mobile and transitory organelles into more static and persistent structures, which loose core SGs components over time, after stress removal (Parker, et al. 2012), (Mackenzie, et al. 2017). Moreover, various constitutive SGs components were found within pathological aggregates in ALS/FTLD spinal cord and cerebral tissues, thus strengthening the idea that SGs are involved in the pathogenesis of TDP-43

proteinopathies (Liu-Yesucevitz, et al. 2010), (Bentmann, et al. 2012), (Volkening, et al. 2009), (Fujita, et al. 2008).

My results indicate that SHSY5Y neuroblastoma cells, differently from HeLA cells, are not prone to form such pathologic-like inclusions, despite they share the neural origin with ALS affected cells. A partial explanation of this apparently contradictory behaviour is related to the SGs nature of such induced TDP-43 containing inclusions. Indeed, TDP-43 is not a constitutive marker of SGs since its incorporation strictly depends on the type of stress inflicted and even more on the cell line (Aulas and Vande Velde 2015), (Dewey, et al. 2012). At this regard, others already reported that HeLA cells are more prone to form SGs incorporating TDP-43, than the neural cell lines SHSY5Y and Neuro 2a, in presence of various stress conditions (Dewey, et al. 2012). Thus, it is possible that also upon the type of stress induced by the overexpression of the protein, the generation of TDP-43 positive SGs is facilitated in HeLA cells with respect to other cell lines. Since some ALS-linked mutants in the C-terminal region of TDP-43 are considered intrinsically prone to form accruals (Johnson, et al. 2009), we assessed also the aggregation-propensity of one TDP-43 mutation: M337V. This mutation is thought to be particularly detrimental in ALS pathogenesis since it was associated with early onset cases of the disease (Sreedharan, et al. 2008), (Gendron, et al. 2013b). Moreover, it was reported that motor neurons experimentally obtained from an ALS patient with the M337V mutation, showed increased levels of endogenous TDP-43 as well as higher amounts of its C-terminal truncated forms of 35 KDa (Bilican, et al. 2012). Despite these evidences, TDP-43 M337V overexpression in HeLA cells was not more prone to form cytoplasmic inclusions respect to the WT protein, thus its use was abandoned after a few analysis.

The low percentages of cells forming TDP-43 positive SGs in the entire cell population overexpressing TDP-43, have been technically limiting, thus most of the observations in our study were carried out by exploiting imaging techniques, which allow to make analysis at single cell level. Therefore, once TDP-43

containing inclusions have been characterized as SGs, I devoted some efforts to find a way to expand the proportion of cells in which SGs incorporated also TDP-43. Among the adopted strategies, I treated cells with various heat shock (HS) and oxidative agents like ARS and Paraquat, which have been reported to boost TDP-43 sequestration within SGs in various cell lines (Dewey, et al. 2012), (Parker, et al. 2012), (McDonald, et al. 2011). Most of these approaches were not able to induce the formation of TDP-43 containing SGs in a larger portion of the cell population. Differently, paraquat treatment promoted TDP-43 recruitment in SGs of a higher fraction of cells, in accordance with the existing literature (Parker, et al. 2012). Nevertheless, this drug treatment also induces DNA damage on its own (Chinta, et al. 2018) in almost the whole cell population regardless of granules generation. This observation led us to discard this treatment because it might complicate the interpretation of the results (data not shown).

Noteworthy, also a few cells transfected with an empty vector showed the recruitment of endogenous TDP-43 into cytoplasmic granules, which is not surprising since TDP-43 has an intrinsic ability to be recruited into stress granules (Aulas and Vande Velde 2015), (Dewey, et al. 2012).

