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“Liver let die: oxidative DNA damage and hepatotropic viruses”

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SUMMARY

Chronic infections by the hepatotropic viruses hepatitis B virus (HBV) and hepatitis C virus (HCV) are major risk factors for the development of hepatocellular carcinoma (HCC). It is estimated that more than 700,000 individuals per year die from hepatocellular carcinoma, and around 80% of HCC is attributable to HBV or HCV infection. Despite the clear clinical importance of virus-associated HCC, the underlying molecular mechanisms remain largely elusive.

Oxidative stress, in particular DNA lesions associated with oxidative damage, play a major contributory role in carcinogenesis, and are strongly linked to the development of many cancers, including HCC. A large body of evidence demonstrates that both HBV and HCV induce hepatic oxidative stress, with increased oxidative DNA damage being observed both in infected individuals and in murine models of infection.

Here, we review the impact of HBV and HCV on the incidence and repair of oxidative DNA damage. We begin by giving a brief overview of oxidative stress and the repair of DNA lesions induced by oxidative stress. We then review in detail the evidence surrounding the mechanisms by which both viruses stimulate oxidative stress, before focusing on how the viral proteins themselves may perturb the cellular response to oxidative DNA damage, impacting upon genome stability and thus hepatocarcinogenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is an increasing global health problem, accounting for more than 90% of primary liver tumours. Worldwide, HCC is the third cause of cancer-related death, responsible for 700,000 deaths per year (Ferlay *et al.*, 2010), and the sixth most common cancer. Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are major risk factors for the onset and progression of HCC (El-Serag, 2002; Simonetti *et al.*, 1992). Globally, up to 80% of hepatocellular carcinoma is attributable to HBV or HCV infection (Perz *et al.*, 2006). The underlying mechanisms remain unclear, although an increasing body of evidence suggests that the viral proteins themselves may contribute directly to tumourigenesis.

Timely and precise repair of DNA lesions is crucial for the maintenance of genome stability. Since genomic instability is a characteristic of all tumours, DNA damage recognition and repair processes represent an important barrier to the initiation and progression of tumourigenesis. Cells in multicellular organisms are continuously exposed to DNA damage arising from a variety of endogenous and exogenous sources. These include reactive oxygen species, ultraviolet light, background radiation, and environmental mutagens. In particular, oxidative stress and elevated reactive oxygen species (ROS) levels are linked with number of human diseases. ROS that accumulate as a result of oxidative stress can directly react with DNA molecules to produce a variety of oxidative DNA lesions, whose repair by components of the base excision repair (BER) and nucleotide excision repair (NER) pathways is crucial in maintaining genome stability.

Oxidative DNA damage may play a pivotal role in hepatocarcinogenesis associated with chronic viral infection. A growing number of publications have concluded that chronic HBV and HCV infection correlates with an increased incidence of oxidative DNA damage (Bolukbas *et al.*, 2005; Demirdag *et al.*, 2003; Farinati *et al.*, 2007a; Fujita *et al.*, 2008; Machida *et al.*, 2006; Nishina *et al.*, 2008). Whilst it is clear that ROS are continuously generated during chronic inflammation over the course of chronic viral infection, increasing evidence suggests that the viral proteins of HBV and HCV may themselves contribute to a state of chronic oxidative stress in infected hepatocytes.

In this review, we will summarise the current knowledge surrounding oxidative stress and DNA damage during hepatotropic viral infection. We will compare and contrast the mechanisms by which the proteins encoded by HBV and HCV induce oxidative stress, before providing an overview of the impact of these viral proteins on oxidative DNA damage and repair, and briefly examining the ultimate consequences on the development of HCC.

OXIDATIVE STRESS

Reactive oxygen species may arise by exposure to exogenous agents such as ionizing radiation or drugs, or may be generated from endogenous sources such as metabolism, apoptosis or inflammation. Oxidative stress represents a shifting of the balance between oxidants (such as ROS) and the cellular antioxidant response, leading to potential cellular damage and contributing to disease. Cells are able to withstand a relatively low level of oxidative damage. However, sustained oxidative stress, arising through the

increased presence of radicals and ROS, or by a lack of antioxidant capacity within the cell, will engender increased damage to lipids, proteins and DNA. A detailed description of the repair of oxidative DNA damage is covered in a subsequent section. ROS are primarily generated in the mitochondria as by-products of cellular metabolism, through electron leakage from the mitochondrial electron transport chain. However, they also play a key role as second messengers in cellular signalling. ROS-induced damage may influence pathway signalling, gene expression, cell cycle, metabolism, and apoptosis. Oxidative stress may also activate oncogenic signalling pathways, ultimately contributing to cellular transformation (Hussain *et al.*, 2003).

Viral infection and oxidative stress: non-specific inflammation

Although this review focuses on how the HBV and HCV viral proteins induce oxidative stress, it is clear that chronic infection by either virus triggers a non-specific immune-mediated inflammation (hepatitis), which is innately linked to oxidative stress. During acute liver injury and hepatic inflammation, ROS are generated by both neutrophils and Kupffer cells (reviewed by (Jaeschke, 2011)), as the principal toxic mediators to induce cell death. Since these cells exist in close proximity to hepatocytes, some ROS (i.e. H_2O_2) are able to diffuse into hepatocytes, although the plasma membrane represents a barrier to the free diffusion of $O_2^{\cdot -}$. Membrane superoxide dismutases are able to convert these $O_2^{\cdot -}$ anions to H_2O_2 in the extracellular space, which are then able to cross membranes and elicit intracellular signalling (Fisher, 2009). Thus, these ROS will enhance and amplify the intracellular effects engendered by the viral proteins themselves, and will also affect neighbouring

(uninfected) hepatocytes (Jaeschke, 2011), although they may also serve to activate intracellular antioxidant defences. Moreover, mitochondrial reactive oxygen species are able to drive proinflammatory cytokine production (Naik & Dixit, 2011), further exacerbating both inflammation and the production of ROS.

OXIDATIVE DNA DAMAGE AND REPAIR

ROS may directly interact with DNA to induce oxidative DNA damage. Oxidative DNA lesions may comprise abasic sites, deaminated or adducted bases, and single stranded DNA breaks (SSBs). These lesions include thymine and thymidine glycol, 5-hydroxylmethyluracil, and 8-hydroxy-deoxyguanosine (8-OHdG). The latter represents the most widely studied of oxidative DNA lesions, and is used as a robust marker of oxidative DNA damage. The accumulation of oxidative DNA lesions is considered mutagenic, and a significant contributory factor to human disease (reviewed by (Sedelnikova *et al.*, 2010)).

The majority of oxidative DNA lesions are repaired by components of the base excision repair pathway (BER), and to a lesser extent, by nucleotide excision repair (NER). The rate of transient oxidative DNA damage is typically balanced by its rate of repair, but chronic oxidative stress may result in permanent genetic damage. Since there are multiple, overlapping pathways for the repair of oxidative DNA damage, perturbation of one component of these pathways is expected to slow but not abolish repair.

