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# *Xenopus* egg extract: A powerful tool to study genome maintenance mechanisms

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ABSTRACT

DNA repair pathways are crucial to maintain the integrity of our genome and prevent genetic diseases such as cancer. There are many different types of DNA damage and specific DNA repair mechanisms have evolved to deal with these lesions. In addition to these repair pathways there is an extensive signaling network that regulates processes important for repair, such as cell cycle control and transcription. Despite extensive research, DNA damage repair and signaling are not fully understood. In vitro systems such as the *Xenopus* egg extract system, have played, and still play, an important role in deciphering the molecular details of these processes. *Xenopus laevis* egg extracts contain all factors required to efficiently perform DNA repair outside a cell, using mechanisms conserved in humans. These extracts have been used to study several genome maintenance pathways, including mismatch repair, non-homologous end joining, ICL repair, DNA damage checkpoint activation, and replication fork stability. Here we describe how the *Xenopus* egg extract system, in combination with specifically designed DNA templates, contributed to our detailed understanding of these pathways.

## 1. Introduction

The integrity of our genome is protected by a large variety of DNA repair mechanisms that counteract the continuous DNA damage resulting from both endogenous and exogenous sources. Malfunctioning of any of these mechanisms can lead to DNA mutations and is often associated with an increased risk of developing cancer (Hoeijmakers, 2009). In addition, defects in specific DNA repair pathways can lead to a large variety of diseases, that are in many cases characterized by developmental defects, premature ageing and cancer predisposition (O'Driscoll, 2012; Ribezzo et al., 2016). At the same time, deficiencies in DNA repair pathways have emerged as a powerful characteristic to enhance cancer therapy based on synthetic lethality (Pearl et al., 2015; Rehman et al., 2010). Understanding the molecular mechanisms of DNA repair pathways is important to develop such therapies, but also to further understand DNA repair deficiency diseases and the mechanisms that keep our genome stable.

Genetics and cell biology have provided many important insights into DNA repair pathways on a cellular and organismal level. However, to understand the biochemistry underlying these pathways, in vitro systems have proven to be very effective. *Xenopus* egg extracts have been used to study a variety of complex cellular processes, such as mitosis, actin metabolism, nuclear transport, apoptosis, DNA replication, and DNA repair (Hardwick and Philpott, 2015). *Xenopus* egg extracts contain a high concentration of proteins required to drive the rapid cell divisions after fertilization of the egg. DNA replication and repair are highly conserved between *Xenopus laevis* and mammalians, making this system well-suited to study these processes in detail. In addition, despite the major advances that have recently been made in the reconstitution of budding yeast DNA replication from purified components, the *Xenopus* egg extract system is currently the only system that enables efficient vertebrate DNA replication to take place outside the cell. This system has also been extensively used to study replication-linked processes, such as checkpoint activation, responses to replication fork stalling and DNA interstrand crosslink repair. Many genome maintenance mechanisms have been studied using *Xenopus* egg extracts, and in this review we will focus on mismatch repair, non-homologous end joining, interstrand crosslink repair, checkpoint activation, and replication fork stability. In particular, we will discuss how the use of specific DNA templates has enabled the recapitulation of these pathways and contributed to the understanding of their molecular mechanisms (Table 1).

## 2. *Xenopus* egg extract

The conventional *Xenopus* egg extract is made by crushing mature *Xenopus laevis* eggs at a moderate speed to produce an unfractionated

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**Table 1**
Summary of repair pathways studied in *Xenopus* egg extracts and the DNA templates that are used for this.

| Pathway | MMR | NHEJ | ICL repair | Damage checkpoint activation | | Fork stalling |
|---|---|---|---|---|---|---|
| | | | | ATR | ATM | |
| **Substrate** schematic | Mismatch | DSB | Interstrand crosslink | | | G-quadruplex |
| **Substrate** detail | - DNA plasmid containing mismatch | - Linearized plasmid DNA<br>- Linear fragments Containing:<br>- 3′ overhang<br>- 5′ overhang<br>- blunt ends | - DNA plasmid containing ICL<br>ICL type:<br>- cisplatin<br>- nitrogen mustard<br>- abasic site<br>- psoralen | - Chromatin DNA, polymerase-helicase uncoupling by aphidicolin<br>- M13 ssDNA circular DNA with annealed primers | - linear dsDNA fragments<br>- Digested dsDNA plasmid | - Chromatin DNA, polymerase-helicase uncoupling by aphidicolin<br>- ssDNA plasmid containing G-quadruplex |
| **Extract** | - HSS<br>- NPE | - LSS<br>- HSS | - HSS+NPE<br>- HSS | - LSS<br>- NPE | - LSS<br>- HSS | - LSS<br>- HSS |
| **Refs** | (Brooks et al., 1989; Kawasoe et al., 2016; Varlet et al., 1990; Varlet et al., 1996; Ghodgaonkar et al., 2013) | (Davis and Chen, 2013; Pfeiffer and Vielmetter, 1988; Di Virgilio and Gautier, 2005; Beyert et al., 1994; Zhu and Peng, 2016; Daza et al., 1996; Thode et al., 1990) | (Deans and West, 2011; Niedernhofer et al., 2005; Räschle et al., 2008; Angelov et al., 2009; Enoiu et al., 2012; Zhang et al., 2015; Le Breton et al., 2011; Fu et al., 2011; Knipscheer et al., 2009; Bluteau et al., 2016) | (Willis et al., 2012; Cupello et al., 2016; Yazinski and Zou, 2016; Dasso and Newport, 1990; Kumagai et al., 1998; Byun, 2005; Michael et al., 2000; Van et al., 2010; Lupardus, 2002; MacDougall et al., 2007; Kumagai et al., 2006; Hashimoto et al., 2006; Kumagai and Dunphy, 2000; Kumagai and Dunphy, 2003; Jones et al., 2003; Lee and Dunphy, 2010; Lee et al., 2007; Lee and Dunphy, 2013; Duursma et al., 2013) | (Yan and Michael, 2009; Bétous et al., 2013; Costanzo et al., 2000; You et al., 2005; Dupré et al., 2006; You et al., 2007) | (Ramírez-Lugo et al., 2011; Couch et al., 2013) |

