Trends in Genetics

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Feature Review

DNA Damage Triggers a New Phase in Neurodegeneration

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

Highlights

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

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

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

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

Being Liquid Helps to Focus

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

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

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

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

Box 1. Stress Granules

Stress granules (SGs) are cytoplasmic membraneless organelles (MLOs) with the main function of slowing down mRNA translation thought to guarantee cell survival upon different insults, such as viral infections, oxidative stress, heat shock, and increased osmolarity [64]. Fundamental constituents of SGs are polyadenylated RNAs, stalled translation complex, and various RNA-binding proteins (RBPs) which bear intrinsically disordered regions (IDRs) that promote extensive weak intermolecular interactions [63]. The RBPs T cell internal antigen 1 (TIA1) and RAS GTP-activating protein-binding protein 1 (G3BP-1) are constitutive SG components, intrinsically able to undergo liquid–liquid phase separation (LLPS) and considered the minimal set of factors needed to nucleate the formation of these MLOs in response to stress [140,141].

SG nucleation is initiated upon polysome dissolution following translation arrest. This exposes mRNA to TIA1 and G3BP-1 and favors their accumulation on the RNA [63]. SG then recruits additional intrinsically disordered RBPs, ultimately promoting phase separation [14,29]. Importantly, this process is reversible, and, once stress is resolved, recovery of translation causes an ATP-dependent SG disassembly [63,64,141].

Beyond their physiological relevance, SGs are becoming objects of interest due to a proposed connection with the pathogenesis of various neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) [63]. Affected tissues of the ALS/FTLD spectrum often harbor TDP-43- and FUS-positive inclusions colocalizing with SG components [10,63,140]. However, although SGs devoid of TDP-43 are reversible, TDP-43 incorporation makes them more persistent after stress removal. It has been reported that TIA1 mutations, in a subset of patients with ALS, are accompanied by less dynamic TDP-43-containing SGs [142]. A recent study found that mitoxantrone, a planar compound known to interact with RNAs, prevents the inclusion of TDP-43 into SGs, thus representing a potential therapeutic strategy for ALS/FTD [143].

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

Glossary

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

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

The Role of RNA in Phase Transition

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

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

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

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

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

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

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

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

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

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

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

Phase Separation Regulates the DNA Damage Response

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

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

Box 2. DNA Damage Response and Repair

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

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

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

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

HR instead requires the formation of single-stranded DNA (ssDNA) at the DSB through a process called 'DNA end resection.' In eukaryotes, resection is initiated by MRE11, the MRN subunit retaining nuclease activity [147]. This nuclease function of MRE11 acts in concert and is regulated by the presence of Ku and replication protein A [153,154]. The recombinase RAD51 is then loaded onto the ssDNA generated to form a nucleoprotein filament that invades the homologous DNA region that is used as a template for new synthesis of the resected DNA [146].

Box 3. DNA Damage Response and RNA

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

RNAPII loading at DSBs results in the transcription of a few kilobases long ncRNA molecules named 'damage-induced long noncoding RNAs' (dilncRNA) [33]. In S/G_2 phase cells, dilncRNA can form RNA-DNA hybrids at resected DNA ends, a structure that favors the recruitment of homologous recombination (HR) factors [90,157]. dilncRNA can be further processed into small ncRNAs called 'DNA damage response RNAs' (DDRNA) by the RNAi machinery factors DROSHA and DICER [32]. A study in *Arabidopsis thaliana* and human cells showed that, upon DSB generation, small RNAs are produced in an ATR-, DICER-, and AGO2-dependent manner [158] and act in association with AGO2 protein [159] and take part in HR and nonhomologous end joining repair [157]. A recent study based on deep sequencing of endogenous RNA generated at multiple DSBs mostly occurring at ribosomal DNA loci in human cells identified two populations of damage-induced ncRNA: one expressed at low level and required for DNA repair and a secondary amplified population loaded onto Argonaute proteins [160]. Once generated, DDRNAs act locally at the site of break through base pairing with complementary dilncRNA [33]. RNA synthesis and DDRNA–dilncRNA annealing support the nucleation of DDR foci downstream of H2AX phosphorylation [36]. Indeed, preventing dilncRNA and DDRNA synthesis by transient RNAPII inhibition or preventing DDRNA–dilncRNA pairing by the use of specific antisense oligonucleotides (ASOs) is sufficient to suppress DDR foci formation and DNA repair, demonstrating that dilncRNA and DDRNA, rather than the mere recruitment of the transcriptional machinery, modulate DDR foci formation and stability [33]. Consistently, DDR foci are sensitive to RNA degradation, similarly to other MLOs, such as nucleoli and SGs [32,40,41].