Base excision repair (Figure 1) is initiated by a DNA glycosylase that recognizes and removes the damaged base, generating either a SSB or an abasic site which requires further processing by an AP endonuclease (APE1). The resultant SSB may be repaired by two alternate pathways: short-patch BER (Figure 1, bottom right), involving Pol β and the DNA ligase III/XRCC1 complex, which inserts a single nucleotide; or long-patch BER (Figure 1, bottom left), which replaces 2-12 nucleotides, and is dependent upon PCNA and Flap Endonuclease 1 (Fen1), and may involve several polymerases (Pol β, δ and ε). The factors governing the choice of long- or short-patch BER remain unclear.

The nucleotide excision repair (NER) pathway (Figure 2), which is generally associated with repair of bulky, helix-distorting adducts, is also able to repair oxidative DNA lesions, albeit to a lesser extent. Detection of bulky lesions, whether in actively transcribed regions (right, panel) or not (left panel) (see Figure 2 for details), leads to recruitment of the TFIIH complex to sites of damage. Members of the TFIIH complex facilitates helix unwinding and excision of the strand containing the adduct, and repair is completed by several proteins which complete gap-filling/ligation. NER is capable of removing all of the oxidative lesions induced by ROS (Kuraoka *et al.*, 2000; Reardon *et al.*, 1997), as NER initiating factors (CSA, CSB, XPC and XPE) rapidly bind to oxidative lesions (Menoni *et al.*, 2012). However, the overall contribution of NER to the repair of oxidative lesions remains to be determined, and it is likely that NER acts as a backup pathway to BER. Interestingly, oxidative stress generated by activated neutrophils, and potentially by kuppfer cells, results in a reduction in nucleotide excision repair

(NER) capacity, suggesting that inflammation may non-specifically reduce NER efficiency (Gungor *et al.*, 2007).

In some circumstances oxidative DNA damage may result in double strand break (DSB) formation, especially if lesions are clustered together on opposite strands. Although DSBs will arise at a lower frequency to SSBs, their rapid repair is crucial to maintaining genetic stability. Repair of DSBs involves either recombination using homologous sequences from undamaged sister chromatids (homologous recombination; HR), or non-homologous end-joining (NHEJ), which can result in small deletions. For a comprehensive view on DSB repair, the reader is directed to an excellent recent review by (Chapman *et al.*, 2012).

In addition, a growing body of literature suggests that cellular tumour suppressors, including those involved in repair of other types of DNA lesions, may play a vital role in regulating oxidative stress. p53 is activated in response to oxidative stress, and is thought to play an antioxidant role in such circumstances. Regulation of both pro- and anti-oxidant genes by p53 is thought to be crucial in regulating the cellular response and cell fate to acute oxidative stress (reviewed in (Vurusaner *et al.*, 2012)). Moreover, the breast cancer susceptibility genes BRCA1 and BRCA2, involved in DNA repair and cell cycle checkpoint progression, also play caretaker roles against oxidative stress. In particular, in response to oxidative stress, BRCA1 upregulates the expression of numerous antioxidant genes (e.g. glutathione-S-transferase, alcohol dehydrogenases) (Bae *et al.*, 2004). BRCA1 over-expression protects against oxidising agents, maintaining the cellular redox balance in the face of oxidative stress. Similarly, BRCA1 may enhance the activity of the antioxidant

transcription factor Nrf2 (Ishikawa *et al.*, 2005). In addition to its well-characterised role in the repair of DSBs by promoting HR, BRCA2 is also critical for the repair of oxidative lesions. Specifically, BRCA2 (as well as BRCA1) is required for the transcription-coupled repair of 8-OHdG lesions, crucial for the prevention of transcription stalling and subsequent mutagenesis (Bae *et al.*, 2004; Le Page *et al.*, 2000).

Thus, members of multiple, overlapping and functionally diverse pathways (namely BER, NER and DSB repair) are required for the effective repair of oxidative DNA lesions resulting from oxidative stress.

HBV, OXIDATIVE STRESS AND DNA DAMAGE

HBV infection and oxidative stress

HBV is one of several closely-related DNA viruses within the family *Hepadnaviridiae*. The viral genome encodes a small number of gene products: a reverse transcriptase and DNA polymerase (pol); a capsid protein (core); envelope proteins L, M and S; and a multifunctional protein (X) involved in replication, oncogenesis, and a myriad of other cellular metabolic dysfunctions. Replication of HBV is complex and proceeds via the reverse transcription of genome-length RNA; thus, HBV is classified as a dsDNA retrovirus (group VII).

Chronic HBV infection is a major etiological factor for HCC: the risk of HCC development in chronic HBV carriers is more than 100-fold greater than in uninfected individuals (Ito *et al.*, 2010). The vast majority of new cases of HBV-associated HCC occur in developing countries, especially sub-Saharan

Africa and Southeast Asia. Although HCC generally occurs in cirrhotic livers, HBV is also able to transform hepatocytes in the absence of chronic inflammation and cirrhosis (Brechot, 2004). During chronic infection, fragments of HBV DNA may integrate into the host genome, preferentially into chromosome 17, creating mutations. These integrated fragments often encode the X protein of HBV (HBx) or truncated preS proteins, the integration of which often correlates with hepatocarcinogenesis (Ding *et al.*, 2012) .

Several groups have shown that HBV infection is associated with oxidative stress in chronically-infected individuals (Bolukbas *et al.*, 2005; Demirdag *et al.*, 2003). Both lipid peroxidation and oxidative DNA damage, markers of oxidative stress, are elevated in patients infected with HBV. Indeed, patients with chronic HBV infection exhibit increased 8-OHdG accumulation (Fujita *et al.*, 2008). Furthermore, *in vitro* HBV replication in hepatoma cell lines (specifically HepAD38 cells) induces oxidative stress (Severi *et al.*, 2006).

A direct role for HBV-encoded proteins in oxidative stress

In addition to non-specific oxidative stress generated by local inflammation in response to viral infection, increasing evidence suggests that the HBV proteins directly regulate cellular ROS production (Figure 3 upper panel), and deleteriously alter intracellular antioxidant defences in HBV-infected cells, causing apoptosis and extensive liver damage and thus engendering accelerated hepatocellular renewal. However, the consequences of these interactions and their impact are yet to be fully understood.

