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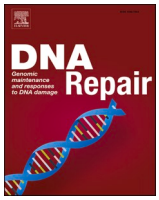

Chromosomal single-strand break repair and neurological disease: Implications on transcription and emerging genomic tools

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ABSTRACT

Cells are constantly exposed to various sources of DNA damage that pose a threat to their genomic integrity. One of the most common types of DNA breaks are single-strand breaks (SSBs). Mutations in the repair proteins that are important for repairing SSBs have been reported in several neurological disorders. While several tools have been utilised to investigate SSBs in cells, it was only through recent advances in genomics that we are now beginning to understand the architecture of the non-random distribution of SSBs and their impact on key cellular processes such as transcription and epigenetic remodelling. Here, we discuss our current understanding of the genome-wide distribution of SSBs, their link to neurological disorders and summarise recent technologies to investigate SSBs at the genomic level.

1. Introduction

1.1. Sources of single-strand breaks

Single-strand breaks (SSBs) are the most common type of DNA lesions, occurring at a frequency of ~10,000 times per cell each day [1,2]. Unrepaired SSBs lead to genome instability as they interfere with critical genetic processes like replication and transcription [3]. SSBs and defects in their repair have been implicated in the development of several diseases including neurological diseases, cancer, and heart failure [3–5]. SSBs can occur either directly via the disintegration of the sugar-phosphate backbone, or indirectly via enzymatic cleavage of the backbone as intermediates or products of DNA repair and/or metabolic processes. Oxidative stress has been implicated in the production of SSBs either directly via the disintegration of the oxidised base / nucleotide or indirectly when the damaged base is removed via the base excision repair (BER) enzymes [6,7].

BER itself can generate SSBs as intermediates of the repair pathway. Following spontaneous base loss or removal of a damaged base by a DNA glycosylase, abasic or apurinic/apyrimidinic (AP) sites are formed. These AP sites, if not properly cleaved and repaired by apurinic/apyrimidinic (AP) endonuclease (APE1), can undergo spontaneous β-elimination forming SSBs [8]. In addition to stochastic base damage, AP sites are formed as a result of programmed epigenetic processes such as cytosine demethylation. 5-methylcytosine (5mC) are actively processed by ten-eleven translocation (TET) proteins. TET proteins utilise molecular oxygen to oxidise 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), successively. 5fC and 5caC are further removed by thymine DNA glycosylase (TDG) leaving AP sites [9,10]. Moreover, a reactive iron form of hydroxyl radical (Fe(IV)-oxo) and superoxide anion radicals ($\bullet O_2-$) are formed during the action of TET proteins [11,12]. These reactive intermediates attack adjacent guanine bases forming 8-oxoguanine (8-oxodG) that are further cleaved by 8-oxodG DNA glycosylase 1 (OGG1) resulting in AP-sites [13,14].

Another epigenetic process that generates reactive oxygen species (ROS), which can result in oxidative SSBs, is histone demethylation. Demethylation and acetylation of histones H3 and H4 is a requirement for transcription activation. Two families of histone demethylases exist: flavin adenine dinucleotide-dependent monoamine oxidases (e.g., lysine-specific demethylase 1 (LSD1)) and the Jumonji (JMJ) family. They generate H_2O_2 and ($\bullet O_2-$) respectively as by-products during their

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action [15,16].

Another common source of SSBs is the aborted activity of cellular enzymes such as DNA topoisomerases. Topoisomerases introduce transient DNA breaks as obligate intermediates of the catalytic cycle to relax tightly wound DNA to help progression of DNA replication and transcription. These breaks are called cleavage complex intermediates, which are normally re-joined by topoisomerases at the end of the cycle [17,18]. However, stabilisation of cleavage complexes can occur due to collisions with DNA polymerases or RNA polymerases. In addition, the presence of nearby DNA lesions, such as AP-sites, intermediates of cytosine demethylation and oxidative breaks can prevent the re-ligation [19–21]. Moreover, abortive DNA ligation reactions can lead to persistent breaks. To exert their function, DNA ligases interact with ATP to form an enzyme–adenylate complex, then transfer the activated AMP to the 5′ phosphate at the nick, and finally form the phosphodiester bond with the release of AMP [22]. However, if they attempt to repair non-ligatable or 'dirty' breaks induced by ROS, abortive intermediates are formed leaving an adenylate group covalently linked to the 5′ phosphate at single-strand nicks. Aprataxin (APTX) was found to resolve these abortive DNA ligation intermediates by catalysing the nucleophilic release of adenylate groups [23]. The different sources of SSBs are summarised in Fig. 1.

2. Single-strand break repair

The SSB repair (SSBR) pathway is a highly orchestrated process that safeguards genomic integrity. General steps of SSBR include SSB detection, DNA end processing, gap filling, and eventually ligation. The repair process is initiated with the recognition of the SSB by sensor proteins such as (poly(ADP-ribose) polymerases (PARPs). Upon binding to the SSB, PARPs are allosterically activated and this catalyses the addition of poly(ADP-ribose) (PAR) chains residues at the break sites, onto themselves and at nearby proteins, creating a scaffold for subsequent repair factors to bind [24]. Initially, a single mono-ADP-ribose (MAR) residue is attached to the target substrate which is then further elongated by PARPs, such as PARP1, to form a poly-ADP-ribose (PAR) chain [24], which can be subsequently removed by PAR glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3) [25]. PARG hydrolyses the ribose-ribose bonds that are within the PAR chains to generate free chains or MAR residues [26]. ARH3, on the other hand, generates MAR residues only [27,28] which are removed by macrodomain-containing proteins that possess ADP-hydrolase activity such as MacroD1, MacroD2 and C6orf130 [29–31]. The accumulation of PAR ADP-ribose chains attracts a multitude of repair factors to the site of the SSB [32,33]. Among these factors, XRCC1 (X-ray repair cross-complementing protein 1) plays a central role. It serves as a scaffold protein that interacts with and stabilises different DNA end-processing enzymes such as tyrosyl DNA phosphodiesterase 1 (TDP1), aprataxin (APTX), apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1) and polynucleotide kinase 3′-phosphatase (PNKP) [34,35].

Following the repair of the DNA ends, SSBR can further proceed via the short-patch or long-patch repair pathway. In short-patch SSBR, the DNA gap is filled by Polymerase β which inserts a single nucleotide followed by nick sealing with DNA ligase (LIG3α). The long-patch SSBR involves the addition of a series of nucleotides, usually around 2–12 bases long, by DNA polymerase β, δ and ε creating a 5'-single-strand flap that is removed by flap endonuclease 1 (FEN1) before ligation by DNA ligase (LIG1) [36–38]. The short-patch SSBR is efficient for repairing minor lesions without altering the DNA sequence extensively. However, the long-patch SSBR is more error-prone and is highly active in post-mitotic neurons [39,40].

Interestingly, SSBR proteins can play a role in the repair of other DNA lesions. BER involving NTH1, APE1, PARP1, XRCC1, and FEN1 have recently been shown to rapidly remove a subset of photodimers