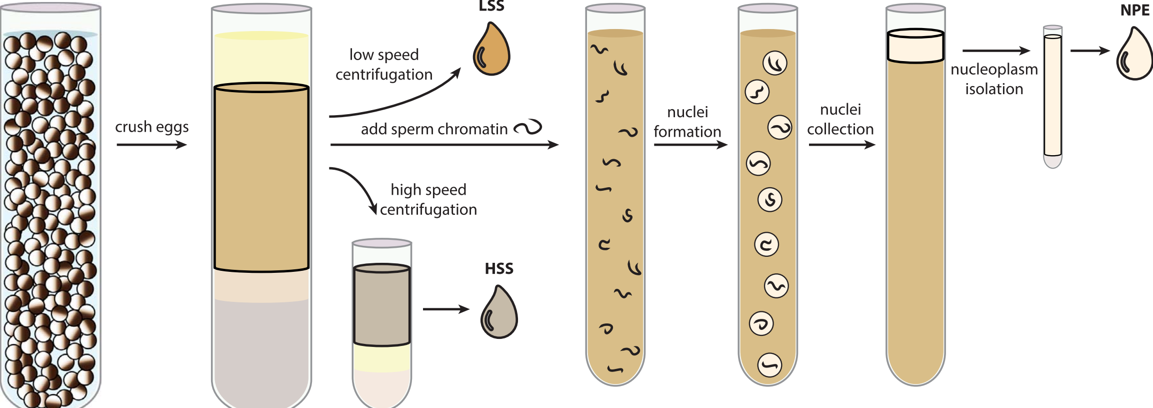


**Fig. 1.** Schematic representation of *Xenopus* egg extract preparation. Unfertilized *Xenopus laevis* eggs are crushed and the crude cytoplasmic faction is collected. A low speed centrifugation (100.000×*g*) step produces cytoplasmic extract including the membranes (LSS). A high speed (260.000×*g*) centrifugation step produces cytoplasmic extract without membranes (HSS). Incubation of the crude cytoplasmic extract with sperm chromatin, in the presence of ATP, induces nuclei formation. Nucleoplasm (NPE) is isolated by centrifugation after collection of the nuclei.

egg cytoplasm extract, called low speed supernatant or LSS (Lohka and Masui, 1983) (Fig. 1). Addition of this extract to demembranated sperm chromatin results in the formation of a nucleus around the DNA and a single, complete round of DNA replication (Blow and Laskey, 1986; Newport, 1987). DNA replication in this system depends on the formation of nuclei, and when membranes are removed by centrifuging the extract at higher speed, the resulting ‘high speed supernatant’ or HSS (Fig. 1) does not support DNA replication. However, also in the absence of active DNA replication, both LSS and HSS support efficient DNA repair, most likely due to the high concentration of repair factors in the extract. Although the LSS extract provided many insights into biological processes, the requirement for nuclei formation to allow

DNA replication presents some limitations. Factors that affect nuclei formation, or that are not imported into the nuclei, cannot be investigated, and small DNA molecules such as plasmids do not replicate efficiently (Blow and Laskey, 1986). To circumvent these issues, a nucleus-free DNA replication system was developed that involves two extracts that are added sequentially to the DNA (Walter et al., 1998). Incubation of the DNA in the membrane-free HSS leads to the assembly of pre-replication complexes (pre-RCs) by loading of ORC, Cdc6, Cdt1, and MCM2-7. Addition of a highly concentrated nucleoplasmic egg extract (NPE) triggers replication initiation and allows a single, complete round of DNA replication (Tutter et al., 2006; Lebofsky et al., 2009). To make NPE, nuclei formed in LSS are harvested and the nucleoplasm is isolated by high-speed centrifugation (Fig. 1). This two-extract system promotes efficient replication of defined DNA substrates such as (modified) DNA plasmids, and has the added advantage that replication initiation is relatively synchronous. Therefore, this is a unique system to study replication-coupled DNA repair processes.

## 3. Mismatch repair

The mismatch repair (MMR) pathway deals with base misinsertions and small inserts or deletions that are introduced during DNA replication. The repair process occurs in roughly four phases: mismatch recognition, identification of the error-containing nascent strand, removal of the mismatched strand, and finally resynthesis and ligation to restore the double helix. Deficiencies in MMR genes increase mutation rates by several orders of magnitude and leads to a predisposition to cancer, most frequently colon cancer (Jiricny, 2013; Li and Martin, 2016).