Over a decade ago, HBx was shown to localise to mitochondria, decreasing mitochondrial membrane potential, and increasing both cytochrome c release and apoptosis (Takada *et al.*, 1999). Moreover, transgenic mice expressing the HBV proteins (including HBx) also display elevated hepatic oxidative stress levels compared to nontransgenic controls, with a concurrent increase in oxidative DNA damage (Hagen *et al.*, 1994). These findings were extended to demonstrate that ROS scavengers were able to inhibit HBx-mediated mitochondrial membrane depolarisation and subsequent apoptosis (Shirakata & Koike, 2003). In agreement, Lee and colleagues demonstrated that HBx alters mitochondrial membrane potential, perturbs mitochondrial electron transport, affects hepatocyte metabolism and increases cellular ROS production (Lee *et al.*, 2004). HBx has accordingly been reported to sensitise cells to apoptosis induced by oxidative stress, mainly through loss of the anti-apoptotic protein Mcl-1 (Hu *et al.*, 2011). In general agreement, HBx was shown to induce apoptosis through regulation of Bcl-XL and Bax (Kim *et al.*, 2008; Miao *et al.*, 2006). It is therefore clear that expression of HBx stimulates intracellular ROS production and impacts upon apoptosis.

The HBV surface antigen (HBsAg) and associated PreS region have also been associated with oxidative stress. Expression of truncated mutants of the HBV PreS/S polypeptide in hepatoma (Huh7) cells induced production of ROS via ER-stress pathways, resulting in oxidative DNA damage (Hsieh *et al.*, 2004; Wang *et al.*, 2005), although these results might be strongly influenced by non-specific ER stress triggered by high levels of HBV protein expression. Hsieh *et al.* (2004) also showed that transgenic mice expressing

these mutants exhibited elevated oxidative DNA damage and up-regulated expression of Ogg1, the DNA glycosylase mainly responsible for the repair of 8-OHdG lesions. Such oxidative damage may play an important role in hepatocarcinogenesis associated with HBV infections, illustrated by elevated nodular profileration and increased tumour development in mice expressing PreS mutants (Wang *et al.*, 2005). In contradiction, examination of a small cohort of patients (n=38) failed to reveal a link between PreS mutation and increased oxidative stress in HBV-infected patients, although HBV infection was again linked to elevated levels of 8-OHdG and Ogg1 expression (Gwak *et al.*, 2008).

Given these data, one would expect that intracellular antioxidant defences would be activated in the presence of the HBV proteins. Accordingly, HBV upregulates the expression of cytoprotective genes containing antioxidant response elements (AREs), both *in vitro* and in HBV-infected liver tissues (Schaedler *et al.*, 2010). However, Schaedler and co-workers demonstrated that this upregulation was independent of ROS, and, in contrast to other studies, suggested that this upregulation might confer survival benefits upon HBV-infected cells, allowing them to survive the sustained oxidative stress found in the infected liver. The discrepancy of Schaedler's findings with other published data (described above) emphasises the difficulty of studying changes in the redox balance occurring during a natural HBV infection, and questions the relevance of using HBV models usually devoid of an immune response.

These studies demonstrate that HBV infection induces extensive oxidative stress and activates intracellular oxidative repair pathways, and suggest that HBx expression (and perhaps that of PreS) is, in part, responsible. However, certain aspects require further clarification, and may be addressed by controlling the level of HBV protein expression to more closely resemble that observed in infected individuals and by validating the results in the context of the whole HBV genome. Although antioxidant treatments seem a promising avenue of therapeutic research for HBV infection, their efficacy is as yet unknown.

HBV and oxidative DNA damage

As detailed above, HBV infection, HBx and PreS induce oxidative stress, which culminates in increased hepatic oxidative DNA damage, with increased levels of 8-OHdG found in HBV-infected patients, in transgenic mice expressing either pre-S mutants or HBx, and in hepatoma cells expressing HBx (Fujita *et al.*, 2008; Gwak *et al.*, 2008; Hagen *et al.*, 1994). A number of studies have suggested that the HBV-encoded proteins, in addition to their role in inducing oxidative stress, may also inhibit cellular DNA repair pathways. Despite the conflictual nature of some of these reports, we attempt to summarise the current state-of-knowledge below.

HBV and the repair of oxidative DNA lesions

A growing body of literature suggests that the HBV proteins may alter the repair of DNA lesions, including oxidative DNA damage (Figure 4). There are, however, conflicting accounts of the effect of HBV on the base excision

repair pathway. Gwak and colleagues (2008) demonstrated that expression of Ogg1 was increased in hepatic tissues from HBV-infected patients, regardless of whether they originated from tumoural or non-tumoural regions (Gwak *et al.*, 2008). In agreement, expression of Pre-S mutants induced Ogg1 expression *in vitro* and in murine models (Hsieh *et al.*, 2004). These studies suggest that infected cells respond to HBV-induced oxidative stress by upregulating cellular glycosylases involved in BER, activating DNA repair (Figure 4, left panel). However, recent studies have demonstrated that HBx inhibits BER initiated by Thymidine glycolsylase (Tdg) (van de Klundert *et al.*, 2012). Whilst this study examined the effects of HBx on BER induced by Tdg *in vitro*, the effect of HBx on accumulation of 8-OHdG remains unstudied, as Tdg acts on G/T mismatches and not on 8-OHdG.

A considerable number of studies report that HBV inhibits NER (Figure 4, right panel). This has in large part been attributed to the HBx protein. Cells expressing HBx render hepatoma cells more sensitive to UV-C, and HBx inhibits global NER in a host-cell reactivation assay (Jia *et al.*, 1999; Lee *et al.*, 2005; Mathonnet *et al.*, 2004). Moreover, HBx inhibits the expression of the TFIIH subunits XPB and XPD, and interacts with and inhibits the function of TFIIH, leading to increased UV-C sensitivity of cells expressing HBx (Jaitovich-Groisman *et al.*, 2001; Qadri *et al.*, 2011). In addition to inhibiting global NER, HBx may also impede NER within transcriptionally active genes (transcription-coupled NER) (Mathonnet *et al.*, 2004). However, since the impact of NER on repair of oxidative DNA damage is unclear (as mentioned above), the impact of HBx-induced NER inhibition upon oxidative damage during HBV infection remains to be studied.

As outlined previously, the p53 tumour suppressor is also involved in the response to oxidative DNA damage. Intriguingly, HBV has been shown to perturb p53 function (Figure 4, lower panel). The interaction between HBx and p53 is well established (Chung *et al.*, 2003; Lin *et al.*, 1997; Wang *et al.*, 1994; Yun *et al.*, 2000), and it seems that this alters the binding of p53 to p53-responsive elements, resulting in aberrant gene expression (Chan *et al.*, 2013). Given these data, one may imagine that interactions between HBx and p53 would negatively impact the repair of oxidative DNA lesions.

In addition to the specific mechanisms detailed above, the frequent random integration of HBV DNA into genes encoding DNA repair and checkpoint proteins (e.g. Wrn, hTERT, Rad17 (Toh *et al.*, 2013)) may also serve to perturb the cellular response to DNA damage. Furthermore, oxidative stress may increase the rate of HBV fragment integration *in vitro* (Dandri *et al.*, 2002). In agreement with this latter remark, this study also suggests that the SSB repair factor PARP-1 may play a protective role against HBV DNA integration, presumably by rapidly repairing breaks which would otherwise favour integration of exogenous DNA.