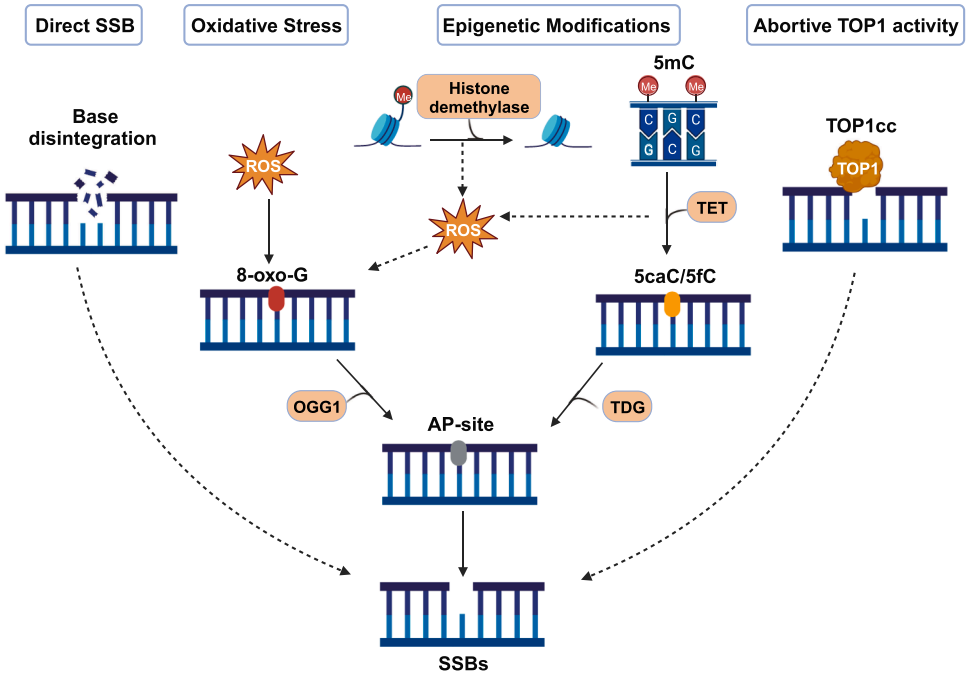

Fig. 1. Sources of SSBs. SSBs can arise either directly via the disintegration of the sugar-phosphate backbone or indirectly due to enzymatic cleavage of intermediates of certain DNA metabolic processes. Oxidative stress can cause direct disintegration of oxidized bases/nucleotides, or it can attack guanine bases forming 8-oxoguanine (8-oxodG) that are cleaved by 8-oxodG DNA glycosylase 1 (OGG1) resulting in abasic sites (AP-sites). Oxidative DNA demethylation of 5-methyl cytosine (5mC) by the ten-eleven translocation (TET) proteins generates 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC are cleaved by thymine DNA glycosylase (TDG) forming AP-sites. In addition, both oxidative DNA demethylation and histone demethylation generate ROS as by-products and can also form 8-oxodG, resulting in AP-sites. Finally, abortive activity of DNA topoisomerase 1 (TOP1) which leads to stabilisation of cleavage complexes (TOP1cc) can occur due to collision with DNA polymerases or RNA polymerases or in the presence of nearby DNA lesions such as AP-sites, intermediates of cytosine demethylation and oxidative breaks. "Created with BioRender.com.".

following UV irradiation in wild-type human cells [41].

2.1. SSBs at non-coding regulatory regions

DNA damage and repair mechanisms have been extensively studied over the past 50 years with the focus on the coding regions of the genome which represent only 2% of the entire genome. Non-coding DNA regions encompass various elements such as promoters, enhancers, introns, and intergenic regions. While they don't directly encode proteins, they play critical roles in gene regulation and chromatin organisation. DNA breaks in non-coding regions can influence gene expression by disrupting transcription factor binding sites, enhancer-promoter interactions, or splicing signals. These disruptions can lead to altered gene expression patterns, potentially impacting the cellular phenotype, and contributing to disease [42].

Genome-wide mapping of SSBs has identified promoters and enhancers as hotspots for SSBs and their associated repair. Thousands of DNA repair hotspots were identified at the enhancers and promoters of post-mitotic neurons [39,40,43]. These hotspots overlap with regions of accessible chromatin identified by ATAC-seq and enriched at regions with high levels of H3K27 acetylation, a histone mark associated with active promoters and enhancers [39]. The sources of damage at these sites and the mechanisms of repair are still not well-characterised. Here, we attempt to explain why the architecture and principles of DNA repair in non-coding regions can be different from coding regions.

2.2. Sources of breaks at promoters and enhancers

Promoters and enhancers are sites of extensive epigenetic reprogramming that renders them hotspots for DNA breaks. Active DNA cytosine demethylation is one mechanism that generates SSBs at these regions. Supporting this hypothesis, SSBs are enriched around transcriptional start sites (TSSs) specifically at GC-rich regions [43], and SSBs repair peaks occur in neuron-specific enhancers at or near C/G nucleotides [40]. In addition, post-mitotic neurons derived from induced pluripotent stem cells (iPSC) had fewer SSBs upon depletion of TDG, however, they were not completely abolished indicating other possible sources of damage [44].

Histone modifications are required for enhancer activity. For instance, removal of methylated histone H3 (H3K27me3) and acetylation of lysine 27 on histone H3 (H3K27ac) are markers of active enhancers that help maintain an open chromatin state and promote enhancer RNA synthesis [45]. Also, demethylation of H3K4me and H3K9me at target loci by histone demethylases occurs at specific loci to facilitate transcription [16]. These demethylation reactions generate ROS which increases the oxidative stress burden at enhancers and their associated promoters. It is unclear whether the breaks at promoters and enhancers are the result of the AP-sites formed after TDG-mediated cleavage of demethylation intermediates (5fC and 5caC) or ROS-mediated oxidative stress resulting from the action of TET enzymes and histone demethylases.

DNA sequence itself can determine the susceptibility of specific loci to oxidative DNA breaks. For example, promoters are G-rich with high propensity of forming G4-quadruplexes which exhibit increased level of guanine bases that are most readily oxidised to 8-oxodG [46]. The mechanism underlying the increased susceptibility of promoters to oxidative DNA breaks is unclear, but it is likely attributed to an increase in the rate of formation of 8-oxodG or a decrease in the repair capacity [47,48]. The persistence of unrepaired breaks has been reported to affect several physiological processes such as transcription, DNA replication as well as the formation of secondary DNA structures such as the three-stranded RNA-DNA hybrids, R-loops, and the guanine rich, G4-quadruplexes [47,49].

2.3. Open chromatin state

Being in open chromatin and continuously exposed to various gene regulatory factors (e.g., transcription factors, enhancer RNAs and chromatin remodelling factors), non-coding regions are predicted to be more vulnerable to the occurrence of DNA breaks. Rapid and efficient repair mechanisms should be employed to protect these important gene regulatory elements. In the meantime, open chromatin state facilitates the repair of these regions. Open chromatin was found to allow the recruitment of the BER proteins to facilitate the repair of 8-oxodG lesions, while chromatin compaction hinders it [50].

2.4. Transcription regulatory events

Promoter and enhancer regions are the sites where multiple transcription regulatory events occur such as enhancer-promoter looping and RNA polymerase II (RNAPII) pause/release. While these events have little direct impact on the coding regions, they can largely affect the promoter and enhancer regions. For instance, cell type–specific gene expression is regulated via interactions between promoters and enhancers that are located far apart via the formation of chromatin loops [51]. The enhancer–promoter looping causes topological constraints, which require Topoisomerase 1 (TOP1) activity to be resolved, increasing the possibilities of endogenous DNA breaks and the demand for repair [52]. Transient DNA breaks (e.g., TOP1-induced breaks or oxidative breaks) at these regions and the recruitment of DNA repair proteins can facilitate the crosstalk between promoters and enhancers. However, accumulation of the unrepaired 8-oxodG makes these regions more prone to DNA breakage and the occurrence of SSBs [53–55].

Moreover, oxidative DNA breaks at promoters and enhancers have been found to increase the pausing index (the proportion of RNAPII molecules at promoters compared with gene bodies) by delaying the release of RNAPII molecules from pause sites [56]. This indicates that DNA breaks at these regions need to be repaired promptly to help transcription progression and the optimal coordination between the repair and transcription machinery is required to avoid the competition between these two essential processes.

2.5. Distinction from transcription-coupled repair (TCR)

Actively transcribed genes are more efficiently repaired compared to the inactive regions [57–59]. The transcription-coupled repair (TCR) mediated by the Cockayne Syndrome Protein B (CSB) and Xeroderma pigmentosum proteins helps to eliminate bulky lesions of DNA (e.g., UV-induced lesions). This overcomes the stalling of elongating RNAPII to help the progression of transcription [60–62]. Despite our knowledge about the repair of the bulky UV-induced lesions and how it coordinates with the transcription elongation, we, surprisingly, know very little about how the mechanisms of repair of the more abundant oxidative breaks and demethylation intermediates at enhancers and promoters and how they are coordinated with transcription initiation.

Recently, the nuclear mitotic apparatus (NuMA) protein was reported to play a role in the coordination between the repair and the transcription initiation machinery. NuMA is a structural protein that promotes nuclear formation, mitotic spindle assembly and stabilization [63,64]. It was found to interact with the oxidative DNA repair proteins TDP1 and XRCC1 in a PARP1-dependent manner. It also interacts with the initiating and promoter-paused Serine-5 phosphorylated RNAPII (p-Ser5 RNAPII) regulating its availability at promoters and enhancers by modulating the extent of its ADP-ribosylation [56]. NuMA plays a protective role against oxidative DNA damage at enhancers and promoters and was found to be enriched at promoter regions at approximately 100 bp on either side of the TSS. NuMA deficient cells exhibited increased oxidative breaks and AP-sites at enhancers and promoters [56].

2.6. Are SSBs causes or consequences of transcription?

Transcription increases the rate of spontaneous and chemically induced mutations in a phenomenon that is referred to as transcription-associated mutagenesis (TAM) [65]. Examples of TAM include C>T substitutions resulting from cytosine deamination in the exposed ssDNA strand [66,67], and TOP1-mediated transcription-dependent signature of (2 to 5 base pair) deletions which have been identified in yeast and mammalian cancer cells [68,69]. Another source of DNA breaks during transcription is R-loop formation which is the RNA/DNA hybrid formed due to the hybridization of nascent RNA to the transcribed DNA strand with a displaced single-stranded DNA that is exposed to damaging agents and nucleases. R-loops also increase the chances of error-prone DNA synthesis [70].