Studying the effects on cell health of cytosolic granules containing wild type TDP-43 is of crucial importance since most of ALS events are sporadic and rarely correlate with genetic variants (Taylor, et al. 2016) although they are all marked by TDP-43 pathological aggregates (Mackenzie, et al. 2007). Furthermore, a great fraction of ALS-linked mutations on TDP-43 was suggested to increase the tendency of the protein to aggregate (Johnson, et al. 2009) supporting the idea that these mutations might have a pathogenic role just for their ability to affect TDP-43 agglomeration. These notions are all consistent with the idea that TDP-43 aggregation could be the major common denominator in the pathogenesis of all ALS subtypes. Moreover such view emphasizes how perturbations of the TDP-43 ability to phase separate that depend on its concentration and on its stabilization

affecting its physical behaviour, could represent one pathogenic mechanism fuelling ALS/FTLD.

Consistently, overexpression of TDP-43 WT has been associated to neurodegenerative phenotypes in model organisms as Drosophila melanogaster (McGurk, et al. 2018) and increased levels of the WT protein correlated with augmented neural toxicity in various rodent models (Wegorzewska and Baloh 2011), enforcing the idea that higher levels of the TDP-43 protein are detrimental for cell health.

To investigate whether TDP-43 positive SGs had some impacts on genome instability, first I analysed the distribution of the DNA break marker γH2AX in cells overexpressing TDP-43. The obtained data clearly indicate that cells characterized by TDP-43 containing SGs had a strong nuclear-wide accumulation of γH2AX, in the absence of any exogenous DNA damaging treatment. However, this kind of genotoxic stress didn't reflect ongoing apoptosis as revealed by the lack of both tunel signals and cleaved caspase 3 positivity. Not even R-loops (reported in C9-ALS cases (Walker, et al. 2017), (Haeusler, et al. 2014)) were found within cells with TDP-43 positive granules, ruling out the possibility that γH2AX accumulation associated with TDP-43 inclusions arises from genomic instability or replicative stresses caused by such RNA:DNA hybrids.

The hypothesis that TDP-43 positive SGs induce replicative stress was also discarded in light of the low levels of Cyclin A observed in cells with pan nuclear γH2AX, indicating that those cells are rather stalled in the G1 phase of the cell cycle. Block of such cells in the G1 phase is consistent with the hypothesis that pan nuclear γH2AX reflects high levels of DNA damage, which in turn activate a G1-S cell cycle checkpoint (Houtgraaf, et al. 2006). In fact DNA damage triggers cell cycle checkpoints, which serve to prevent DNA replication or mitosis of cells with unrepaired DNA (Jackson and Bartek 2009), (Branzei and Foiani 2008), (Houtgraaf, et al. 2006).

Importantly, pan nuclear γH2AX signals of cells with TDP-43 positive SGs were drastically reduced after inhibiting ATM and DNA-PK, indicating that such kinases are the ones responsible for H2AX phosphorylation. Consistently, the diffuse, nuclear wide activation of ATM in cells with TDP-43 positive SGs was also confirmed by directly analysing the distribution of pATM. Indeed, in addition to be primarily implicated in γH2AX generation within nuclear foci around DSBs (Lavin 2008), (Harper and Elledge 2007), (Meek, et al. 2008) ATM and DNA-PK kinases were found to be responsible for pan nuclear γH2AX signals after the generation of IR-induced clustered DNA damage (Meyer, et al. 2013). These data support the scenario that such strong and diffuse γH2AX signal arises from a huge amount of DNA damage widely distributed in the nucleus. Unfortunately, the small fraction of cells marked by TDP-43 positive SGs impeded us to exploit tools like the comet assay, otherwise useful to evaluate the amount of physical DNA damage in a more homogenous cell population, (Collins 2004). Trying to overcome this limitation we are setting up the following approach: we plan to use the DNA damage in situ ligation followed by Proximity Ligation Assay (diPLA) protocol that allows DSBs detection at the single cell level, by imaging (Galbiati, et al. 2017). By this tool, using antibodies against biotin and γH2AX I will compare the amounts of DSBs between cells with and without TDP-43 positive SGs in the same cell population.