From these studies, it is apparent that HBV proteins (notably HBx) interact with components of both BER and NER pathways, implying that the function of these pathways in the repair of oxidative lesions may be perturbed during HBV infection, ultimately contributing to the elevated levels of 8-OHdG observed in HBV-infected patients.

HCV, OXIDATIVE STRESS AND DNA DAMAGE

HCV infection and oxidative stress

HCV is a member of the Flaviviridae family of enveloped, positive-single strand RNA viruses. The positive-sense RNA genome acts as a template for viral genome replication, and is also translated into a polyprotein which is cleaved by both host and virally-encoded proteases to generate 10 proteins: the capsid (core) protein; envelope glycoproteins E1 and E2; the p7 ion channel; and 6 non-structural proteins (NS2-NS5B).

Chronic infection by HCV is a major risk factor for the onset and progression of HCC (El-Serag, 2002; Saito *et al.*, 1990). Chronic HCV infection is responsible for approximately a third of HCCs, and has become the principal cause of HCC in most industrialized areas. Cirrhosis appears to be an important nonspecific determinant of HCC occurrence in HCV-infected patients, and very few cases of HCC without cirrhosis have been reported in these individuals (Simonetti *et al.*, 1992).

Oxidative stress and elevated reactive oxygen species (ROS) production are frequently observed during chronic HCV infection. As with HBV, they are thought to play a central role in HCV-associated HCC. Elevated levels of oxidative DNA damage (namely 8-OHdG), 4-hydroxynoneal, and increased lipid peroxidation have been observed in HCV-infected patients (Farinati *et al.*, 2007b; Kato *et al.*, 2001; Konishi *et al.*, 2006; Mahmood *et al.*, 2004; Shackel *et al.*, 2002). HCV infection has been associated with an almost fourfold increase in 8-OHdG levels compared to uninfected controls, and HCV infection induces higher levels of oxidative stress than does HBV (Farinati *et al.,* 2007*b*). In line with this, antioxidant therapies are able to

alleviate to some extent the level of hepatic damage in chronic HCV infection, although there is no clear evidence that antioxidants alone are useful therapeutic agents in these patients (reviewed by (Singal *et al.*, 2011)).

HCV proteins directly induce oxidative stress

A considerable body of experimental evidence demonstrates a direct role for the HCV proteins in inducing oxidative stress (Figure 3 lower panel), from a variety of different models (reviewed in (Ivanov *et al.*, 2013; Simula & De Re, 2010)). Indeed, FL-N/35 mice transgenic for the entire ORF of HCV (thus expressing the entire complement of viral proteins) exhibit elevated ROS levels (Higgs *et al.*, 2012; Nishina *et al.*, 2008), and expression of the HCV polyprotein induced ROS production (Piccoli *et al.*, 2007). Expression of several individual HCV proteins have also been linked with overproduction of ROS, as detailed below. Although a causative role of HCV-associated oxidative stress in the development of HCC in murine models has yet to be shown, several studies suggest that oxidative stress induced by the HCV proteins may trigger genomic instability, eventually leading to HCC.

HCV core and oxidative stress

Although core (and other HCV proteins) primarily localises to the endoplasmic reticulum, it also associates with mitochondria (Korenaga *et al.*, 2005). Several studies have demonstrated that expression of the HCV core protein induces oxidative stress, in a variety of experimental systems. Mice transgenic for core, or for core, E1 and E2, demonstrate increased oxidative stress and enhanced ROS production (reviewed in (Wang & Weinman, 2006,

2013)). Moriya and colleagues showed that core induced a shift in the hepatic oxidant/antioxidant state, leading to mitochondrial damage and possibly contributing to the onset of HCC in their core-transgenic mice (Moriya *et al.*, 2001). Korenaga *et al.* demonstrated that core protein associates with mitochondria and remains associated with the mitochondrial outer membrane in core-E1–E2 transgenic mice, leading to a disruption of mitochondrial electron transport complex 1, the generation of ROS and oxidation of the glutathione pool (Korenaga *et al.*, 2005). Thus it seems that expression of the HCV core protein provokes mitochondrial dysfunction, leading to oxidative stress, coupled with activation of components of the intracellular superoxide scavenging system, including catalase and glutathione (Koike, 2007).

Similar results have been obtained from study of HCV-infected cell cultures, or of tumoural cells expressing core. Expression of core under the control of a tetracycline-regulated promoter induced oxidative stress and lipid peroxidation in HeLa and Huh7 cells (Okuda *et al.*, 2002). Such expression efficiently induced a cellular antioxidant response, and increased expression of antioxidant genes (Li *et al.*, 2002). Expression of core (as well as E1 and NS3) in Huh7 cells leads to an increase in reactive oxygen species (ROS), and a decrease in mitochondrial permeability (Machida *et al.*, 2006; Pal *et al.*, 2010). Overexpression of core in Huh7 hepatoma cells also increased mitochondrial Ca^{2+} uptake, perhaps explaining the increased cytochrome c release by mitochondria in response to Ca^{2+} in the presence of core (Li *et al.*, 2002). Similar observations have also been made *in vivo* in core-transgenic mice (Korenaga *et al.*, 2005). It is therefore clear that increased mitochondrial uptake of Ca^{2+} induced by HCV core stimulates ROS production, leading to

435 the modification of electron transport components, and inducing cellular
436 oxidative stress.

437 *The HCV non-structural proteins and oxidative stress*

438 Numerous studies have linked the HCV non-structural proteins,
439 especially NS3 and NS5A, to oxidative stress. The vast majority of these have
440 involved expression of a single viral protein in isolation, although expression
441 of the non-structural HCV proteins also induces ROS (Boudreau *et al.*, 2009;
442 Rivas-Estilla *et al.*, 2012). Expression of the NS3 protease enhances ROS
443 production in hepatoma cells (Machida *et al.*, 2006; Pal *et al.*, 2010). Similarly,
444 NS5A expression in hepatoma cells induces mitochondrial ROS production,
445 Ca^{2+} release and activates downstream kinases (Gong *et al.*, 2001; Machida
446 *et al.*, 2006; Pal *et al.*, 2010). Overproduction of ROS has also been observed
447 in NS5A-transgenic mice (Wang *et al.*, 2009).

448 Expression of both NS4B and NS5A also induce ER stress (Gong *et*
449 *al.*, 2001; Li *et al.*, 2009). In agreement, Asselah *et al.* (2010) reported that ER
450 stress markers were activated in biopsies from HCV-infected patients.
451 However, transgenic mice expressing low levels of the HCV proteins do not
452 exhibit ER stress (Lerat *et al.*, 2009). Taken together, these data suggest that
453 ROS production through HCV-induced ER stress might be linked to HCV
454 infection rather than HCV protein expression (Asselah *et al.*, 2010).