Recently, there has been an emerging theme that some forms of DNA damage are required for specific physiological functions rather than being completely undesirable. For instance, DNA breaks at promoters and enhancers are formed in response to specific stimuli and help transcriptional activation by acting as nucleation points for binding of various DNA damage response proteins, which in turn leads to local chromatin remodelling, changes in chromatin topology, and eventually activates transcription [53]. The interactions between DNA SSBs and transcription are well-characterised [71].

TOP1 DNA nicking activity has been found to be a prerequisite for ligand-dependent enhancer activation and enhancer RNA (eRNA) synthesis. Signal-dependent enhancer activation temporally precedes activation of its cognate promoter and increases eRNA transcription. TOP1 was found to form long-lived DNA breaks at androgen receptor-regulated enhancers which are accompanied by the recruitment of the DNA repair machinery, including ataxia telangiectasia and Rad3-related protein (ATR) and the MRE11–RAD50–NBS1 (MRN) complex, followed by additional components of the DNA repair machinery [72]. In addition, Topoisomerase 1 cleavage complex (TOP1cc) has been identified as an epigenomic signature that is enriched at acutely activated enhancers and is induced by 17β-estradiol, dihydrotestosterone, tumor necrosis factor alpha or neuronal depolarization. TOP1cc is recognized by the DNA damage sensor protein Ku70 mediating signal-dependent enhancer activation. Ku70 acts to tether a heterochromatin protein 1 gamma (HP1γ)–mediator subunit Med26 complex (HP1γ–Med26) to facilitate the serine 5 phosphorylation of RNAPII promoting transcriptional elongation of enhancers [73].

Moreover, TOP1 has emerged as a critical component of the transcriptional machinery at promoters. TOP1 was found to interact with the transcription factors, TFIID and TFIIA, during the pre-initiation complex (PIC) assembly and assist nucleosome disassembly to maintain open chromatin at promoters [74–76]. TOP1 acts predominantly at medium output promoters with paused RNAPII. Some paused promoters were sensitive to camptothecin (CPT) which is a TOP1 inhibitor, revealing the role of TOP1 in RNAPII promoter-proximal pausing [77]. Recently, it has been revealed that DNA relaxation of TOP1 is tightly coordinated with pause-release of RNAPII. TOP1 activity at promoters is strongly dependent on the kinase activity of bromodomain-containing protein 4 (BRD4), a protein that phosphorylates RNAPII at serine-5. BRD4 binds both TOP1 and RNAPII helping to manage the level of supercoiling to overcome the torsional stress opposing transcription, facilitates elongation and preserves negative supercoiling that assists promoter melting at the TSS [78,79].

Other mechanisms for SSBs that can activate transcription have been reported. AP sites in the promoters of several genes were reported to regulate transcription [80–82]. 8-oxodG can function as a regulatory or epigenetic marker in gene expression processes. 8-oxodG in the G-rich promoters of different genes can activate transcription via the BER pathway by inducing a transition in the DNA structure that leads to a G-quadruplex conformation. Furthermore, the oxidatively generated 8-oxodG resulting from H_2O_2 produced via the action of LSD1 determines the recruitment of OGG1 and its ability to activate transcription [82–85]. The recently identified SSB hotspots at enhancers and promoters and their association with the intermediates of DNA cytosine demethylation may suggest a possible role for these programmed breaks in the regulation of transcription [40,43,44]. However, the mechanisms of this regulation and the specific functions exerted by these breaks need further investigations.

Now, it has become clear that a tight coordination is required between the transcription and repair machinery to help maintain genomic stability and gene expression levels. This can be achieved via proteins that can facilitate both repair and transcription. A recently reported possible coordination mechanism is mediated by NuMA. NuMA is enriched at the promoters and enhancers of immediate early response genes (IERGs), proinflammatory genes and paused genes which need to respond promptly to stress and DNA damage [56]. Another link between SSBR and transcription is mediated by the scaffold protein XRCC1 which is recruited to SSBs via the action of PARP1 and/or PARP2 activity through direct interaction between poly(ADP-ribose) and the central BRCT domain in XRCC1. XRCC1 then facilitates the recruitment and assembly of SSBR proteins including POLβ, LIG3, PNKP and APTX [86–89]. The assembly of this protein complex is required to limit PARP1 activity during BER, thus preventing its hyperactivity and trapping on BER intermediates [90]. PARP1 hyperactivity was found to reduce transcriptional recovery after DNA damage via the recruitment and enhanced activity of the ubiquitin-specific protease USP3. Increased USP3 activity reduces global levels of monoubiquitinated histones including histone H2A and H2B monoubiquitination at K119 and K120 (H2AmUb and H2BmUb, respectively). Maintaining normal levels of monoubiquitinated histones is important for transcription activation [91]. Interestingly, the global levels of H2BK120ub were markedly reduced upon NuMA depletion. Whether NuMA plays a similar role like XRCC1 to prevent PARP1 trapping and increased USP3 activity is unknown [56]. Fig. 2 illustrates the roles of NuMA and XRCC1 in the coordination of SSBR and transcription.

3. Consequences of mutations in protein coding regions of SSBR factors

In neuronal cells, DNA breaks can form either due to exposure to exogenous sources such as chemicals or radiation or endogenously where it arises from physiological processes such as cellular metabolism, transcription and demethylation which result in oxidative stress that leads to the formation of SSBs. The formation of two adjacent SSBs leads to a DSB which is normally repaired via homologous recombination (HR). Since HR requires a sister chromatid as a template for repair, it is a key pathway to repair the DSBs that arise in the replicating neural progenitor cells when cells are in the S and G2 phases of the cell cycle [92]. However, post-mitotic neuronal cells are non-replicating and hence they are unable to utilise the error-free HR pathway and depend on the error-prone Non-Homologous End Joining (NHEJ) to repair these DSBs through ligating incompatible DNA ends [93].

There is clear evidence that germline mutations arising in the coding regions of different DDR players, particularly the proteins involved in sensing and processing SSBs, cause neurological disease. Accumulation of unrepaired DNA strand breaks not only leads to a malfunctioning DDR but can also result in the formation of the deleterious DSBs and affect other cellular processes such as transcription [94–96]. Mutations in the coding region of genes encoding for SSBR proteins have been reported in five proteins to date. These are summarised in Table 1. The consequence of these mutations and defective proteins has been associated with neurodegenerative disorders characterised by cerebellar ataxia and oculomotor apraxia which are summarised in Table 2.

The cerebellum is highly vulnerable to the effect of this repair deficiency due to the increased level of neurogenesis that takes place postnatally and its high oxygen demand, which increases its sensitivity to oxidative stress and hence, is highly affected by the defects in the repair proteins [93]. Since SSBs are formed at a very high frequency in the