The mechanism of mismatch repair has been studied in *Xenopus* egg extracts since the late 1980s, initiated by a study by Brooks et al. in 1989. This study showed that a mismatch induces local DNA synthesis spanning up to a few hundred nucleotides around the mismatched site (Brooks et al., 1989). While this study used an HSS egg extract to support mismatch repair, a more recent study demonstrated that a nuclear extract (NPE) is more efficient in MMR (Kawasoe et al., 2016). Mismatch-containing plasmid DNA templates are commonly generated using single stranded phagemids that are converted to double stranded plasmids by annealing a mismatched second strand, or by extension from a mismatched primer. Repair of these mismatches is highly efficient in egg extract and occurs independently of replicative DNA synthesis.

The mismatch-containing sequences are often designed in such a way that repair can be monitored by the generation or loss of recognition sites for specific restriction enzymes. Using this method, Varlet et al. found that mismatch repair in *Xenopus* egg extracts is not equally efficient for all possible mismatches and seems to be most efficient for GT and AC mismatched pairs (Varlet et al., 1990). This has also been observed in mammalian in vitro systems, indicating that the MMR mechanism is highly conserved (Holmes et al., 1990; Thomas et al., 1991).

A major unresolved issue in the mismatch repair field is the identity of the strand discrimination signal. The repair machinery must determine which of the two DNA strands is the newly synthesized strand and therefore contains the mismatched nucleotide. Early experiments in egg extract showed that the presence of a nick on one of the two strands increased the efficiency of repair of this nicked strand, indicating that a nick can serve as such a strand discrimination signal (Varlet et al., 1996). It is tempting to reason that this nick can be used as a starting point for repair synthesis, however, this study showed that this is not the case. Repair synthesis occurred close to, and on either side of, the mismatch regardless of where the nick was positioned. This indicates that the nick has a signaling role rather than serving as a starting point for strand removal, as was also more recently observed (Kawasoe et al., 2016). Consistent with this, it was found in human cell extracts that the nick that serves as strand discrimination signal is not necessarily the starting point of strand removal and repair synthesis. The MMR factor MutLα is an endonuclease that makes several additional nicks, on either side of the mismatch, after recognition of the mismatch containing strand (Kadyrov et al., 2006). This is specifically important if the strand discrimination nick is 3′ of the mismatch, since the exonuclease that removes the mismatched strand only acts from 5′ to 3′ end.

While a nick has been shown to serve as a strand discrimination signal in several eukaryotic in vitro systems (Holmes et al., 1990; Thomas et al., 1991), there are other mechanisms that can serve this purpose. It has been demonstrated that ribonucleotides that are erroneously built into the DNA during replication can help to identify the nascent strand (Lujan et al., 2013; Ghodgaonkar et al., 2013), likely by producing a nick as an intermediate of repair. The base excision repair (BER) pathway also creates nicks during repair that could potentially serve to discriminate between DNA strands. A recent study used human cell and *Xenopus* egg extracts to show that processing of oxidized guanines that are mismatched to an adenine, can also facilitate MMR (Repmann et al., 2015). This study used a plasmid template containing an oxidized guanine ($G^o$) opposite to a C or an A, in addition to a GT mismatch in the vicinity. Processing of the mismatched A opposite the $G^o$ by the MutY-homologue (MYH) resulted in enhanced MMR of the GT mismatch. However, the presence of a C opposite the $G^o$ did not enhance MMR, indicating that $G^o$ processing by the glycosylase OGG1 is inhibited and no strand discrimination signal is generated. This suggests an elegant mechanism to ensure MMR takes place on the nascent strand that contained the mismatched A and not on the parental strand containing the $G^o$ (Repmann et al., 2015).

In addition to these mechanisms based on the presence or regeneration of nicks, it has also been suggested that the directional loading of PCNA can provide strand discrimination information (Umar et al., 1996; Pluciennik et al., 2010). Using NPE extract and a mismatched plasmid template loaded with PCNA in an orientation-specific manner, Kawasoe et al. recently showed that PCNA can serve as such a strand discrimination signal even in the absence of nicks on the template DNA (Kawasoe et al., 2016). In addition, this study showed that the mismatch repair factor MutSα, which directly interacts with PCNA, helps to retain PCNA on the DNA until repair is finished.

## 4. Non-homologous end joining

Double-strand breaks (DSBs) are repaired by two main mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). HR promotes error-free repair by using sequence information from a sister chromatid, while NHEJ simply ligates the two ends of the DNA together, which often leads to deletions or insertions. Some aspects of HR have been studied in *Xenopus* egg extracts, but NHEJ has been more extensively studied using this system. During non-homologous end joining, the two ends of the DSB are first bound by the Ku70-Ku80 (Ku70/80) heterodimer, which is followed by the recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA end ligation, if necessary preceded by DNA end-processing, is mediated by DNA ligase 4, its essential cofactor XRCC4, and the XRCC4 paralogs XLF and PAXX (Radhakrishnan et al., 2014; Chiruvella et al., 2013). Defects in NHEJ have been linked to immunodeficiency syndromes and cancer development (O'Driscoll, 2012; Davis and Chen, 2013).