Importantly, cells treatment with the DNA damaging agent NCS, a radiomimetic drug used to generate DNA DSBs (Kuo, et al. 1984), revealed that in cells with TDP-43 positive SGs, DDR activation was compromised at different levels. Indeed the accumulation within nuclear foci of both the apical DDR kinase ATM and the downstream DDR mediator 53BP1, was selectively impaired in TDP-43 overexpressing cells marked by TDP-43 positive SGs.

It is also important to highlight that the generation of TDP-43 containing SGs was not always accompanied by TDP-43 nuclear clearance, suggesting that the observed DDR impairment as well as the diffuse ATM activation, are strictly

linked to the formation of SGs that incorporate TDP-43. This outcome is in accordance to the so-called gain of function theory formulated to explain a facet of ALS pathogenesis (Johnson, et al. 2009), (Zhang, et al. 2009). In fact such theory specifically ascribes to SGs, direct roles in ALS-linked toxicity and our study might propose a similar role on DDR impairment.

In recent years my group discovered a novel class of small ncRNA termed DDRNAs that are directly involved in the first steps of DDR (Francia, et al. 2012). DDRNAs are processed by DICER and DROSHA RNases, which are historically involved in miRNAs biogenesis. Because DICER and DROSHA physically and functionally interact with TDP-43 (Kawahara and Mieda-Sato 2012), (Di Carlo, et al. 2013) and since defects in miRNAs generation were widely reported in ALS (Emde, et al. 2015), (Taylor, et al. 2016) it is interesting to address if these RNases are compromised in terms of proteins levels, activity or localization in cells with TDP-43 cytosolic inclusions. Indeed, a possible scenario is that DROSHA/DICER alterations affect DDRNA biogenesis and thus DDR activation and DDR foci formation upon TDP-43 proteinopathies. Accordingly, a decrease in DICER activity has been already associated to the generation of SGs containing also AGO-2 (Emde, et al. 2015). Recently, DROSHA was instead reported to be a component of SGs specifically formed in motor neurons (Markmiller, et al. 2018). Thus, we analysed DROSHA localization in cells with TDP-43 positive SGs and we noticed that although DROSHA was not recruited into SGs, its nuclear levels significantly decreased, suggesting that DDRNAs biogenesis could be strongly dampened in the same cells.

Intriguingly, DDR impairment and DROSHA reduction associated with the generation of TDP-43 containing SGs, didn’t occur in ARS treated cells, forming SGs devoid of TDP-43. These data indicate that neither the mere overexpression of TDP-43 nor solely the generation of SGs, are able to drastically affect the response to induced DSBs as well as to reduce DROSHA nuclear levels. These detrimental effects are rather the result of TDP-43 positive SGs generation.

The possible causes of DROSHA reduction were investigated, although they still remain uncertain. We supposed that DROSHA decrease could be due to the activation of the p38 MAPK activating the calpain protease, or to the proteasome-mediated degradation (Ye, et al. 2015). At one side we couldn't observe any differences in the activation of the p38 MAP-kinase between cells with and without TDP-43 positive SGs, thus ruling out the possibility that DROSHA was degraded by calpain. Additionally, not even proteasome inhibition by MG-132, performed in parallel to TDP-43 overexpression, was able to recover nuclear DROSHA levels.

Given that the tested hypothesis were not validated, it will be necessary to take into account other possibilities to gain insights about how DROSHA is selectively reduced in cells with TDP-43 positive SGs. An option is that DROSHA could be cleared through the autophagy pathway, as reported for Spinal muscular atrophy (SMA) motor neurons (Goncalves, et al. 2018). This hypothesis is in line with the evidences that perturbations of the autophagy pathway are associated to ALS (Protter and Parker 2016). Moreover, alterations on this clearance pathway could be responsible for the cell retention of SGs, since their resolution is indeed mediated by autophagy (Buchan, et al. 2013). Alternatively the nuclear drop of DROSHA levels could be justified with a decreased expression of the gene or with a different regulation of its alternatively spliced isoforms (Link, et al. 2016).