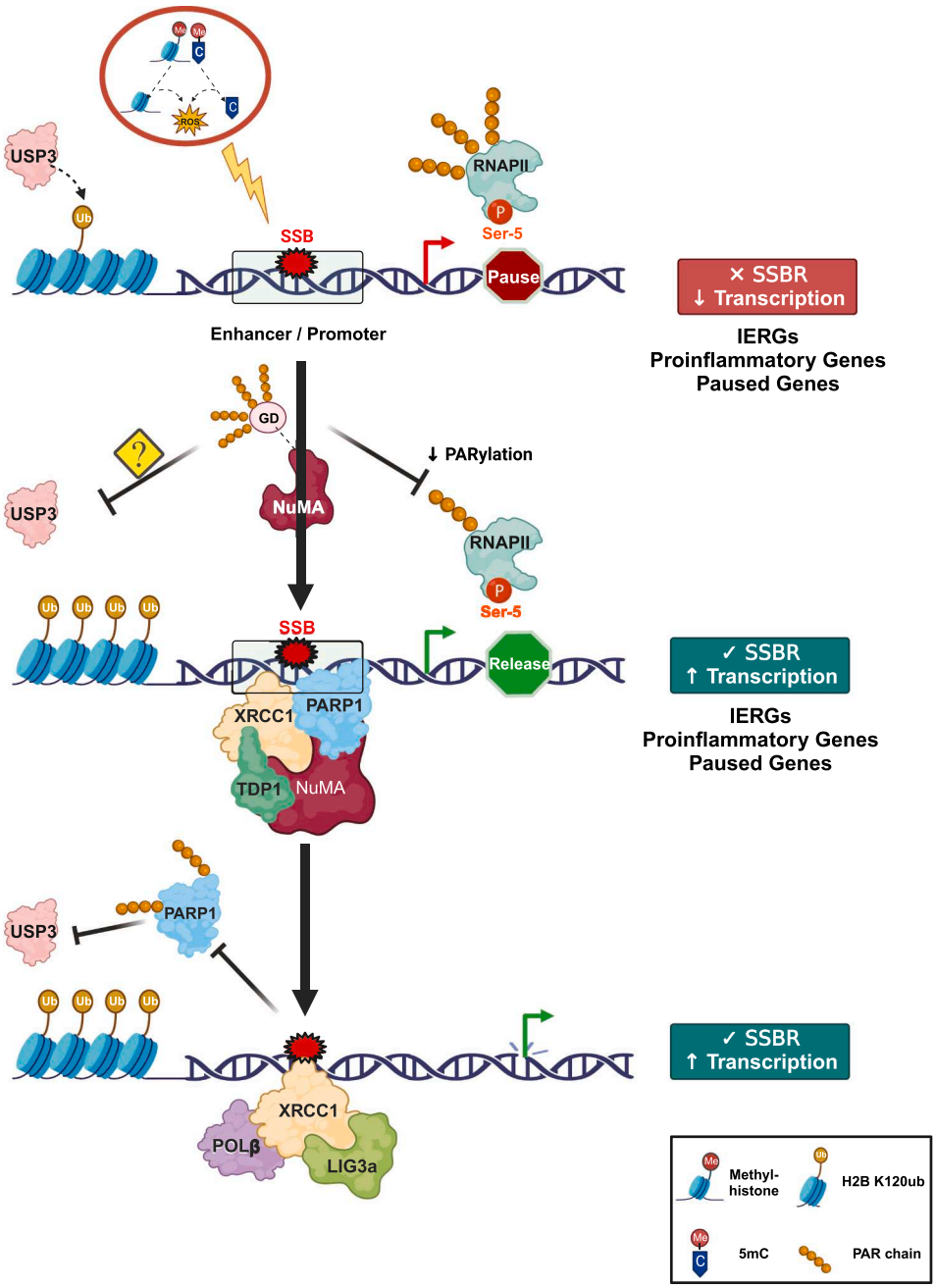

Fig. 2. The role of XRCC1 and NuMA in coordinating SSBR and transcription initiation. Non-coding DNA regions are exposed to multiple sources of SSBs including cytosine demethylation, histone demethylation and ROS. DNA damage reduces transcription activity due to increased RNAPII pausing via increased PARylation and the recruitment of histone deubiquitinase ubiquitin-specific protease 3 (USP3) which decreases the global levels of histone H2B monoubiquitination at K120 (H2BK120ub) which is important for transcriptional activation. NuMA is enriched at the promoters and enhancers of immediate early response genes (IERGs), pro-inflammatory genes and paused genes that need to respond promptly to stress. NuMA interacts with the SSBR proteins, TDP1 and XRCC1, in a PARP1-dependent manner facilitating the process of repair. Moreover, it increases the availability of RNAPII at promoters and facilitates its release from pausing to activate transcription. NuMA limits RNAPII PARylation upon DNA damage, possibly by acting as a PAR 'sink' or an enrichment factor for PAR-degrading enzymes. XRCC1 facilitates the recruitment and assembly of a multi-protein complex including POLβ, LIG3. The assembly of this protein complex limits PARP1 activity during SSBR preventing its hyperactivity and trapping. This helps to maintain normal levels of H2BK120ub via limiting the activity of USP3, thus promoting transcription. NuMA depletion was found to reduce H2BK120ub levels. Whether NuMA inhibits USP3 in a similar way to XRCC1 is still unknown. "Created with BioRender.com.".

cells, they tend to affect the terminally differentiated neurons and have a potential effect on transcription [93,94,96]. Consistently, the expression level of these SSBR proteins, according to the GTEx RNA-Seq v8 dataset, is higher in the cerebellum compared to other brain regions, which further supports the importance of their role in protecting the cerebellum from SSBs. This was also true for another recently identified SSBR player, NuMA, whose expression level shows a similar pattern (Fig. 3). However, there are no neurodegenerative disorders reported to date with mutations in the gene encoding NuMA [56].

The phenotypes observed in patients harbouring mutations in the SSBR proteins tend to be more specific to the nervous system, in contrast to patients with defects in DSBR proteins who often exhibit extra-neurological manifestations [93]. However, a perplexing aspect remains, which is how mutations reported to be in a single gene can be attributed to multiple phenotypes as detailed in Table 2. Monogenic diseases are mostly an exception as most diseases do not support the one

Table 1
SSBR proteins associated with neurological disorders.

Mutated Protein	Primary SSB Substrate	Mutation in gene	Mutation in protein	Variant effect	Neurological Disorder	Reference
TDP1	3'-Phosphotyrosyl termini (TOP1 errors)	c.1478A>G (exon 14)	p.H493R	Loss-of-function mutation	Spinocerebellar ataxia with axonal neuropathy (SCAN1)	[97,98]
XRCC1	All SSBs	c .1293 G>C (exon 11)	p.K431N	Affects splicing, inducing premature stop codons / non sense-mediated mRNA decay and / or missense mutation	Ataxia with oculomotor apraxia-5 (AOA5)	[99,100]
		c .1393 C>T (exon 12)	p.Q465 *	Nonsense-mediated mRNA decay		
APTX	5-AMP termini (DNA ligase errors)	167insT (exon 2) / 689insT (exon 5)	p.V56 * / p.V230 *	Frameshift with premature stop codon	Ataxia with oculomotor apraxia-1 (AOA1)	[101, 102]
		c .95 C>T (exon 2) / c .617 C>T (exon 5)	p.P32L / p.P206L	Missense mutation		
		318delT (exon 3) / 840delT (exon 6)	p.106 * / p.280 *	Frameshift with premature stop codon		
		c .266 T > G (exon 3) / c .788 T > G (exon 6)	p.V89G / (p.V263G	Missense mutation		
		c.G837A (exon 6)	p.W279X	Nonsense mutation		
		c.G596A (exon 5)	p.R199H	Missense mutation		
PNKP	3'-Phosphate termini (ROS, TOP1 errors)	c .526 C>T (exon 5)	p.L176F	Point mutation	Microcephaly with early onset seizures (MCSZ)	[103]
		c .975 G>A (exon 11)	p.E326K	Non-conservative amio acid change		
	5'-Hydroxyl termini (TOP1 errors)	1250_1266dup GGGTCGCCCATCGACAAC (17 bp duplication in exon 14)	p.T424GfsX48	Frameshift		
		g.5646_5662del (17 bp deletion in intron 15)		Disrupts mRNA splicing (skips exon 15)		
		c.[1123 G>T]; [1253_1269dupGGGTCGCCATCGACAAC]	p.[(Gly375Trp)]; [(Thr424Glyfs*49)]	Point mutation; Duplication	Ataxia oculomotor apraxia-4 (AOA4)	[104]
		c.[1123 G>T]	p.[(Gly375Trp)]	Point mutation		
		c.[1221_1223del]; [1549_1550insTGTACTGC]	p.[(Thr408del)]; [(Gln517Leufs*24)]	Stop-gain		
		c.[1221_1223del]; [1315_1329delinsGGGT]	p.[(Thr408del)]; [(Arg439Glyfs*51)]	Stop-gain		
		c.[1123 G>T]; [1322_1323insAGCCG]	p.[(Gly375Trp]; [Gly442Alafs*27)]	Point mutation; Stop-gain		
		c. C1549T (exon 17)	p.Gln517ter	Nonsense mutation	Charcot –Marie Tooth disease Type 2B2 (CMT2B2)	[105]
PARP1	All SSBs	c .384 T > A (exon 2)	p.C128TERM	Stop-gain	Cerebellar ataxia with dystonia/parkinsonism and oculomotor dyspraxia	[106]

For APTX, the nucleotide and amino acid numbers for the gene mutations are based on both the short and long isoforms of APTX.

gene-one disease model. Instead, different phenomena, which are not mutually exclusive, play a role in mediating the observed phenotype. These include penetrance which can be related to age, gender or ethnicity, expressivity, and pleiotropy of the mutant allele [107]. Variant expressivity can manifest as differences in how the mutated protein is expressed across different neuronal subpopulations [108]. According to genome-wide association studies, 4.6% of genetic variants and 16.9% of genes are pleiotropic. Both phenomena are commonly observed in neurological disorders [109].

Exons compromise about 1–2% of the human genome and are commonly sequenced using whole exome sequencing (WES) to identify mutations present in the protein-coding regions believed to be responsible for disease. It is believed that approximately 85% of mutations in the exome would be responsible for disease [110,111]. Hence, compared to whole genome sequencing (WGS), WES is used as it is a cost-effective alternative tool that aids in the diagnosis of disease [112]. However, recent research particularly that from the ENCODE project is shedding light on the importance of investigating the non-coding regions [113, 114]. Mutations in these non-coding regions can affect the expression of genes under their regulation thus, affecting the production and function of the corresponding proteins. Such mutations in the non-coding genome are less likely to be captured by WES and would therefore necessitate utilising WGS.