Non-homologous DNA ends are readily joined in *Xenopus* egg extract, as was first demonstrated in the late nineteen eighties by Pfeiffer and Vielmetter (Pfeiffer and Vielmetter, 1988). This study, and most of the following studies, used linearized plasmid DNA containing 3′ or 5′ single-stranded overhangs or blunt ends. Both LSS and HSS egg extracts support efficient NHEJ (Di Virgilio and Gautier, 2005). End joining in *Xenopus* egg extract is mostly error-free, and the process involves end-alignment or overlap and filling in of the gaps

(Beyert et al., 1994; Zhu and Peng, 2016). While NHEJ in human cell extracts seems to follow the same mechanisms (Daza et al., 1996), the efficiency is much higher in *Xenopus* egg extracts, possibly due to the high concentration of Ku proteins (Labhart, 1999).

*Xenopus* egg extracts have proven to be extremely useful to examine the roles of specific proteins in NHEJ. In 1990, even before any of the currently known alignment factors were identified, an important study provided evidence that there must be a protein that keeps the DNA ends perfectly aligned during end joining (Thode et al., 1990). This model was based on the observation that the filling in of a DSB end containing a 3′ overhang can precede ligation, suggesting that DNA synthesis starts from the other 3′ DSB end and passes the nick. This can only occur when the ends are perfectly aligned. Soon after Ku70/80 was identified, it was confirmed that it also plays a role in the end joining observed in *Xenopus* egg extracts (Labhart, 1999). However, this study did not rescue the NHEJ defect after Ku70 depletion with recombinant protein, a necessary control to rule out unspecific effects. In follow up work it was shown that a Ku-dependent mechanism ligates ends with non-compatible 3′ overhangs by forming a 2 nt overlap by non-canonical base pairing, from which the fill-in of the remaining gaps is initiated (Sandoval and Labhart, 2002). A role for Ku70 was later confirmed by several other reports (Di Virgilio and Gautier, 2005; Zhu and Peng, 2016; Graham et al., 2016), and DNA-PK was also shown to be required for NHEJ in egg extract (Di Virgilio and Gautier, 2005; Gu et al., 1996, 1998). Finally, a very recent report used *Xenopus* egg extracts to show that p97 and Ku80 ubiquitylation are required for unloading of Ku from DSBs after repair has finished (van den Boom et al., 2016).

MRE11 is a subunit of the MRE11-Rad50-Nbs1 (MRN) complex, and while this protein has an important role in NHEJ in *Saccharomyces cerevisiae*, in vertebrates this was controversial. Di Virgilio and Gautier reported that depletion of MRE11 from cytosolic egg extract does not affect the efficiency or kinetics of NHEJ, nor does it affect its fidelity (Di Virgilio and Gautier, 2005). However, this study used immunodepletion to remove MRE11 from extract, and it is difficult to fully exclude the possibility that the small amount of MRE11 remaining is sufficient to support NHEJ. In addition, these experiments were performed on clean DNA ends produced by restriction enzymes, while in vivo substrates could be more complex. In support of this, recent studies in *Xenopus* egg extract showed that MRE11 is required for joining of ends with 5′ bulky adducts (Liao et al., 2016) and during an alternative pathway of NHEJ (alt-NHEJ) where resection is required (Taylor et al., 2010). In mammalian systems, it has now been shown that MRE11 contributes to alt-NHEJ and also to some extent to canonical-NHEJ (Rass et al., 2009; Xie et al., 2009).

A single-molecule study using *Xenopus* egg extract recently elucidated a 2-step mechanism for synapsis of the DNA ends during NHEJ (Graham et al., 2016). First, a thorough analysis using immunodepletions and rescue experiments, showed a requirement for Ku70/80, DNA-PKcs, XLF, XRCC4 and Lig4, for the end joining of blunt ended linear DNA substrate. This further validates the *Xenopus* egg extract system as a physiologically relevant in vitro system to study NHEJ. Single-molecule experiments were performed in egg extract to monitor the interaction between Cy3-labeled blunt ended DNA molecules tethered to a glass surface and Cy5-labeled DNA molecules in solution. This revealed two types of interaction between the DNA ends: a long-range interaction in which the Cy5–labeled DNA was tethered to the Cy3-labeled DNA on the surface, at a distance too large for FRET to occur, and a short-range interaction in which a FRET signal could be detected between the dyes present on each end. The long range interaction was short lived and dependent on Ku70/80 and DNA-PK but not its catalytic activity, while the short range interaction, in addition to these factors, required the catalytic activity of DNA-PK, as well as Lig4, XLF and XRCC4. These results show that the DNA ends are brought together for ligation in a multi-step process that requires different NHEJ factors at each stage (Thode et al., 1990). How each factor contributes to these different interaction modes, and how the transition between the two modes is facilitated remains to be further investigated. In addition, it will be of great interest to study the ligation of DNA ends that are modified, or carry 3′ or 5′ extensions, using this system.