455 Recently, we demonstrated both in HCV patients' biopsies and in the
456 FL-N/35 mouse lineage (transgenic for the entire ORF of an HCV genotype 1b
457 isolate, and expressing the full repertoire of HCV proteins at a low level in the

liver), that hepatic c-Myc expression is elevated, increasing ROS production, and that NS5A is involved in this process (Higgs *et al.*, 2012). It is well established that expression of the proto-oncogene c-Myc can induce ROS production (Dang *et al.*, 2005; Graves *et al.*, 2009; Karlsson *et al.*, 2003; Ray *et al.*, 2006; Vafa *et al.*, 2002). We demonstrated that the increased ROS production induced by c-Myc is, at least in part, associated with transcriptional deregulation of cytochrome P2C9 (CYP2C9), a component of the mitochondrial respiratory chain cytochrome P450 (CYP450).

Limited evidence also suggests that the HCV non-structural proteins, together with core, repress hepcidin expression in a ROS-dependent manner, altering iron metabolism (Miura *et al.*, 2008). Importantly, NS5A-induced ROS production may also impact on glucose production, since an NS5A-dependent decrease in the phosphorylation of the transcription factor Foxo1 and subsequently increased glucose production was decreased by *N*-acetyl cysteine (Deng *et al.*, 2011). It is probable, therefore, that oxidative stress induced by HCV impacts on several other HCV-associated pathologies, including diabetes.

HCV and ROS detoxification

Since oxidative stress is a hallmark of HCV infected cells, the excess ROS produced are clearly inefficiently detoxified. In an attempt to examine the impact of HCV on ROS detoxification pathways, several publications have examined the impact of core and the other HCV proteins on the Nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway, which is of crucial importance in the regulation of intracellular oxidation. When associated with

small proteins (sMaf), Nrf2 positively regulates the transcription of genes containing antioxidant response elements (ARE) in their promoters. (Hirotsu *et al.*, 2012). Recently, Carvajal-Yepes *et al.* showed that, in hepatoma cells harbouring JFH1 replicons, HCV core triggers the delocalization of sMaf proteins from the nucleus to the endoplasmic reticulum where they bind HCV NS3. This ultimately restrains Nrf2 from entering into the nucleus and thereby inhibits the induction of Nrf2/ARE-regulated genes, thus resulting in lower expression of cytoprotective genes (Carvajal-Yepes *et al.*, 2011). In contradiction, other published results showed that the overexpression of core, NS3 and NS5A enhances Nrf2 expression (Ivanov *et al.,* 2011), and that the Nrf2/ARE antioxidant pathway is activated in cells infected with HCV *in vitro*, providing an anti-apoptotic protection mechanism (Burdette *et al.*, 2010). Further study is therefore necessary to determine how HCV impacts on the Nrf2/ARE pathway.

Collectively, these studies clearly demonstrate that several of the HCV proteins (notably core, NS3 and NS5A) induce oxidative stress through a variety of pathways, thus increasing the likelihood of oxidative DNA damage. ROS-induced apoptosis and oxidative DNA damage may both contribute to carcinogenesis, by on the one hand increasing compensatory hepatocellular proliferation to create a mitogenic and mutagenic environment, whilst on the other hand inducing further heritable genetic damage.

HCV and oxidative DNA damage

As described above, the role of HCV and the viral proteins in inducing oxidative stress is well established, and results in the increased 8-OHdG

levels found both in HCV-infected patients, and in HCV-infected cells *in vitro*. We have also observed increased levels of single-stranded DNA damage in mice transgenic for the entire complement of HCV proteins (Higgs *et al.*, 2012). In a manner similar to HBx, several studies have indicated that, in addition to their role in inducing oxidative stress, HCV core, NS3 and NS5A may also have deleterious consequences on the repair of oxidative lesions (amongst other types of DNA damage) (Figure 4).

HCV and the repair of oxidative DNA lesions

There is a relative paucity of studies examining the impact of HCV on the DNA damage response. Moreover, given the importance of HCV in inducing oxidative DNA damage, there are surprisingly few reports concerning HCV and BER. HCV core has been suggested to inhibit the DNA glycosylase activity responsible for excision of 8-OHdG, although the mechanisms remain unclear, since core fails to interact with or perturb the expression of the BER components (Machida *et al.*, 2010a). Pal and colleagues found that expression of the Neil1 DNA glycosylase (which has marginal activity towards 8-OHdG) was perturbed in HCV-infected cell cultures and biopsies from HCV-infected patients, with a concomitant reduction in Neil1-specific glycosylase activity, and increased 8-OHdG levels (Pal *et al.*, 2010). Together, these two reports provide the only preliminary evidence that HCV may have deleterious consequences on BER.

In a similar manner, there are limited reports that HCV deregulates NER. We have shown that hepatocytes of mice expressing the HCV proteins exhibit reduced NER repair by means of a plasmid reactivation assay (Higgs

et al., 2010). Although unconfirmed, it is possible that the reduced expression of Gadd45β, a p53-response gene linked to NER, plays a contributory role. Intriguingly, these mice also develop hepatic steatosis (as do many HCV-infected individuals), which, since NER is diminished in steatotic livers (Schults *et al.*, 2012), may suggest that HCV-induced steatosis contributes to the observed decrease in NER.

The Ataxia telangiectasia mutated (ATM) kinase plays a key role in the cellular response to DSBs, and also plays an important role in the response to oxidative DNA damage (reviewed in (Chen *et al.*, 2012)). ATM is necessary for repair of ROS-induced DSBs in non-replicating cells (Guo *et al.*, 2010; Woodbine *et al.*, 2011). Previous reports have suggested that HCV interacts with ATM, and perturbs its function (Ariumi *et al.*, 2008; Machida *et al.*, 2010b). Thus, it is tempting to speculate that HCV may also disrupt the repair of DSBs arising from both clustered oxidative lesions as well as exogenous sources.

A substantial body of evidence demonstrates that various HCV proteins alter p53 signalling and function *in vitro* (Alisi *et al.*, 2003; Deng *et al.*, 2006; Kao *et al.*, 2004; Kwun & Jang, 2003; Lan *et al.*, 2002; Smirnova *et al.*, 2006; Yamanaka *et al.*, 2002). From these studies, it is apparent that core, NS3 and NS5A interact with p53, and this interaction seems to inhibit p53 activity, although the reported consequences are sometimes contradictory. However, these findings are yet to be repeated *in vivo* in the various transgenic or humanised murine models currently available, and these observations may be a consequence of the use of hepatoma cells and/or of over-expressed

proteins in the majority of these studies. However, it is clear that the impact of the HCV proteins on p53 function, especially during DNA repair, is worth further investigation.

From these data, it is apparent that HCV proteins interact with several DNA repair factors, and may therefore contribute to the elevated levels of 8-OHdG and SSBs observed in HCV-infected individuals by negatively regulating the repair of oxidative DNA lesions. Clearly future work must focus on whether HCV perturbs these processes, and on the precise mechanisms involved.