4. Investigating SSBs using molecular and cell biology tools

In the above-mentioned neurological disorders, it was evident that patients had elevated levels of DNA damage, and this was found to play a role in the pathogenesis of these disorders. While WES was utilised to identify the mutations in the genes that were implicated in these diseases, different molecular and biochemical assays were utilised to investigate the extent of DNA damage and in particular, the accumulation of SSBs.

One of the earliest tools used for the detection of SSBs was the elution of radioactively labelled DNA from a cellulose membrane or sedimentation through a sucrose gradient under denaturing conditions to release small DNA fragments formed as a result of the SSBs [115]. Nick translation is currently used instead where labelled nucleotides are incorporated at the template 3'OH groups via a DNA polymerase with 5'− 3'-exonucleolytic activity and hence the position of the DNA break can be determined. To increase the sensitivity of this technique, SSBs SensiTive Recognition of Individual DNA Ends (sSTRIDE) was developed where nick translation using biotinylated nucleotides to label the DNA lesions is combined *in situ* with proximity ligation assay (PLA) using anti-biotin antibodies from two different species to detect the SSBs [116]. Another technique that also utilises *in situ* labelling of the 3'-OH ends to detect SSBs by fluorescence microscopy is Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) [117].

Table 2
Clinical presentation of the SSBR-associated neurological disorders.

Mutated Protein	Disease	Cerebellar ataxia	Cerebellar atrophy	Axonal/sensory neuropathy	Seizures	Oculomotor apraxia	Microcephaly & developmental delay
TDP1	Spinocerebellar ataxia with axonal neuropathy (SCAN1)	√	√	√	√		
XRCC1	Ataxia with oculomotor apraxia-5 (AOA5)	√	√	√		√	
APTX	Ataxia with oculomotor apraxia-1 (AOA1)	√	√	√		√	
PNKP	Microcephaly with early onset seizures (MCSZ)				√		√
	Ataxia oculomotor apraxia-4 (AOA4)	√		√		√	
	Charcot-Marie Tooth disease Type 2B2 (CMT2B2)	√		√		√	
PARP1	Cerebellar ataxia with dystonia/ parkinsonism and oculomotor dyspraxia	√				√	

High Performance Liquid Chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) is considered to be the gold standard method to identify and quantify the different DNA lesions [118]. However, it requires expensive equipment, high technical expertise and can be time-consuming as several steps are required from culturing the cells to isolating the DNA and processing it prior to running it on the HPLC-MS/MS and subsequently, data analysis [119,120].

The comet assay or single cell gel electrophoresis is a more commonly used tool in the laboratories to provide a semi-quantitative measure of the level of DNA damage [121–124]. It involves the embedding of cells in agarose, followed by lysing them and then using an electrical field to allow the migration of the damaged DNA out of the nuclei, to produce a tail. This tail is then visualised using a dye and the tail moment which is calculated from the tail length and DNA intensity reflecting the DNA content is used to give an estimate of the level of DNA damage in cells [125]. Conducting the electrophoresis under alkaline conditions (pH $>$ 13) allows unwinding of the supercoiled DNA to detect SSBs. In order to investigate oxidative DNA damage specifically, incubation of the lysed cells with the bacterial endonucleases, formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (ENDO III) has been reported [126]. Compared to the HPLC-MS/MS, it requires inexpensive equipment and is a relatively simple technique that can be performed in most molecular biology laboratories.

Another commonly used technique is to use antibodies or reagents in combination with fluorescent microscopy to detect damaged DNA lesions such as 8-oxodG directly [127,128] or indirectly through targeting specific SSBR proteins such as XRCC1 and LIG3 [129–131] and associated post-translational modifications such as ADP-ribosylation [132–134]. Although these allow detection and monitoring of the SSBs, they have several drawbacks. These include technical challenges associated with the staining protocol and chromatin accessibility, in addition to their reliance on the existence and functioning of the SSBR proteins. Moreover, some reports have suggested that DNA damage markers could sometimes be present at non-damaged sites, giving rise to false positive results [135,136].

While the techniques described above allow identifying and quantifying the formation of SSBs, they do not provide insights on where in the genome this damage happens. To address this concern, fluorescence *in situ* hybridization (FISH) and quantitative polymerase chain reaction (qPCR)-based approaches have been utilised to investigate DNA damage across specific genes [137–139]. Ligation-mediated PCR (LMPCR) can be used to detect oxidatively damaged DNA through cleavage of the 8-oxodG residues using the bacterial Fapy DNA glycosylases or the *E. coli* endonuclease III to convert it into a SSB with a 5'-phosphate group. This is followed by a primer extension step with a gene-specific primer to generate a blunt end to which linkers are ligated and the resulting fragment is then amplified via PCR. To investigate the sequence of these lesions, the DNA fragments are run on a sequencing gel then transferred to nylon membranes and a gene-specific probe is used to visualise them [140]. These can be used to investigate a limited number of regions or loci within the genome; however, they are not affected by the presence of the DNA lesions and the polymerases are able to proceed with the amplification process regardless of the type of DNA lesion present.

5. Investigating SSBs using next-generation sequencing tools

The sequence of events from DNA damage formation to the initiation of a disease may be clearly understood in several disorders since the molecular mechanisms of how the different DNA repair pathways function have been studied extensively. However, complete understanding of the underlying mechanisms remains unknown as the consequence of these defective repair players on the accumulation of DNA breaks in the genome remains to be elucidated, thereby, limiting the capacity to intervene therapeutically [141]. Recent interest has arisen with the development of DNA-damage mapping tools that facilitate identifying the regions of the genome with increased susceptibility to DNA breaks. This is opening the door to improving our understanding of the role that these damaged DNA lesions play in the pathogenesis of these disorders and providing insights on the functional consequences of these damaged lesions.

Initial tools provided insights on the distribution of damage at the chromosomal levels [142]. Since the resolution of these tools was quite low, they did not provide insights on whether these breaks were happening at specific genomic loci or at specific genes [143]. However, with the current advances in the next generation sequencing technologies, higher resolution mapping of these DNA breaks is now possible. The techniques developed to date, either detect specific DNA lesions or repair intermediates or they label the DNA ends after a DNA break has been formed. In the following section, the different DNA damage sequencing methods that have been developed to detect SSBs will be described, in addition to a comparison between the different tools from a technical perspective (Table 3) and an overview of the DNA damage distribution, advantages and limitations of each tool (Table 4) [144,145].

5.1. Mapping 8-oxodG

One of the most common adducts that is formed as a result of oxidative stress, is the oxidation of guanine base into 8-oxodG [146]. Several techniques have been developed that map the genome-wide distribution of 8-oxodG across various organisms such as human, mouse and yeast cells. While the different techniques have different principles underlying them, they all suggest that the distribution of 8-oxodG is not stochastic in the genome and that it is enriched at open regulatory regions in the chromatin and those involved in active transcription. **OxiDIP-Seq** involved the utilisation of an 8-oxodG antibody to immunoprecipitate the DNA fragments that contained 8-oxodG prior to library preparation and sequencing [147]. In **OG-Seq**, the 8-oxodG residues were conjugated to a biotinylated probe that harboured a terminal amino group and a polyethylene glycol linker (BTN). The labelled DNA fragments were then enriched via a pull down with streptavidin-coated magnetic beads before being subjected to