## 5. Interstrand crosslink repair

DNA interstrand crosslinks (ICLs) are toxic DNA lesions that covalently connect the two strands of the DNA. ICLs can be formed endogenously by byproducts of cellular metabolism such as aldehydes, but can also be induced by ICL inducing chemicals, such as Mitomycin C, nitrogen mustards and cisplatin. Because ICLs prevent strand separation and inhibit DNA replication and transcription, they are extremely toxic to rapidly dividing cells, which is why ICL inducing agents are often used in cancer chemotherapy (Deans and West, 2011). In cells, most ICLs are repaired in S-phase, while a minor pathway acts in G1. Until less than a decade ago, little was known about the molecular mechanism of ICL repair. Genetics had implicated structure-specific endonucleases, translesion polymerases, and HR factors in this repair process and a model was postulated that involved replication fork collision, ICL unhooking by dual incisions, TLS past the unhooked ICL and HR to restart the replication fork (Niedernhofer et al., 2005). However, this model was not experimentally confirmed and it did not explain a role for the Fanconi anemia proteins in ICL repair. Fanconi anemia (FA) is a cancer predisposition disorder caused by a defect in any of the 21 currently known FA genes. Cells of FA patients are extremely sensitive to ICL inducing agents, suggesting that the FA pathway plays a role in ICL repair.

Understanding the molecular mechanism of ICL repair was hampered by the lack of a system to study it biochemically, but this changed in 2008 when replication-coupled ICL repair was recapitulated in *Xenopus* egg extract (Räschle et al., 2008; Ben-Yehoyada et al., 2009). Since then, this system has made extensive contributions to our understanding of the molecular mechanism of ICL repair. The system makes use of plasmid DNA templates that contain a site-specific ICL in combination with HSS and NPE egg extracts, that allow replication to start simultaneously on each plasmid (Knipscheer et al., 2012). This enables the dissection of the different stages of ICL repair. Importantly, a direct readout for ICL repair is possible by the regeneration of a restriction site that is blocked by the ICL (Räschle et al., 2008). Several different types of crosslinks can be induced in plasmids such as nitrogen mustard-like, MMC-like, psoralen/UV, abasic-site or cisplatin ICLs (Ben-Yehoyada et al., 2009; Angelov et al., 2009; Enoiu et al., 2012; Zhang et al., 2015; Semlow et al., 2016).

Replication-dependent ICL repair in *Xenopus* egg extracts is most intensively studied using cisplatin and nitrogen mustard-like crosslinks and is initiated when replication forks from either side converge at the ICL (Räschle et al., 2008) (Fig. 2, step 1 and 2). Although one study indicated that some ICL processing can take place upon fork arrival from one side, it was not clear whether this leads to ICL repair (Le Breton et al., 2011). A more recent study showed that dual fork collision is a prerequisite for ICL repair in *Xenopus* egg extract (Zhang et al., 2015). After fork convergence and stalling of the forks 20–40 nucleotides from the ICL, one of the replication forks resumes DNA synthesis and stalls again when it is only one or a few nucleotides from the crosslink (Räschle et al., 2008) (Fig. 2, step 3a). This ‘approach’ step can only occur once the CMG helicase is unloaded from the DNA, a step that depends on fork convergence (Zhang et al., 2015; Fu et al., 2011). In addition, another study showed that the HR factor BRCA1 (FANCS) has an unexpected early function in ICL repair in promoting this CMG unloading step (Long et al., 2014). Although it remains to be seen whether fork convergence is required for the repair of all ICLs in mammalian cells, this mechanism does have the important advantage that ICL repair only starts once DNA replication is completed. This