CONCLUSIONS AND FUTURE PERSPECTIVES

The causative link between HBV and HCV infection and oxidative DNA damage is well established. Although a number of mechanistic insights concerning the ability of virus-infected cells to repair such damage have been provided from *in vitro* and *in vivo* models, it is still not possible to present a final picture of the effect of the HBV or HCV viral proteins on such repair processes. Therefore, at present it is probably easier to list the areas surrounding these viruses and DNA damage which remain to be studied, rather than those that have been studied.

Clearly, further detailed studies need to be carried out on the ability of cells expressing the viral HBV or HCV proteins to repair oxidative DNA lesions, since sustained oxidative damage probably plays a contributory role in the development of virus-associated HCC. Given the importance of both viruses in inducing oxidative DNA damage, there is currently a relative lack of

literature on the impact of the viral proteins on BER. On the other hand, despite a number of publications describing the ability of both viruses to perturb NER, it is unclear whether this impacts significantly upon oxidative repair. The eventual impact on virally-associated pathogenesis, including hepatocellular carcinoma, must also be studied, although this work is hampered by the lack of current suitable *in vivo* models for both HBV and HCV (reviewed by (Lerat *et al.*, 2011)). Increased future understanding of how these viruses regulate host cellular DNA repair pathways in response to oxidative stress will be invaluable in the development of new strategies for the treatment and prevention of chronic liver diseases.

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FIGURE LEGENDS

Figure 1: Repair of ROS-induced DNA lesions. The majority of oxidative lesions are repaired by the BER pathway. Oxidative damage is recognised by a glycosylase (Ogg1 or Neil1 in the case of 8-OHdG), which removes the altered base. Depending upon the glycosylase involved, this will result in either a single-stranded break (SSB) or an abasic (AP) site. The latter is then processed by the Ape1 endonuclease, giving an SSB. These breaks are then repaired by two subpathways of the BER pathway: short-patch (bottom right) or long-patch (bottom left). During short-patch BER, DNA polymerase β fills the gap with a single nucleotide, and the nicked DNA is ligated by XRCC1 and LigIII. In the alternative long-patch repair, SSBs repair involves the replacement of 2-12 nucleotides. This is dependent upon synthesis of a new 2-12bp fragment by either Pol β, Pol δ or Pol ε, and the concomitant displacement of a 5' DNA flap. This flap is then removed by the enzyme Flap Endonuclease 1 (FEN1). Ligation of the nick involves PCNA, LigI and LigIII. The choice between short-patch repair and long-patch repair (dotted arrows) is yet to be understood, and is complicated by the high degree of redundancy between the two pathways.

Figure 2: Alternative repair of oxidative damage by NER. An unknown proportion of oxidative lesions may be repaired by nucleotide excision repair (NER). Two NER subpathways exist, which differ in their ability to detect helix-distorting regions arising anywhere within the genome (global genome NER; left), or those lesions which arise in the transcribed strands of expressed genes (transcription coupled NER; right). In both cases, repair is effected

through the actions of detector proteins (XPC and XPE or CSA and CSB) which recruit TFIIH and its components to the site of damage. TFIIH, XPB and XPD act in conjunction with XPF and XPG to unwind the DNA surrounding the lesion and to excise one strand of the unwound bubble. The final stages of repair involve gap filling by Pol δ or Pol ε, and DNA ligation involving LigIII and XRCC1.

Figure 3: Sources of oxidative stress induced by the HBV (upper panel) and HCV (lower panel) viral proteins. As a result of the innate immune response, chronic infection with HBV or HCV will induce chronic inflammation, eliciting ROS production and creating oxidative DNA lesions. Both viruses, and their proteins, may cause ER stress or induce lipid accumulation, which in turn leads to oxidative stress and oxidative DNA lesions. In addition, HBx and HCV Core and NS5A stimulate ROS by perturbing mitochondria function. HCV NS5A also stimulates c-Myc transcription, leading to perturbation of cytochrome function and thus mitochondrial ROS, and directly increasing oxidative stress. In the case of HBV, the resultant oxidative DNA lesions may contribute to HBV genome integration if left unrepaired. The impact of either virus on the cellular detoxification of ROS remains unclear (denoted by a '?').

Figure 4: Interactions between HBV and HCV proteins and the cellular actors of oxidative DNA repair. (Upper panel): HBV infection upregulates the activity of the BER glycosylases Neil1 and Ogg1 (green arrows), probably as a consequence of increased oxidative stress. In contrast, the HCV proteins inhibit the activity of the Neil1 glycoslyase, and HCV core decreases the

repair of oxidative DNA lesions (red lines). HBx also inhibits the activity of a third glycosylase, Tdg, although the impact of Tdg on oxidative DNA lesions is unclear. HBx also inhibits NER by reducing expression of the NER proteins XPB and XPD, as well as inhibiting transcription-coupled NER. In addition, the HCV proteins also seem to inhibit NER, perhaps as a consequence of reduced Gadd45β expression. (Lower panel): A subset of oxidative lesions are converted into DSBs. HCV non-structural proteins NS3, NS4A and NS5B are reported to interact with the DSB sensor ATM and inhibit its function. Both HBx and the HCV core, NS3 and NS5A proteins also inhibit p53 (red lines), although there are suggestions that Core and NS5A may also stimulate p53 activity (green arrows).