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

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

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

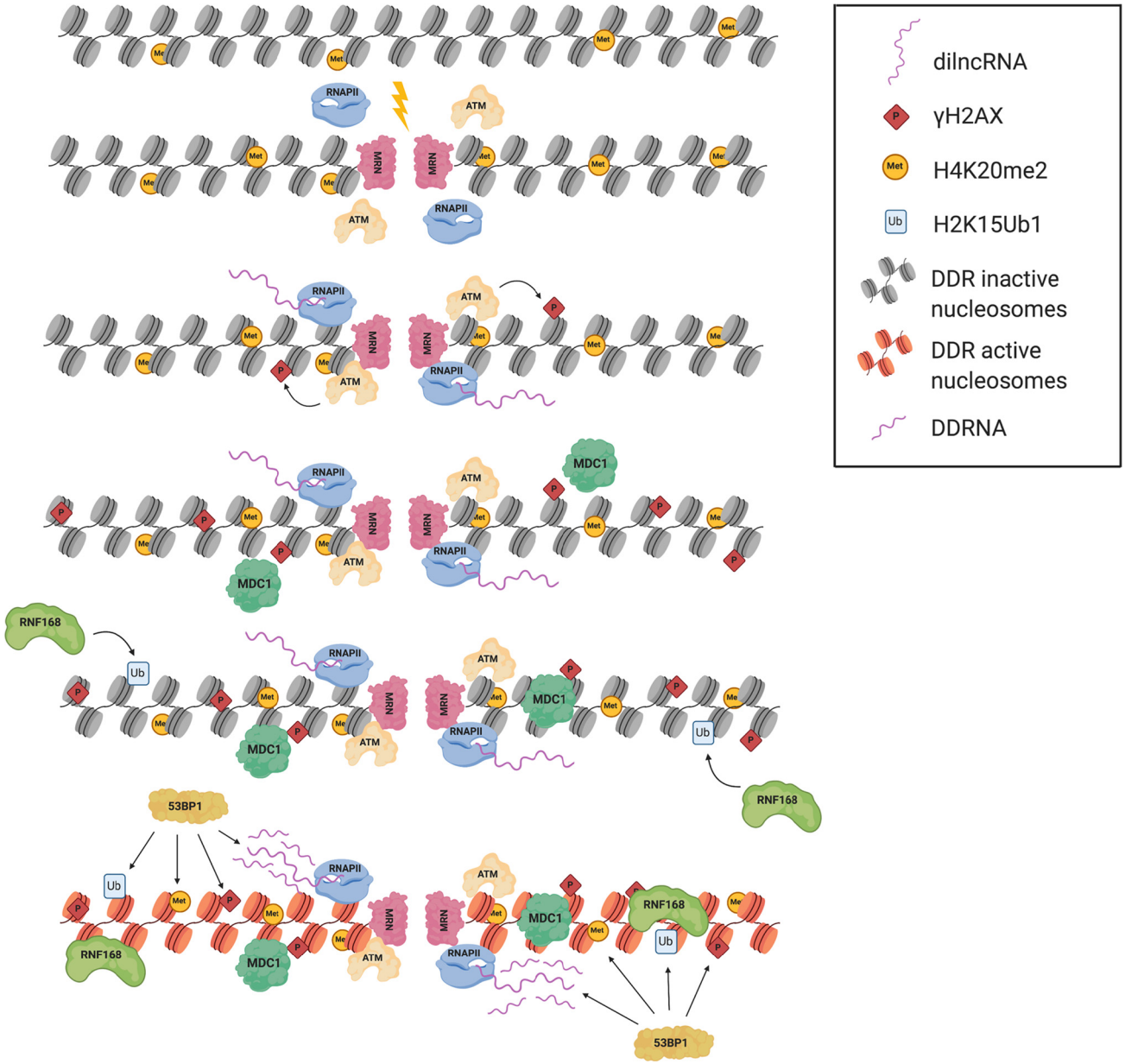

Figure 1. Early Events in the DNA Damage Response (DDR) Signaling Pathway Contributing to 53BP1 Foci Formation. Upon DNA double-strand break formation, MRN is recruited at DNA ends and recruits, independently, the RNA polymerase II (RNAPII) machinery and Ataxia-Telangiectasia Mutated (ATM) kinase to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