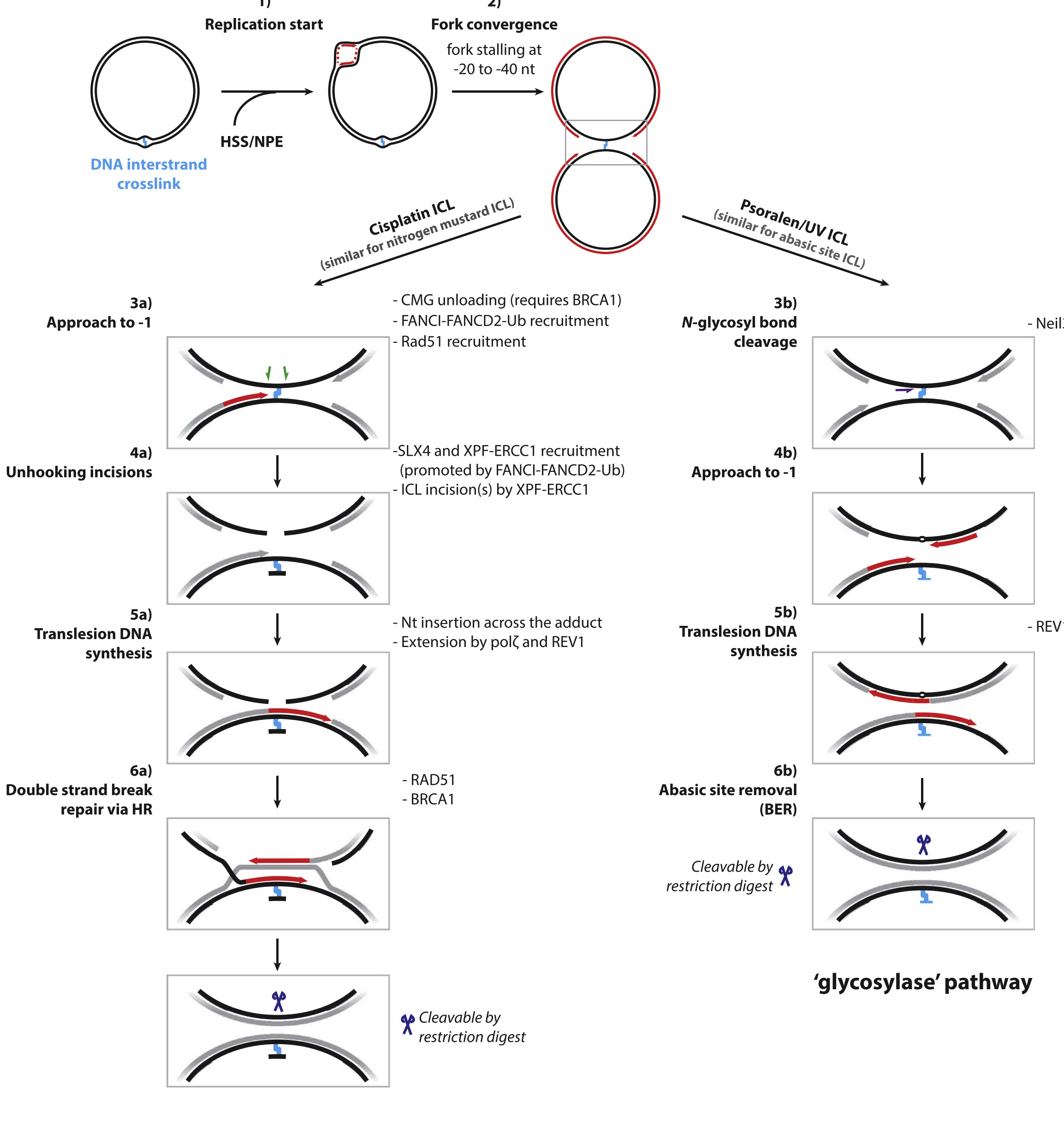


**Fig. 2.** Model for DNA replication-dependent ICL repair in *Xenopus* egg extract. ICL unhooking can occur via nucleolytic incisions on the parental strand ('incisions' pathway, left), or via cleavage of the *N*-glycosyl bond of one of the crosslinked nucleotides ('glycosylase' pathway, right). Both pathways require replication fork convergence to initiate repair. Repair steps are indicated on the left of each pathway, proteins involved on the right. See text for detailed explanation of repair steps.

avoids unreplicated regions or replication fork collapse due to the inability of CMG reloading during S-phase.

CMG unloading and approach of one of the replication forks to the ICL is followed by endonucleolytic incisions on the parental strand on either side of the ICL that effectively 'unhook' the ICL from one of the DNA strands (Fig. 2, step 4a). This repair step requires the activation of the Fanconi anemia pathway by ubiquitylation of the FANCI-FANCD2 complex (Knipscheer et al., 2009). Work in other systems has shown that this is mediated by a ubiquitin E3 ligase complex consisting of 8 FA proteins and the UBE2T(FANCT) ubiquitin conjugating enzyme (Kottemann and Smogorzewska, 2013). In follow-up studies using *Xenopus* egg extract, it was shown that ubiquitylated FANCI-FANCD2 is recruited to the site of the ICL, where it promotes the recruitment of an 'incision complex', consisting of the structure specific endonuclease XPF(FANCQ)-ERCC1 and the nuclease scaffold protein SLX4(FANCP) (Klein Douwel et al., 2014). XPF-ERCC1 is responsible for making at least one of the unhooking incisions, and possibly both (Klein Douwel et al., 2014).

Once the ICL is unhooked from one of the strands, lesion bypass across the adduct on the opposite strand restores the integrity of one of the sister molecules (Fig. 2, step 5a). Lesion bypass most likely takes place in two steps: first a nucleotide is inserted across from the unhooked ICL by an unknown polymerase, then the strand is extended by polymerase ζ in collaboration with REV1. This is based on the finding that depletion of REV7, the regulatory subunit of polymerase ζ, as well as depletion of REV1, both cause an arrest of lesion bypass after a nucleotide has been inserted across from a cisplatin ICL (Räschle et al., 2008; Budzowska et al., 2015). Interestingly, in contrast to cisplatin ICLs, a nitrogen mustard ICL did not require REV7 for lesion bypass (Räschle et al., 2008). Nonetheless, REV7 was recently identified as an FA gene (FANCV) confirming the importance of this protein in ICL repair (Bluteau et al., 2016).

The molecule containing the DSB is regenerated by homologous recombination using the restored sister molecule (Long et al., 2011) (Fig. 2, step 6a). Rad51 is a critical component of HR during ICL repair but may also have a function in early stages of the repair process (Long et al., 2011). This has been suggested because Rad51 (FANCR) is recruited to the ICL even before unhooking incisions have taken place. This would be analogous to the dual role of BRCA1(FANCS) that functions in CMG unloading and presumably also in HR during ICL repair (Long et al., 2014). The sister molecule that contains the adduct is not efficiently repaired in *Xenopus* egg extract and will not become available for restriction digest (Deans and West, 2011).