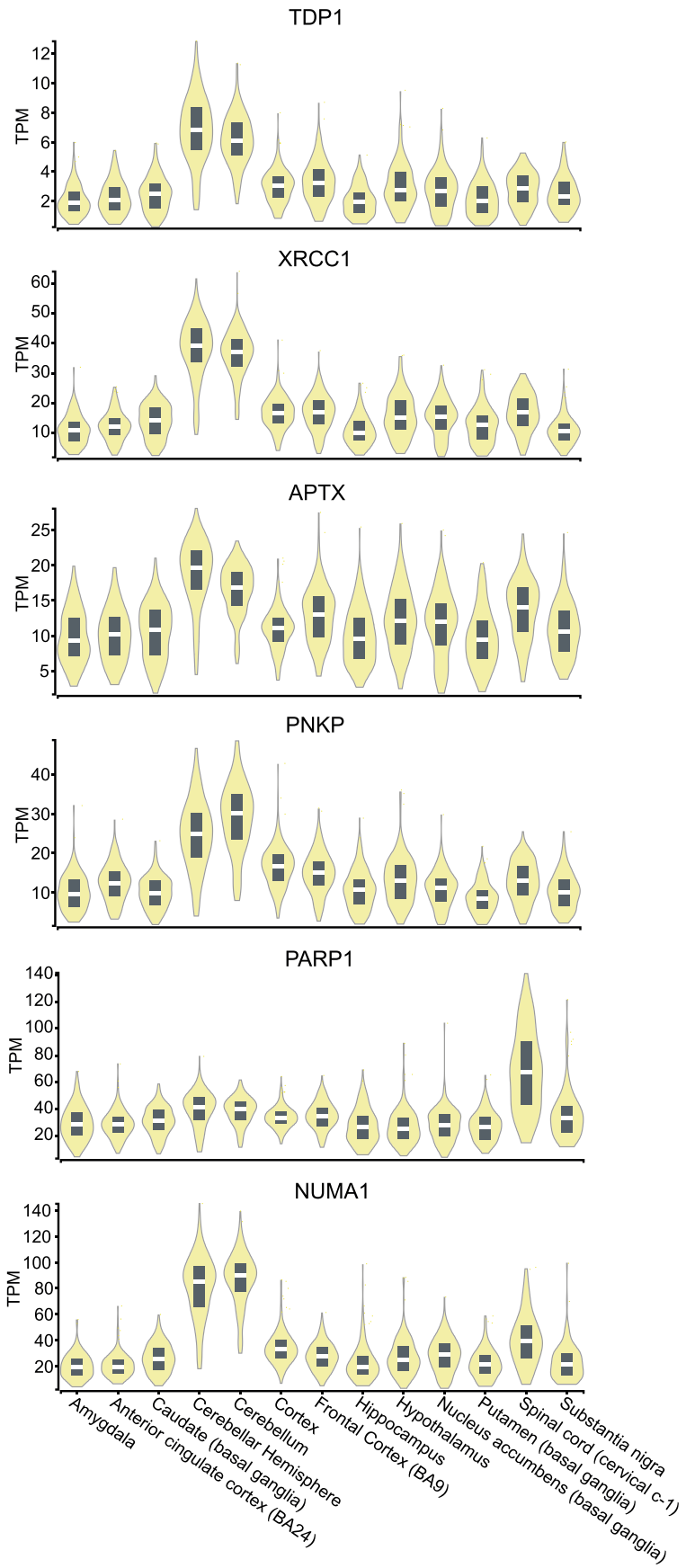

Fig. 3. Expression level of SSBR proteins across different brain regions. Violin plots showing the expression level of TDP1, XRCC1, APTX, PNKP, PARP1 and NUMA1 in Transcripts Per Million (TPM) in various brain regions from the GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2).

Table 3
Technical comparison between different SSB-mapping tools.

Technique	DNA source / and number	DNA Fragmentation Method and fragment size	Sequencing details (Platform, read length)	Resolution	Analysis pipeline
OxiDIP-Seq	MCF10A and MEFs (3T9-MycER) (10 μg DNA)	Sonication using Bioruptor Plus UCD-300 (200-800 bp)	Illumina HiSeq2000 (50 bp single-end)	~150-300 bp	NGS-QC toolkit, Bowtie and BWA, SAMtools, BEDTools, MACS
OG-Seq	MEFs (1 x 10^7 cells or 30 μg DNA) treated with K_2IrBr_6, Oligomers with 8-oxodG	Covaris sonicator (150 bp)	Illumina HiSeq (125 cycle paired-end v 4)	150 bp	NovoAlign, MAC2, BEDTools
Click-code-seq	*Saccharomyces cerevisiae* (5 μg DNA), synthetic oligonucleotides containing 8-oxodG	Covaris S220 Ultrasonicator	Illumina MiSeq (150 bp, single-end)	single-nucleotide	Bowtie2, SAMtools, BedTools,
enTRAP-Seq	MEFs, fluorescent 250 bp labelled oligonucleotides containing 8-oxodG (4 μg DNA)	Enzymatic digestion with NEBNext dsDNA Fragmentase (100-1000 bp)	Illumina HiSeq 5X Ten	100-1000 bp	Bowtie2, MACS2, BEDTools
RADD-Seq	U2OS treated with $KBrO_3$ (2.2 μg DNA)	Covaris S220 Focused-ultrasonicator (150 bp)	Illumina HiSeq 2500 (50 bp single-end)	~150 bp	Bowtie2, SAMTools, BEDTools
AP-Seq	HepG2, RPE-1, SH-SY5Y (7-10 μg DNA)	Diagenode Bioruptor (250-300 bp)	Illumina HiSeq 2000 (125 bp paired-end)	250-300 bp	Bowtie2, SAMtools,
snAP-Seq	*Leishmania major*, HeLa (5 μg DNA)	Covaris M220 system (450 bp)	Illumina Miseq and NextSeq	single-nucleotide	bwa, SAMtools, IGVtools, Deeptools, MACS2,
Nick-Seq	Purified DNA from *Escherichia coli* and *Salmonella enterica* serovar Cerro 87 bacteria (1 μg DNA)	*Nci*I, *Hin*dIII and *Xho*I; or *Sal*I, Xba1 and *Nde*I restriction enzymes	Illumina NextSeq 500 (75 bp, paired-end)	single-nucleotide	Galaxy (Trim Galore, Bowtie2, Bamtools, BEDtools) Scripts to identify SSB sites from intersection between signals from both experiments
GLOE-Seq	Purified DNA or agarose embedded nuclei from *Saccharomyces cerevisiae*, HCT116 $\geq$ 7 x 10^5	Sonication (200-300 bp) Yeast - digested with *Bsr*DI, Nb.*Bsr*DI or *Not*I	Illumina, $\geq$ 35 nt (single end)	single-nucleotide	GLOE-Pipe (customized) to determine break sites and statistical significance
SSiNGLe	K562, mouse N2a, HeLa, human PBMCs $\geq$ 1 x 10^6	MNase Digestion (150-500 bp)	Helicos SMS, > 25 nt; Illumina, 150 nt (paired-end)	Single or few nucleotides	Custom scripts for filtering, read identification and determining break sites
SSB-Seq	HCT116 8 x 10^7 cells, 500 μg DNA	Sonication with Diagenode Bioruptor (200-400 bp)	Illumina, 36 nucleotides (single-end)	200-400 bp	Illumina Analysis Pipeline (image analysis and base calling), Bowtie2
DENT-Seq	Biotinylated oligonucleotides with a single nick, Plasmid, Genomic DNA from E. coli type B cells and human cells 300 ng (Plasmid and E. coli genomes) 10 μg (Human genomic DNA)	dsDNA Fragmentase	Illumina MiniSeq (paired end)	Single nucleotide	Cutadapt, Bowtie2, SAMtools, MACS2
Repair-Seq	Post-mitotic induced pluripotent neurons 5-7 x 10^5 cells, 2-5 μg DNA	Covaris M220 (350-450 bp)	Illumina NExtSeq 500 (paired end)	350-450 bp	Blue Collar Bio (Atropos, bowtie2, biobambam2, MACS2, Homer)
SAR-Seq	Post-mitotic induced pluripotent stem cell-derived glutamergic neurons, rat cortical neurons, Murine pre-B cells (2-4 x 10^7 cells)	Covaris S220 (150-200 bp)	Illumina NextSeq 550 (75 bp single-end)	150-200 nucleotides	Bowtie2, BEDtools, SAMtols, MACS
ddN S1 END-Seq	Post-mitotic induced pluripotent stem cell-derived glutamergic neurons embedded in agarose plugs	Covaris S220 focused ultrasonicator (175 bp)	Illumina NextSeq 550 Series (75 bp single-end) or Illumina HiSeq	single-nucleotide	Bowtie2, BEDtools, PeakAnalyzer

sequencing [148]. The first technique developed that could detect oxidative DNA breaks at a single-nucleotide resolution was **Click-code-Seq**. It relied on the substitution of an 8-oxodG residue with a synthetic modified O-3'-propargyl-dGTP, followed by a click DNA ligation reaction to label the modified nucleotide with a code sequence followed by biotinylating it. This was then utilised to tag the site of damage [149]. Another technique that was developed to map the 8-oxodG residues indirectly was enzyme-mediated trapping and affinity precipitation of damaged DNA and sequencing (**enTRAP-Seq**). It relied on the enrichment of the 8-oxodG-containing DNA fragments using a His-tagged OGG1 K249Q mutant, which lacked the glycosylase activity of OGG1 and was trapped in the presence of sodium borohydride on the DNA at the sites containing 8-oxodG. Using immobilised metal affinity chromatography (IMAC) with Magnetic-His Ni-Particles, the DNA fragments containing 8-oxodG were purified and eluted in the presence of imidazole, before being subjected to library preparation and sequencing [150].