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

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

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

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

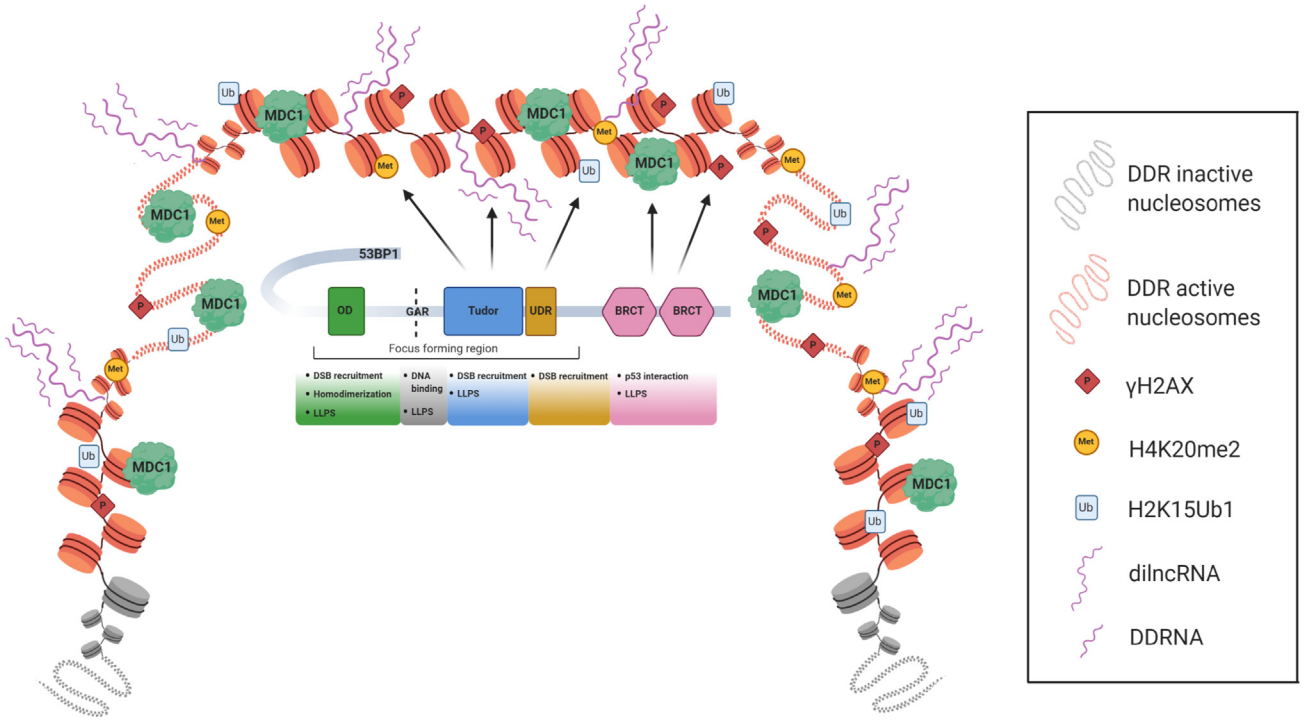

Figure 2. C-terminal Region of 53BP1 With Different Domains and Their Functions Highlighted. DNA damage response (DDR) active chromatin provides multiple sites of interaction with 53BP1, which not only recruits it but also drives its retention and accumulation at sites of damage, thus facilitating its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

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

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

Phase Separation in Neurodegenerative Diseases: A DNA Damage Connection

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

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

Altered LLPS Is Involved in Neurodegeneration

ALS

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

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

Although sharing many functional and structural similarities with FUS, TDP-43 has been implicated in DNA repair only very recently. TDP-43 colocalizes with DDR factors at DSBs and

interacts with core components of NHEJ repair machinery as the XRCC4–ligase IV complex [79]. Importantly, neuronal cells lacking nuclear TDP-43 displayed increased genome instability and reduced survival compared with controls [79]. It was also reported that the ALS-linked *TDP-43*^{Q331K} mutation, previously described to weaken TDP-43 association with nucleic acids and to increase its propensity to form cytosolic aggregates [80], is associated with increased DNA damage accumulation, both in spinal cord tissues of sporadic patients and in cellular model systems [58]. In particular, such a mutation causes TDP-43 nuclear exclusion and hampers NHEJ, leading to defective DSB repair and persistent DNA damage [58]. Recently, TDP-43 aggregates have been shown to possess the ability to be transferred from one neuronal cell to another, suggesting how aberrant LLPS occurring in a cell might also affect DSB repair in the surrounding cells [81]. Overall, these findings indicate a link between ALS-related aberrant TDP-43 phase separation and deficient DNA repair.

hnRNPA1 has been implicated in genome maintenance for its participation in telomere metabolism [82]. hnRNPA1 is reportedly modified both by PARP and DNA-PK [83–85]. PARylation promotes segregation of hnRNPA1 along with TDP-43 into liquid-like assemblies, and PARP inhibition mitigates hnRNPA1- and TDP-43–mediated neurotoxicity in cultured cells and *Drosophila* models of ALS [83].