For years it was thought that the FA pathway-dependent ICL repair mechanism was the only replication-dependent ICL repair mechanism, however, very recently a second mechanism was identified using *Xenopus* egg extracts (Semlow et al., 2016). This study showed that plasmids carrying psoralen/UV- and abasic site-derived ICLs are repaired independently of the FA pathway. However, this pathway is dependent on DNA replication and requires two replication forks to converge at the ICL. Interestingly, in this repair mechanism, the ICL is not unhooked by dual incisions on the parental strand and thereby avoids formation of a DSB. Instead, the glycosylase Neil3 unhooks the two DNA strands by breaking the *N*-glycosyl bond between the sugar and the base of one of the crosslinked nucleotides (Fig. 2, step 3b). This generates one sister molecules that contains an abasic site, and another sister molecule that contains a normal nucleotide or an adduct depending on the chemical nature of the ICL. TLS is required to bypass the abasic site and the adducted nucleotide (Fig. 2, step 5b). Because no DSB is formed during this process, it does not require homologous recombination. While this mechanism is faster and less complex compared to the incision-dependent mechanism, it is also likely to be more mutagenic because it involves bypass of an abasic site (Semlow et al., 2016). These findings also raised some important questions. Neil3 is a bifunctional glycosylase that breaks *N*-glycosyl bonds but also contains lyase activity, which promotes cleavage of the phosphodiester backbone. Semlow et al. suggest that the lyase activity of Neil3 is inhibited during replication-dependent ICL repair because this would lead to a double-strand break and these are not observed during ICL unhooking. A possible alternative explanation could be that lyase activity of Neil3 acts after the bypass of the AP site by TLS. To settle this issue, it would be interesting to make a separation of function mutant of Neil3 that is an inactive lyase, but active glycosylase. Another important question that arises from this work is how these two mechanisms act on ICLs in cells. Importantly, FA pathway deficiency causes the serious disorder Fanconi anemia, indicating that not all ICLs can be repaired by the Neil3 pathway. This is consistent with the initial work in *Xenopus* egg extracts showing that replication-dependent repair of a cisplatin ICL is fully dependent on the FA pathway (Knipscheer et al., 2009). It is tempting to speculate that the preferred pathway choice is predominantly determined by the chemical nature of the ICL and the degree of DNA distortion it induces. However, it is still unclear what the main source of ICLs in cells is. Abasic site ICLs have been suggested to form in vivo, but there are also strong indications that aldehydes may produce the majority of endogenous ICLs (Langevin et al., 2011). How aldehyde-induced ICLs are repaired is currently unclear, but based on genetic experiments it seems very likely that these are, at least in part, repaired by the FA pathway. Future experiments are required to identify the nature of endogenous ICLs and the mechanism(s) used to repair them.

In addition to these replication-coupled ICL repair mechanisms, there is also a replication-independent ICL repair mechanism that has been studied in HSS *Xenopus* egg extracts (Ben-Yehoyada et al., 2009; Williams et al., 2012). These studies use an MMC-like ICL-containing plasmid as a template and repair is quantified by a quantitative PCR on the reaction products. Replication-independent repair does not depend on the FA pathway, REV7, or Rad51 but does require polymerase κ and PCNA (Ben-Yehoyada et al., 2009; Williams et al., 2012). Other methods have indicated the requirement of REV1, polymerase ζ and polymerase η in this repair pathway, as well as several factors involved in nucleotide excision repair (Enoiu et al., 2012; Shen, 2006; Shen et al., 2009). Although the study by Ben-Yehoyoda et al. showed that the FA pathway is not directly involved in replication-independent ICL repair in HSS/NPE, it also shows that the FA core complex does play a role in activation of an ATR-dependent checkpoint response after ICL damage. This is consistent with work in mammalian cells that show that certain FA factors are recruited to ICLs independent of DNA replication (Shen et al., 2009). In contrast, experiments in *Xenopus* egg extract previously indicated that the recruitment of FA factors requires DNA replication (Sobeck et al., 2009). While the upstream role of the FA proteins in checkpoint activation requires further investigation, ATR signaling has been shown to be important for activation of the FA pathway (Andreassen et al., 2004; Ho et al., 2006; Ishiai et al., 2008).

Also, the repair of DNA-protein crosslinks (DPCs) has been studied in egg extract (Duxin et al., 2014). In this study, a substrate for DPC repair was created by covalently attaching the methyltransferase *Hpa*II to a plasmid DNA template site-specifically. DPC repair in *Xenopus* egg extract is dependent on DNA replication and is initiated by replication fork stalling at the DPC. Then, partial degradation of the DPC allows bypass of the remaining adduct by the translesion polymerase ζ. There are strong indications that the protease that degrades the crosslinked protein is the metalloprotease SPRTN/DVC1 (Stingele et al., 2016; Vaz et al., 2016), but this has not yet been shown in the *Xenopus* egg extract system. If immunodepletion of SPRTN from *Xenopus* egg extracts inhibits DPC repair, this system would be well-suited to study the biochemical details and regulation of this pathway.

## 6. Damage checkpoint activation

DNA damage triggers a cellular response referred to as the DNA damage response (DDR). The DDR coordinates cell cycle checkpoints and DNA repair, and can induce cell senescence or apoptosis. Defects in this pathway often lead to genomic instability and cancer predisposition (Hanahan and Weinberg, 2011). The two key kinases in the DDR are the ataxia telangiectasia mutated (ATM) and the ATM and Rad3-related (ATR). ATM primarily responds to double-strand breaks (DSBs), while ATR responds to primed single stranded DNA (ssDNA) (Marechal and Zou, 2013). Both ATM and ATR signaling pathways have been studied in *Xenopus* egg extract and we will give a brief overview of a subset of these studies. More elaborate reviews and protocols have been published elsewhere (Garner and Costanzo, 2009; Costanzo et al., 2004; Srinivasan and Gautier, 2011; Willis et al., 2012; Cupello et al., 2016).