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Figure 1

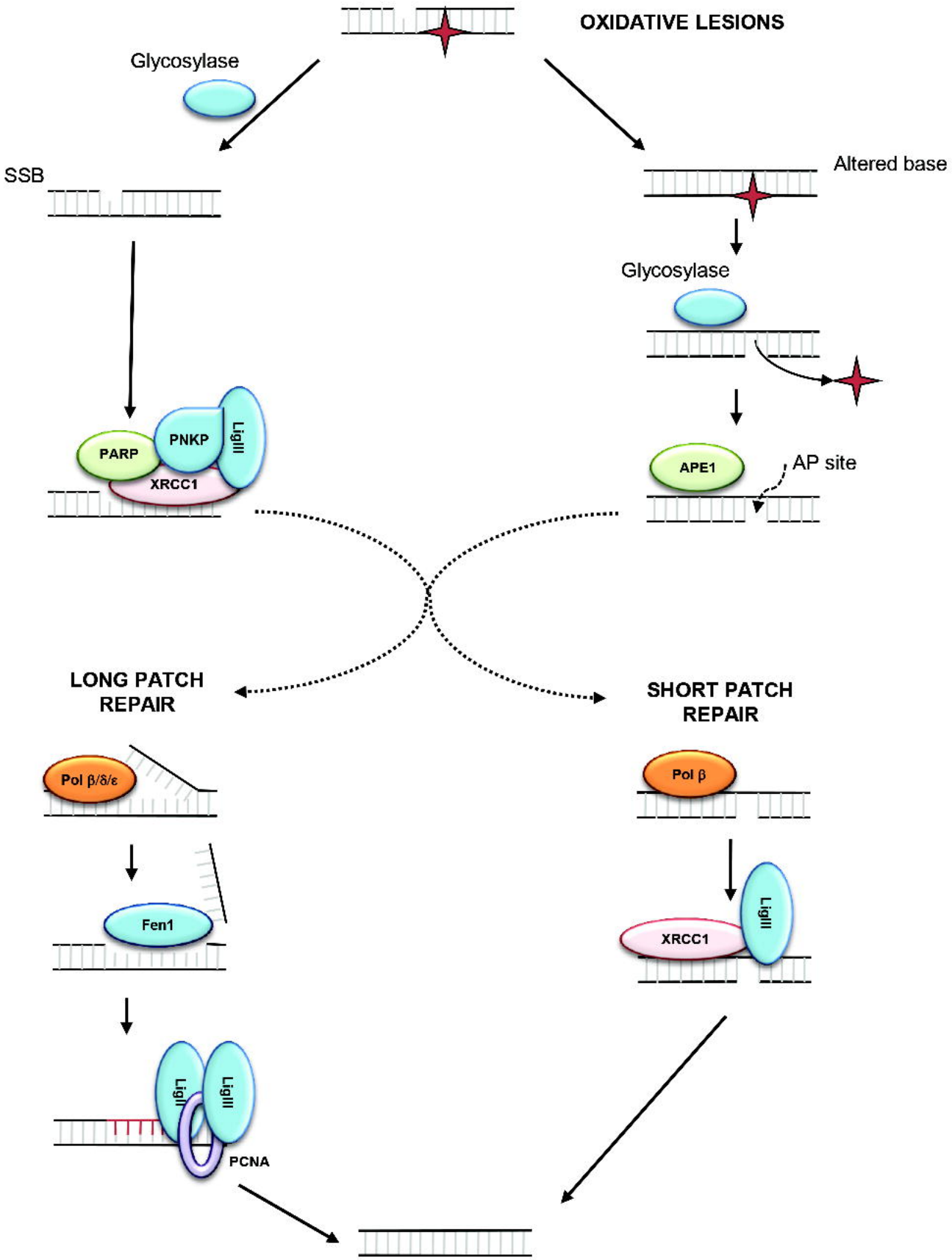
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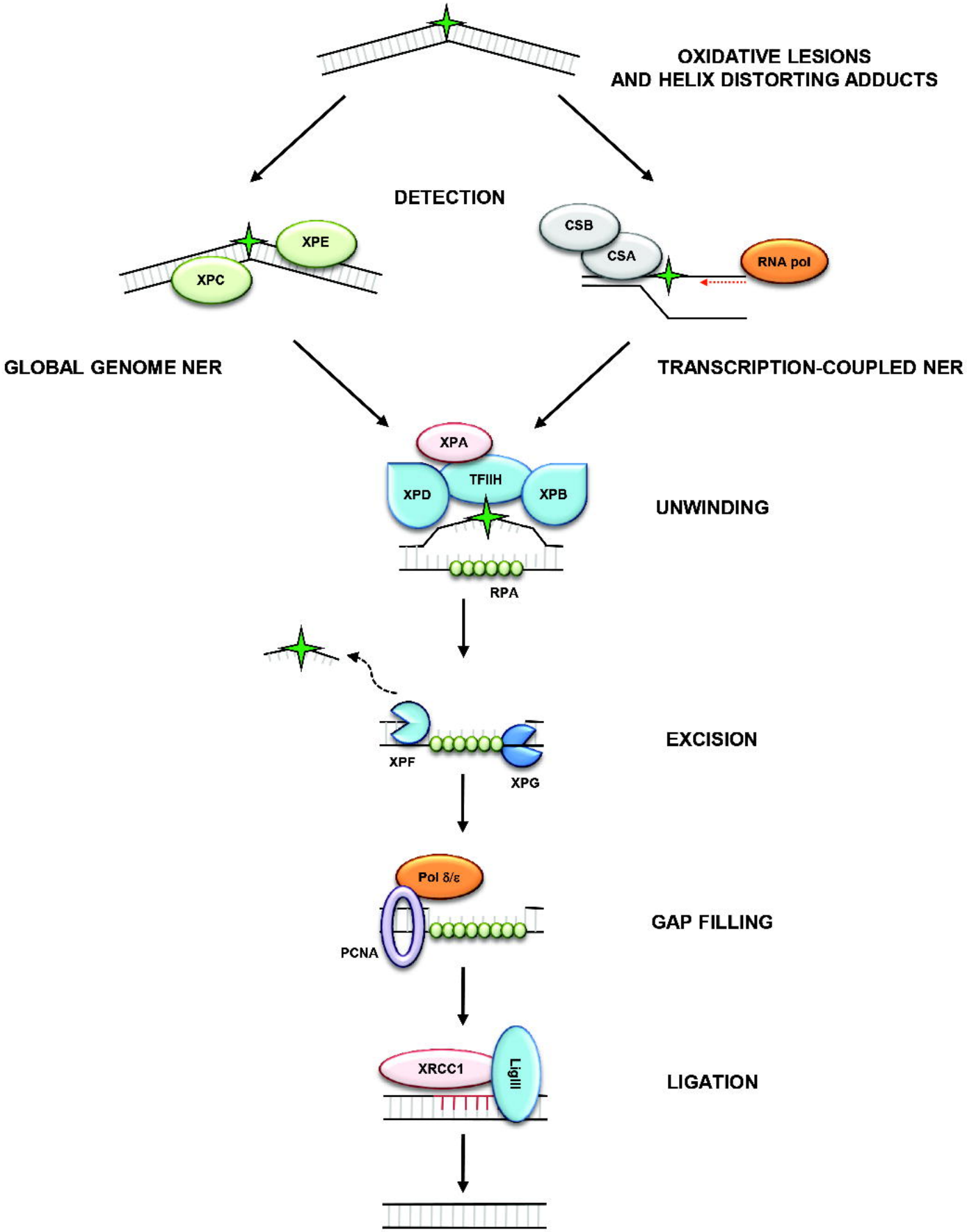
Figure 2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TFIIH
XPD
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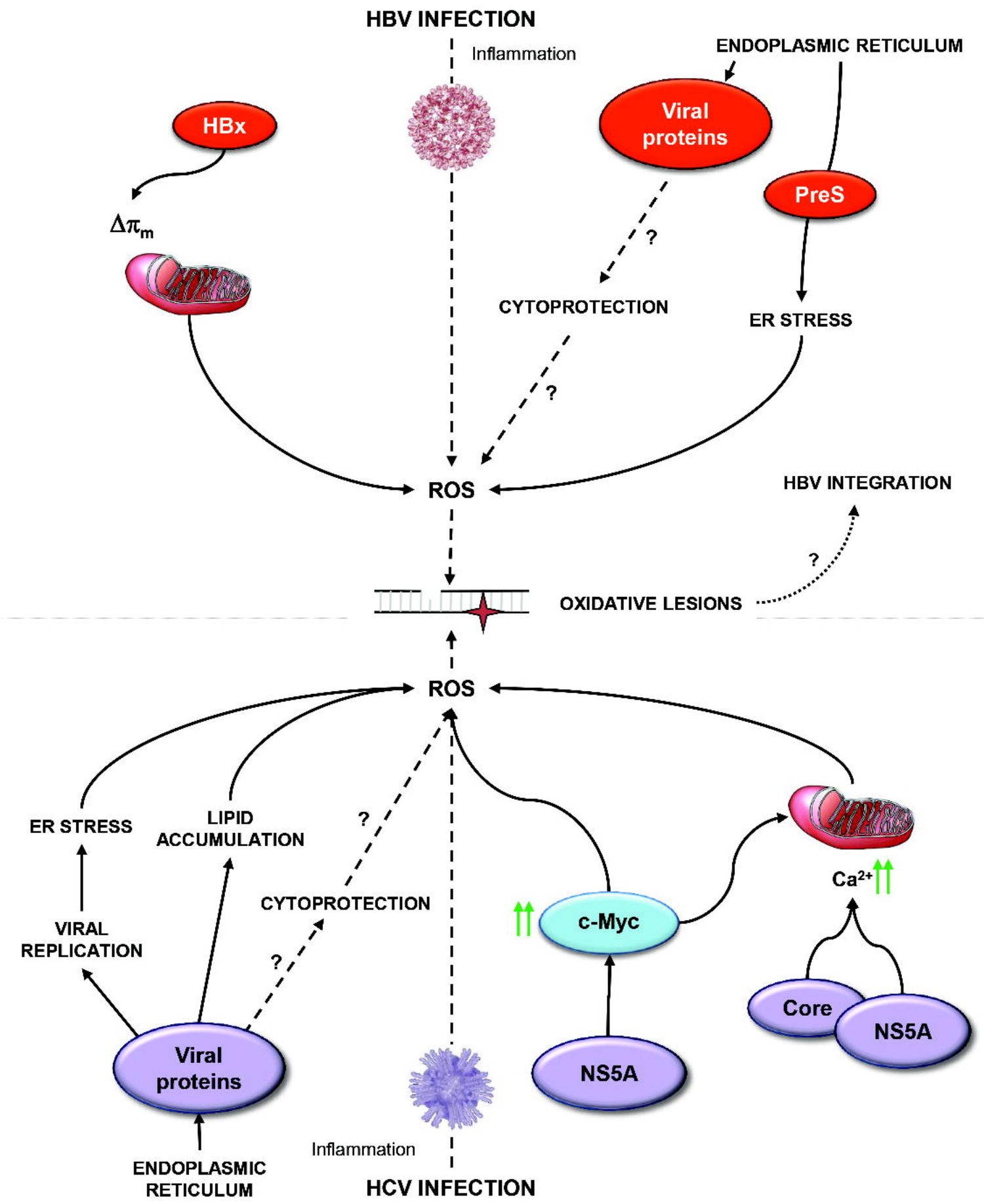
LIGATION