5.2. Mapping AP sites

Apurinic/apyrimidinic (AP) sites or abasic sites are the positions in the DNA strand where a purine or a pyrimidine base has been lost, either in response to DNA damage or as base excision repair intermediates or because of spontaneous hydrolytic reactions. The techniques used to map AP-sites demonstrated that AP-sites were enriched at regions implicated in transcription, replication, and genomic loci with open chromatin conformation. However, the genome-wide distribution of these AP-sites was found to be stochastic and due to the cellular heterogeneity within a population, identification of specific hotspots of AP-sites remains challenging. In repair-assisted damage detection sequencing (**RADD-Seq**), the DNA was extracted and *in vitro* digestion of the 8-oxodG residues was performed using hOGG1 leaving an AP site. The AP site was then cleaved using Endonuclease IV resulting in the formation of a gap in the DNA strand. This was then followed by a displacement synthesis step by Bst DNA Polymerase and then gap filling with biotinylated dUTP using Taq DNA Polymerase. The DNA was then sonicated prior to being pulled down by Protein G beads conjugated to anti-biotin antibody, followed by library preparation and sequencing [151]. Another similar technique which was conducted in several cell lines was **AP-Seq** where the AP-sites in the genome were labelled using a biotinylated aldehyde-reactive probe (ARP) and then pulled down with streptavidin-coated magnetic beads, prior to sequencing. This allowed the detection of the pre-existing AP-sites within the genome [152]. In order to detect AP-sites that are formed due to the presence of 8-oxodG,

Table 4
Comparison in terms of damage distribution, advantages, and limitations between different SSB-mapping tools.

Technique	Signal definition	DNA damage distribution	Advantages	Limitations
OxiDIP-Seq	8-oxodG uniquely-mapped peaks	Increased at a subset of promoters, gene bodies, open chromatin regions, DNA replication origins of active long genes Decreased at TTS	Direct detection of damaged base with a resolution of several 100 base pairs	Short half-life of 8-oxodG Binding affinity of antibody is impaired by DNA secondary structures Low resolution, cannot determine precise genomic regions Damaged lesion captured after sonication causing the introduction of artificial breaks
OG-Seq	Normalised peaks for enriched over input and with respect to the distribution of different genomic elements	Increased at TSS, 3'UTR, 5'UTR and open chromatin regions Decreased at intergenic regions	Validated using oligonucleotide containing a single 8-oxodG Damaged lesion captured prior to sonication reducing the introduction of artificial breaks	Short half-life of 8-oxoG Binding affinity of antibody is impaired by DNA secondary structures which introduces bias in the data Low resolution, cannot determine precise genomic regions
Click-code-seq	Depth at each genomic position with 1-based coordinates	Increased at heterochromatin, telomeres, nucleosomes, regions with decreased RNAPII occupancy Decreased at euchromatin, transcription start and termination sites, DNase hypersensitivity sites (DHS) and autonomously-replicating sequences, acetylated and methylated histones	High specificity for 8-oxodG through utilising FpG *in vitro* to remove the 8-oxodG Can be coupled with different glycosylases (Fpg, OGG1, TDG) to investigate distribution of lesions processed by them	Non-specific detection of sites containing fapy-guanine and methyl-fapy-guanine since they are substrates for Fpg Damaged lesion captured after sonication which can introduce DNA breaks
enTRAP-Seq	Normalised peaks of the enrichment relative to the input	Increased at promoters, 5'UTR, open chromatin regions, CpG islands, G4 quadruplexes Decreased at closed heterochromatin	Increased affinity and specificity due to utilisation of specific repair enzymes / glycosylases Validated using oligonucleotide containing a single 8-oxodG Enzymatic digestion avoids introduction of DNA breaks otherwise introduced by sonication	Lack of single nucleotide resolution to identify damage sites
RADD-Seq	Normalised read counts for enriched samples in each 200 bp window over the corresponding input	Increased at gene bodies, at highly expressed genes and in regions with less condensed chromatin Decreased near TSS	Validate sequence specificity using Nt.*Bsp*QI nicking enzyme in human keratinocytes and optically using the Rapid-RDD protocol	Cannot differentiate between PCR duplicates and two independent breaks at the same loci
AP-Seq	Normalised fold change of the enriched sample over the input	Increased at introns, transposable elements, G4 quadruplexes, telomeres and repetitive sequences Decreased at promoters, exons, termination sites, chromatin loop anchors	Can be coupled with different glycosylases (Fpg, OGG1, TDG) to investigate distribution of lesions processed by them *In vitro* digestion with glycosylases overcomes short half-life of 8-oxodG Damaged lesion captured prior to sonication reducing the introduction of artificial breaks	Cannot provide nucleotide resolution since lesion position is lost during sonication Cross-reactivity with other aldehyde containing bases such as 5-fC and 5-fU
snAP-Seq	Normalisation by RPKM at single-nucleotide resolution and enrichment of peaks rather than single sites compared to the input DNA	Increased at promoters, exons, intergenic regions	Can be coupled with different glycosylases (Fpg, OGG1, TDG) to investigate distribution of lesions processed by them Can be used to investigate thymine modifications	Requires the synthesis of a probe that is not commercially available and requires access to a lab with expertise in organic chemistry Can detect DNA modifications containing other aldehyde groups
Nick-Seq	Normalised read count ratios to the neighbouring nt and control	Increased at F1 origin, pUC origin and ampicillin resistance gene	Increased accuracy and confidence in signal since data is combined from two experiments Can be adapted to detect any lesion that can be converted to a nick with a 3'OH group	Not applicable for eukaryotic cells (complex genome) Not suitable for detecting pre-existing SSBs Requires a certain signal penetrance level to facilitate detection by both techniques
GLOE-Seq	Normalised number of reads initiating opposite each genomic position	Decreased at TSS, increased at TTS	Simultaneous mapping of SSBs and DSBs genome-wide at nucleotide resolution Maps Okazaki fragments with few cells Can be adapted to detect any lesion that can be converted to a nick with a 3'OH group Decreased background due to direct labelling of 3'OH groups in intact genomic DNA prior to fragmentation	Only detects endogenous SSBs with 3'OH group Not suitable for SSBs spaced at < 100 nt Processing in agarose plugs could lead to loss of DNA fragments < 1000 bp Cannot differentiate between PCR duplicates and two independent breaks at the same loci
SSiNGLe	Number of reads initiating opposite each genomic position	Increased at TSS, enhancers, exons, transcription regulatory elements, active histone marks, satellite repeats, CTCF binding sites and early replication forks Decreased at introns	Simultaneous mapping of SSBs and DSBs genome-wide at nucleotide resolution Sequencing on SMS does not require PCR amplification,	Only detects endogenous SSBs with 3' OH group i.e., unable to detect 3'PO_4 groups due to MNase digestion Unable to accurately map SSBs adjacent to genomic regions rich in poly(dA)

(*continued on next page*)

Table 4 (*continued*)

Technique	Signal definition	DNA damage distribution	Advantages	Limitations
			avoiding problems associated with PCR duplicates Can be adapted to detect any lesion that can be converted to a nick with a 3'OH group	Cannot differentiate between PCR duplicates and two independent breaks at the same loci Limited availability of the Helicos SMS platform Presence of homopolymeric runs of nucleotides at read ends complicates downstream Illumina analysis
SSB-Seq	Normalised read pileup, based on average track length of sequenced fragments	Increased at TSS	Potential to detect rare events through extending the nick translation reaction to increase the degree of labelling	Not fully validated hence lacking information on sensitivity and sequence bias is available Cannot provide nucleotide resolution since lesion position is lost during random fragmentation and library preparation steps Uncontrolled nuclease S1 trimming
DENT-Seq	Sites with observed transition mutation rate is attributed to dPTP and dKTP incorporation and within 5 nt from other sites	Not reported	Specifically detects SSBs Incorporation of the mutational signal in the analysis prevents false-positive results Can be adapted to detect any lesion that can be converted to a nick with a 3'OH group	Inability to detect nicks with low penetrance as it requires a certain signal penetrance level to facilitate detection
Repair-Seq	Normalised read counts of Repair-Seq signal over input	Increased at TSS, 5'UTR, gene bodies	Suitable for non-replicating cells	DNA fragmentation prior to labelling of break sites could introduce artefacts Does not directly capture SSBs as it utilises EdU incorporation to infer the position of DNA breaks
SAR-Seq	SAR-Seq peaks that were enriched at least 10-fold above the background (input)	Increased at regions of open chromatin, active enhancers, exons, introns and intergenic regions	Labelling of the break sites prior to DNA fragmentation reduces the chance of introduction of artefact DNA breaks Suitable for non-replicating cells	Does not directly capture SSBs as it utilises EdU incorporation to infer the position of DNA breaks
ddN S1 END-Seq	Calculating the distance between peak summit on the negative strand and the closest peak summit on the positive strand	Increased at enhancers, SSBs with C/G nucleotides	Can be applied for the detection of DSBs produced by a variety of mechanisms Increased sensitivity can be achieved by processing multiple agarose plugs per sample	Cannot detect endogenous SSBs unless chain-terminating dideoxynucleotides (ddNTPs) prior to S1 nuclease digestion are added Decreased sensitivity and increased background as it cannot distinguish between stochastic breaks and background if the breaks are not recurrent at the same position

in vitro digestion with OGG1 was conducted prior to labelling the DNA with the ARP and this was referred to as **OGG1-AP-Seq** [56,152]. In a similar fashion to AP-Seq, **snAP-Seq** also involved labelling of the aldehyde-containing nucleotides such as 5-formyl-uracil (5-fU) and AP-sites with biotinylated hydrazino-iso-Pictet-Spengler (HIPS) probe and then enriching them using streptavidin-coated magnetic beads, in a similar fashion to AP-Seq. However, in order to increase its selectivity and resolution to single nucleotide, a site-specific cleavage step under alkaline conditions was incorporated in the protocol [153]. These techniques are versatile and can be coupled with different glycosylases to investigate the genome-wide distribution of the damaged DNA lesions processed by them.