A contribution to DNA damage repair has also been proposed for Ataxin-2, another ALS-associated gene, because it has been reported to facilitate the resolution of **R-loops**, nucleic acid structures prone to DNA damage accumulation [86]. A similar example has been reported in spinal muscular atrophy (SMA), a neurodegenerative disorder in which R-loop accumulation results in DNA damage accumulation and genomic instability [87]. Zinc finger protein 1 (ZPR1) deficiency results in pathological R-loop accumulation with ZPR1 overexpression shown to provide neuroprotection and rescue of SMA disease [88]. It is possible that R-loop formation may also take place in MLOs generated through LLPS. Interestingly, R-loops generated at sites of damage have also been shown to facilitate DNA repair in yeast and mammalian cells [89,90].

Recently, it has become apparent that the most common genetic signature of familial ALS (45% of the cases) is the G_4C_2 repeat expansion in the *C9ORF72* gene [59]. Notably, repeat-containing RNAs, synthesized from both strands of the *C9ORF72* locus, may undergo a noncanonical translation process called 'repeat-associated non-AUG (RAN) translation,' which generates different types of dipeptide repeat (DPR) proteins [91]. In particular, the highly toxic arginine-rich DPRs have recently been shown to phase separate *in vitro* and, when ectopically expressed in cells, to hamper the liquid dynamics and functions of MLOs, such as SGs and nucleoli [92,93]. Interestingly, DPR expression was found to trigger cell death by impairing signaling of the key DDR factor ATM in ALS motor neurons [56] and to hamper DNA damage repair pathways in cultured cells [94]. Because *C9ORF72*-derived DPR proteins are often present in pathogenic cytoplasmic inclusions of patients with ALS and patients with frontotemporal dementia (FTD) [91], it is easy to imagine a causative link between the accumulation of such DPR deposits and neurodegeneration associated with DDR defects (see Figure 4 for schematic representation).

AD and PD

AD is the most common neurodegenerative disorder, and it is associated with brain lesions constituted mainly by extracellular amyloid-β deposits and intracellular tangles of the hyperphosphorylated microtubule-associated protein tau (*MAPT* gene) [95]. Tau is an IDR-containing protein that plays fundamental roles in synaptic plasticity [96,97] and has recently been reported to undergo LLPS both *in vitro* and in cultured mouse neurons [97–100]. Tau is a highly soluble protein with a very low propensity to form condensates, but it can phase separate reversibly upon phosphorylation or

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

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

HD

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

A Role of RNA Phase Separation in DDR in Neurodegeneration

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

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

Concluding Remarks

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

It is noteworthy that RNA generation at DSBs and its role in fueling LLPS within DDR foci may also contribute to isolating individual DNA lesions and preventing dangerous translocations and unscheduled recombination events by controlling immiscibility of different DDR foci generated from different RNA sequences. Only when damage persists may heterochromatin-associated PTMs be deposited and transcription suppressed, potentially allowing persistent DDR foci to merge [130]. Intriguingly, if, as reported for LAF-1 [29], RNA length can influence droplet viscosity, with long RNA increasing it and short RNA reducing it, it is tempting to imagine dilncRNAs and DDRNAs, their shorter processed forms, being able to differentially modulate DDR foci properties, perhaps over time.

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

Outstanding Questions

How does LLPS affect DDR signaling and neurodegeneration?