Figure 3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HBx
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HBV INTEGRATION
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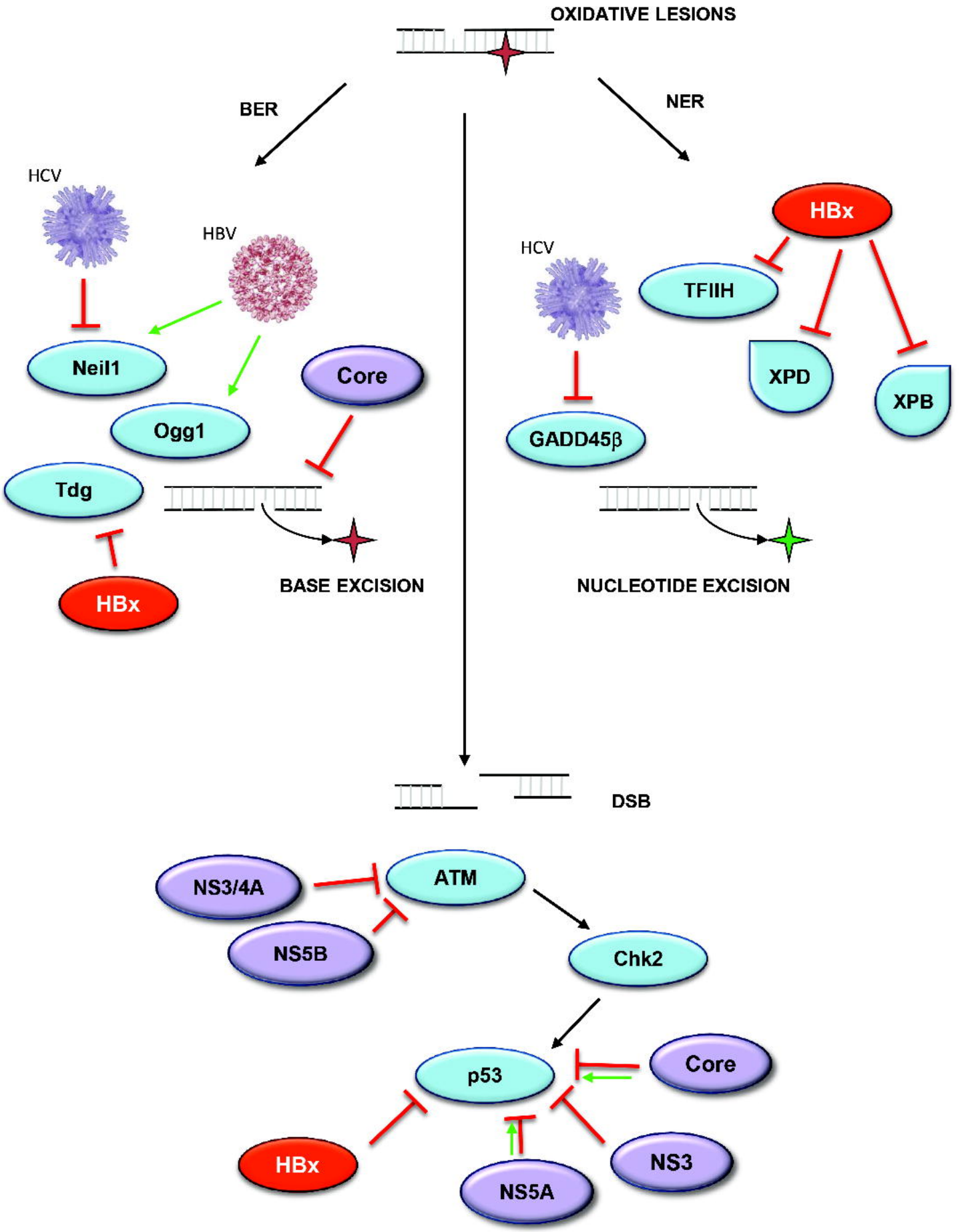
Figure 4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XPD
XPB
GADD45β
NUCLEOTIDE EXCISION
DSB
NS3/4A
ATM
NS5B
Chk2
p53
Core
HBx
NS5A
NS3