Overall the observed effects on DDR and DROSHA levels, associated to cells with TDP-43 positive SGs, support the gain of function theory according to which, TDP-43 recruitment in cytosolic inclusions could be toxic *per se*, in ALS pathology (Johnson, et al. 2009).

Noteworthy, our observations in cell cultures are supported by a pilot experiment conducted in Drosophila melanogaster by one of our collaborator working at the Sapienza University of Rome, Dr. Gianluca Cestra. In particular, it has been observed that overexpression of human TDP-43 (hTDP-43) in flies eyes leads to the following degenerative phenotypes: ommatidia and pigmentation loss and appearance of vitreous and black zones likely due to the activation of cell death.

These effects are in line with our idea that increased levels of the wild type protein could be toxic also in absence of ALS-linked mutations and are consistent with previous studies taking advantage of Drosophila melanogaster models (McGurk, et al. 2018). Even more intriguingly, such degenerative phenotypes of the Drosophila eye are partially but significantly reversed following NBS1 knock down. As described above, NBS1 is the component of the MRN complex directly involved in ATM recruitment and activation at DNA damaged sites (Difilippantonio and Nussenzweig 2007). Thus, these preliminary data suggest that also in an *in vivo* model, TDP-43 overexpression boosts the signalling leading to ATM activation that is responsible at a certain extent for the degenerative phenotypes. Obviously it remains to be determined if also in vivo, activation of ATM arises from the generation of SGs containing TDP-43.

Additionally, preliminary crosses between opportune fly strains show that DICER overexpression is able to recover the degenerative phenotype of the eyes due to hTDP-43 overexpression. Such result strengthens the idea that alterations of the DDRNA biogenesis machinery could be relevant in inducing DDR defects that drive neurodegeneration.

On the other hand, we also showed that TDP-43 depletion impairs at some extent DDR foci accumulation after DSBs induction, suggesting that the protein itself might play a physiological role in supporting the activation of the DDR cascade at sites of DNA damage. However, differently from what happens in presence of TDP-43 inclusions, TDP-43 knock down didn't impact on DROSHA protein levels, indicating that loss of TDP-43 might impair DDR activation with a mechanism different from DROSHA down-regulation. This might suggest that TDP-43 also plays a direct role at DNA damaged sites. Certainly, the negative impact on DDR of TDP-43 inactivation by siRNA is consistent with the already demonstrated role of TDP-43 in preventing the transcription-induced DNA damage (Hill, et al. 2016).

Moreover, whether confirmed, the potential role of TDP-43 in DDR will contribute to expand the list of functional similarities with FUS (Kapeli, et al. 2016), (Kim, et al. 2010), (Wang, et al. 2011), another relevant ALS-linked RBP that form aggregates in a minor fraction of ALS cases, delineating a common pathogenic mechanism for both proteins, in ALS.

A direct role of TDP-43 in DDR is also supported in this study, by the analysis performed in fALS fibroblasts harbouring the G294V mutation on the Gly-rich domain of TDP-43. Such mutation was found in both fALS and sALS cases (Conforti, et al. 2011), was associated with signs of cognitive impairment (Corrado, et al. 2009) and was reported to confer amyloidogenic properties to the protein (Sun, et al. 2014). Preliminary experiments show that fALS fibroblasts have higher levels of endogenous 53BP1 foci. Moreover after DNA damage induction, 53BP1 foci accumulation didn't significantly increase relative to basal conditions, differently from what happens in control fibroblasts. In the same cells instead, γH2AX foci formed with comparable amounts, suggesting that the dose of damage induced was equal in both cell samples. However, in contrast to the observations made by another group (Sabatelli, et al. 2015), we didn't detect any mis-localization of TDP-43 in the cytoplasm of G294V cells with respect to the control ones. These different results might be due to the high rate of variability existing among fibroblasts of different individuals.