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

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

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

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

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

Author contributions

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

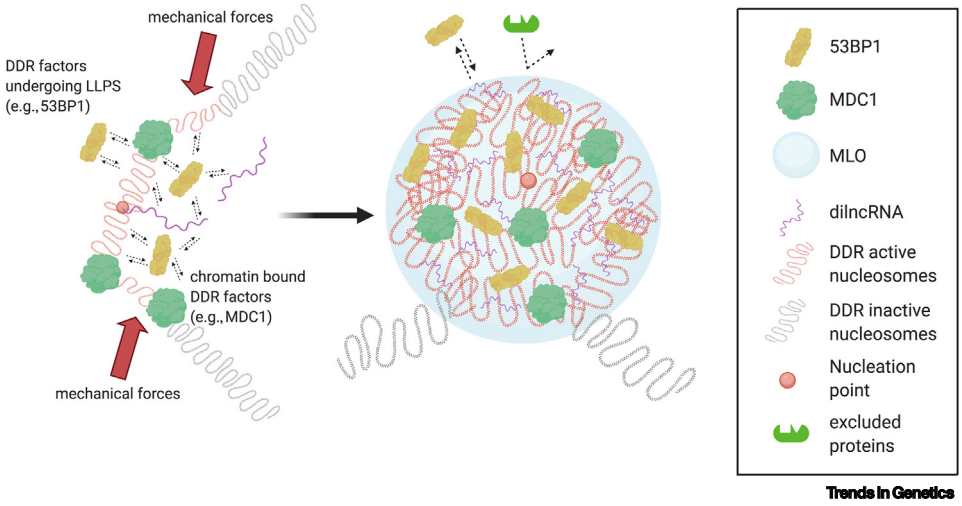

Figure 3. RNA and Modified Chromatin Promote Membraneless Organelle (MLO) Formation. Starting from the nucleation point (orange sphere) provided by the DNA double-strand break, the synthesis of damage-induced long noncoding RNAs (dilncRNA)/DNA damage response RNA (DDRNA) and different chromatin modifications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

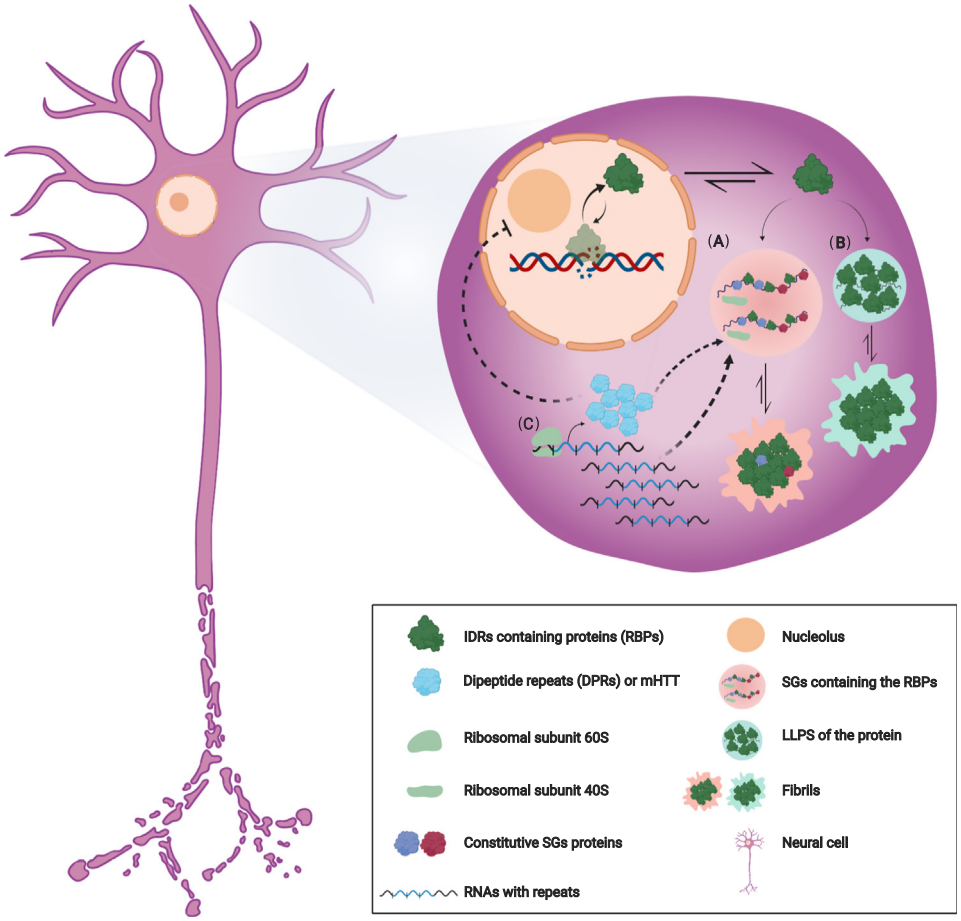

Figure 4. Liquid–Liquid Phase Separation (LLPS) of DNA Damage Response (DDR)-Related Factors or Expanded RNAs May Trigger Neurodegeneration. Several intrinsic causes (i.e., altered protein structure, aberrant post-translational modifications, and accumulation of RNAs) and extrinsic stressors can induce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

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

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