5.3. Mapping nicks and SSBs

SSBs are the most abundant DNA damage lesions and repair intermediates in the genome and mapping them across the genome reveals that they are enriched within regulatory elements [154] such as promoters [155] as well as in the leading strand during DNA replication following the misincorporation of ribonucleotides [156]. **Nick-Seq** was developed to detect SSBs through capturing the 3'OH ends of the broken DNA strand at a single-nucleotide resolution [157]. The genomic DNA is extracted from bacteria and the pre-existing 3'-OH groups are blocked with dideoxy-nucleotide triphosphates with the help of a terminal transferase. The technique is used to map various lesions, so accordingly, the DNA is incubated with a suitable enzyme to convert the lesions into 3'-OH ends. For instance, endonuclease IV is used to convert the formed AP sites to SSBs, and the DNA is then processed in two parallel ways. The 5'-ends of the first set of DNA is modified with α-thio-deoxy-nucleotide triphosphates, resulting in the formation of a phosphorothioate DNA fragment which is resistant to hydrolysis. Nucleases are then added to degrade the non-labelled DNA while the labelled DNA is subjected to library preparation and sequencing. The second set is subjected to poly(dT) tailing with the help of the terminal deoxynucleotidyl transferase (TdT) which facilitates annealing of the oligo(dA) primers to synthesise cDNA libraries. The combination of results from both libraries allows the generation of single-nucleotide resolution maps of the lesions of interest [158]. To map pre-existing 3'OH groups and other base lesions that could be processed to generate a free 3'OH group, genome-wide ligation of 3'-OH ends followed by sequencing (**GLOE--Seq**) was developed. The genomic DNA is denatured by heating it and the 3'-ends of the SSBs are ligated to a biotinylated adaptor containing a single-stranded hexanucleotide overhang. The ligated DNA is then sheared and the DNA fragments containing SSBs are pulled down using streptavidin-coated beads, converted to double-stranded DNA and then ligated to another adaptor that facilitates PCR amplification for library preparation followed by sequencing [156]. Single-strand break mapping at nucleotide genome level (**SSiNGLe**), on the other hand, was performed in multiple cell lines and it involved crosslinking the cells with formaldehyde followed by nuclei isolation [43,154]. The genomic DNA was then fragmented with MNase *in situ*, followed by labelling the 3' ends of the SSBs with a poly A tail using terminal transferase. The DNA was then subjected to sequencing on both, the Helicos Single Molecule Sequencing and Illumina HiSeq which facilitated single-nucleotide

resolution for the mapping of the break sites. In **SSB-Seq**, genomic DNA was extracted and the SSBs were tagged with digoxigenin-labelled dUTP using a nick translation reaction by DNA Polymerase I. The DNA was then sonicated, and the labelled DNA fragments were enriched with an anti-digoxigenin antibody, purified and then subjected to library preparation followed by sequencing [155,159]. Following the development of SSB-Seq, Degenerate and Enrichment Nick Translations followed by Sequencing (**DENT-Seq**) was designed to allow the detection of the nick sites at a single-nucleotide resolution [160]. It relies on nick-translation in the presence of degenerate nucleotides (dPTP and dKTP) which results in the formation of a specific mutational spectra in close proximity to the SSBs and a biotin tag to allow for the enrichment of the DNA fragments. A combination of the mutational signal and the reads adjacent to the nicks permit the precise determination of the nick site at a single-nucleotide resolution while maintaining strand-specificity.

5.4. Mapping Repair Sites

Apart from OGG1-AP-Seq, none of the above-mentioned techniques were employed in neuronal cells until two techniques that are quite similar in principle were reported in post-mitotic neurons derived from induced pluripotent stem cells were developed, namely, **Repair- Seq** [39] and synthesis associated with repair sequencing (**SAR-Seq**) [40]**.** Both techniques relied on the incorporation of EdU in the non-replicating neuronal cells at sites of DNA synthesis to label the regions in the genome where DNA repair is taking place. The EdU was then biotinylated and the sonicated DNA fragments were then purified using streptavidin-coated magnetic beads followed by library preparation and sequencing. An adaptation of the END-Seq that was reported for mapping DSBs [161] was developed by Wu *et al.* whereby they utilised S1 nuclease to convert the SSBs in the genome of the post-mitotic neurons to DSBs and was referred to as **ddN S1 END-Seq**. Since the repair of the SSBs was found to be rapid, dideoxynucleotides were incorporated prior to the S1 nuclease digestion step to prevent further DNA synthesis and to be able to accurately map these regions. The DNA ends were then ligated to biotinylated adaptors and purified with streptavidin-coated magnetic beads prior to sequencing [40].

6. Single-cell DNA Sequencing

Significant progress has been made in the last decade to analyse the genome of single cells. Single-cell tools that rely on examining the RNA and protein expression profile have enhanced our understanding of cellular heterogeneity. However, numerous fundamental biological questions necessitate the development of single-cell DNA sequencing (scDNA-Seq) tools. It is characterised by three main features: fidelity, co-presence and phenotypic association [162]. Fidelity refers to the ability of scDNA-Seq to overcome the limitations of bulk DNA sequencing tools that rely on sequencing thousands to millions of cells, at a genome-wide scale to detect features in the DNA present in a small subset of cells. Despite advances in increasing sequencing depth and coverage, bulk DNA sequencing methods cannot distinguish between mosaic features that occur at a frequency below 0.5% and sequencing errors, which are otherwise distinguishable with scDNA-Seq [163,164]. The co-presence feature of scDNA-Seq allows associating the different mosaic DNA features to the same cells or a subset of cells that they were present in. This information would be lost when samples are homogenised in bulk DNA-seq methods. This feature gives rise to the third capability of scDNA-Seq techniques which is phenotypic association, whereby simultaneous single-cell phenotyping whether with transcriptomic, proteomic or histological profiling is conducted to correlate between the DNA feature and the cell type and/or state where it was present. While this may decrease the resolution of the scDNA-Seq [165], several multi-omic scDNA-Seq tools are currently being developed for numerous applications [166,167].

The previously discussed DNA mapping tools all relied on bulk DNA sequencing. To date, there are no scDNA-Seq tools that have been reported to map sites of DNA breaks. However, single-cell profiling of 5mC has been developed which covers around 5–40% of the haploid genome [168]. Cell type-specific and dynamic methylation information can be obtained, and this allows the classification of cell types and transient cell populations. This has been employed to understand embryonic and brain development, hematopoiesis and cancer [162,169]. Profiling of the methylome at the single-cell level using single-nucleus methylcytosine sequencing (snmC-Seq) and single-cell combinatorial indexing for methylation analysis (sci-MET) opened the door to distinguishing between different brain cell types and identifying regulatory elements that are distinct for specific cells [170]. With the continued advancement in the tools developed for mapping SSBs using bulk-sequencing, the need for understanding the heterogeneity across the different cell types necessitates the development of scDNA-Seq tools that would allow the labelling and capturing of either the SSBs directly or their repair intermediates, in a similar fashion to that developed to capture the 5mC.

Despite all the aforementioned advantages of scDNA-Seq, they also still have several limitations. Similar to bulk DNA sequencing techniques, only a minor portion of the input DNA is captured and sequenced, which requires DNA amplification prior to sequencing. This can introduce errors that eventually complicates the downstream analysis [171].

7. Concluding remarks

Recent advances made in the tools available to study the extent of DNA damage and the mechanisms of SSBR, particularly in neuronal cells has allowed increased understanding of how genome integrity is maintained in neurons. Further research is still required to advance our understanding of the nature of SSBs, their genome-wide distribution and their functional consequences to develop new therapeutic routes for neurological disorders and also harness this knowledge to develop new anti-cancer therapeutics. Increasing the application of the genome-wide, nucleotide-resolution DNA mapping tools to investigate neurodegenerative disorders offers insights on the underlying disease mechanisms as it allows detection of the specific locations of DNA damage accumulation. Moreover, in conjunction with WES and WGS tools, they can allow drawing associations between the presence of these breaks and the genetic mutations, which modify disease onset, risk and severity.

CRediT authorship contribution statement

Antar Sarah: Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Abugable Arwa A:** Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **El-Khamisy Sherif F:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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