Overall these data bring us to speculate that also in the absence of TDP-43 containing inclusions, mutations on TARDBP could alter the activation of a proper response to DNA damage.

The possibility to use ALS derived fibroblasts to search for ALS diagnostic biomarkers is very attractive since differently from other cell types, they can be easily obtained with non-invasive procedures from live ALS patients. Therefore it would be useful to analyze more fALS fibroblasts with different mutations on TARDBP, and possibly deriving from different patients to enforce these data and to gain a major comprehension of the role of TDP-43 in DDR. Indeed as said

before, a limitation associated with the use of fibroblasts is the known great inter-variability of this cell type (Wagner, et al. 2014) that hinders the interpretation of the obtained results. Altered DDR foci accumulation after TDP-43 depletion and in ALS cells with the G294V mutation however suggest that TDP-43 itself is implicated in some aspects of the DDR signaling cascade, validating the idea that also TDP-43 loss of function might contribute to drive ALS/FTLD pathogenesis.

Altogether, our results led us to propose that DDR alterations are a novel, relevant mechanism driving ALS/FTLD pathogenesis. DDR impairment and possibly also defects of DSBs repair mechanisms due to loss of nuclear DROSHA, could explain part of the still obscure pathogenic mechanisms underlying the ALS/FTLD spectrum of disorders.

Conclusions and perspectives

My study, implicates DDR as a novel ALS/FTLD pathogenic mechanism strongly related to the onset of TDP-43 containing inclusions, that are the unquestioned hallmark of these neurodegenerative diseases. However to render more relevant the preliminary results obtained in HeLA cells, it will be necessary to transpose the above observations in ALS model organisms and/or in cells of neural origin obtained from ALS patients.

To these aims and with the help of our collaborators, at the moment we are going to exploit Drosophila models of the pathology to gain insights about the DDR contribution to the ALS pathogenesis. Additionally we are generating iPSCs from the same control and fALS fibroblasts we used to obtain the reported preliminary observations and we plan to differentiate them into motor neurons. In these differentiated motor neurons from ALS patients, we will study DDR activation. Furthermore, we will try to expand the number of fALS and sALS fibroblasts and related iPSCs cell lines to understand at which extent the DDR related effects depend on TDP-43 function, genetic variants and level of aggregation.

We will also expand the study of the effects of TDP-43 positive SGs on DDR, in HeLA cells. Indeed it could be useful to understand if DDR alterations also reflect DSBs repair defects in cells with TDP-43 positive SGs. To evaluate if such TDP-43 containing granules also hinder DNA repair, we will overexpress TDP-43 in HeLA cells endowed of a dedicate fluorescent-based reporter system, known as the Traffic Light Reporter, which allows to measure in the same cell population the relative efficiencies and balance between HR and NHEJ (Certo, et al. 2011).

To further characterize the role of TDP-43 in DDR we are investigating if similarly to FUS it localizes to DSBs, by setting up dedicated ChIP and diPLA protocols in cellular systems of locus-specific DNA damage. Moreover it will be interesting to understand if also TDP-43 itself could affect DSBs repair mechanisms in addition to the DDR signalling. To this aim I will test TDP-43 involvement in both NHEJ and HR repair pathways by employing the DR-GFP HR reporter system and the well-established EJ5-GFP NHEJ reporter assay (Gao et al. 2014; Bennardo et al.

2008) respectively. Moreover, we will investigate the preferred pathway choice in cells depleted for TDP-43, by using the mentioned Traffic Light Reporter system. I will measure NHEJ and HR repair rates in these cell systems after depletion of TDP-43 as well as in untreated cells. Altogether the planned experiments will lay the basis for a deeper comprehension of which is the role of TDP-43 function in the general biology of DDR and at which extent TDP-43 anomalies count in the pathogenic mechanism affecting DDR in TDP-43 proteinopathies *in vivo*.

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