### 6.1. ATR

ATR is recruited to RPA bound ssDNA via its interaction partner ATRIP. In addition, the Rad9-Hus1-Rad1 (9-1-1) complex is recruited to ssDNA-dsDNA junctions and binding of Topoisomerase IIβ-binding protein 1 (TopBP1) to both ATR and the 9-1-1 complex is important to activate ATR. Many additional factors can influence ATR activation and, once activated, ATR phosphorylates numerous downstream effector proteins that play a role in genome maintenance, including Chk1 and RPA (Yazinski and Zou, 2016).

The ATR signaling pathway can be activated in *Xenopus* egg extract using a variety of DNA substrates. Already in the early nineties, aphidicolin, an inhibitor of polymerase α, ε, δ, and ζ, was used to prevent the completion of DNA replication in LSS. This study showed that the presence of unreplicated DNA induces a cell cycle checkpoint that inhibits the entry into mitosis (Dasso and Newport, 1990). Later, similar experiments showed that this checkpoint is mediated by Chk1 phosphorylation (Kumagai et al., 1998). Using the nuclear egg extract NPE and plasmid DNA it was demonstrated that aphidicolin induces the ATR signaling pathway by uncoupling the replicative helicase from

the polymerases, thereby generating single-stranded DNA (Byun, 2005). In addition to DNA unwinding, polymerase α is also required for the activation of ATR (Byun, 2005; Michael et al., 2000), most likely by generating primers on ssDNA and thereby creating additional ssDNA-dsDNA junctions (Van et al., 2010). Other DNA lesions, such as UV and MMS, can also induce ATR signaling in egg extract, likely by uncoupling the replicative helicases from the polymerases (Lupardus, 2002). Interestingly, DNA interstrand crosslinks also induce Chk1 phosphorylation, even though this helicase-polymerase uncoupling is not possible (Räschle et al., 2008; Ben-Yehoyada et al., 2009). The DNA structures that are required for ATR activation are further characterized by using M13-based circular ssDNA templates with primers annealed to it. These studies showed that ssDNA-dsDNA junctions with a free 5′ end activate ATR, and that ATR activation can be enhanced by larger ssDNA regions or additional 5′ ends (Van et al., 2010; MacDougall et al., 2007).

The activation of ATR by interaction with TopBP1 has been studied in detail using LSS extract in combination with aphidicolin. TopBP1 uses its ATR activation domain (AAD) to interact with and activate ATR (Kumagai et al., 2006; Hashimoto et al., 2006). The interaction is further enhanced by ATR-dependent phosphorylation of the AAD domain (Hashimoto et al., 2006). An additional regulator of the ATR signaling pathway, Claspin, was also identified in *Xenopus* egg extract and mediates the phosphorylation of Chk1 (Kumagai and Dunphy, 2000, 2003).

The 9-1-1 complex is recruited to ssDNA-dsDNA junctions by the Rad17-dependent RFC (replication factor-C)-like complex (Jones et al., 2003) and Rad17 may directly mediate the interaction between the 9-1-1 complex and TopBP1 (Lee and Dunphy, 2010). Furthermore, direct interaction between the Rad9 component of the 9-1-1 complex and TopBP1 is required for ATR activation, although this interaction does not seem to be required for TopBP1 recruitment to ssDNA (Lee et al., 2007; Lee and Dunphy, 2013; Duursma et al., 2013). It has been suggested that TopBP1 loading precedes 9-1-1 recruitment to ssDNA/dsDNA junctions and is mediated by polymerase α (Yan and Michael, 2009). In addition, a recent study using M13-based ATR activating DNA structures, showed that the MRN complex is important for the recruitment of TopBP1 and ATR signaling (Duursma et al., 2013). This was later also demonstrated using aphidicolin treated chromatin in egg extract (Lee and Dunphy, 2013). This was a surprising finding as the MRN complex had previously only been implicated in ATM signaling.

Finally, translesion synthesis (TLS) polymerases have also been shown to play a role in ATR signaling in *Xenopus* egg extract. Upon generation of long stretches of ssDNA after addition of aphidicolin, polymerase κ was implicated, together with polymerase α, in generating small stretches of dsDNA that are required to load the 9-1-1 complex (Bétous et al., 2013). In addition, REV1 does not seem to be involved in loading of the core activating complex RPA-ATR/ATRIP-TopBP1-9-1-1 but seems to function downstream in activation of Chk1 (DeStephanis et al., 2015).

### 6.2. ATM

At the site of a double-strand break (DSB), ATM is recruited and activated by interaction with the MRN complex, which also localizes to these lesions. Activated ATM then phosphorylates histone H2AX that subsequently recruits MDC1. This initiates a ubiquitylation cascade at the site of damage, mediated by the ubiquitin ligases RNF8 and RNF168, leading to the recruitment of other DSB regulators such as 53BP1 and BRCA1. When a sister chromatid is present, in late S or G2 phases, DSBs are repaired via homologous recombination (HR). This is initiated by activation of DNA end resection, leading to the removal of the Ku70/80 heterodimer from the DNA ends. In the absence of a sister chromatid, DNA end resection is inhibited and the DSB is repaired via non-homologous end joining (NHEJ). Importantly, the activation of ATM by DSBs leads to the phosphorylation of many downstream effectors, such as Chk2 and p53, which not only mediate DNA repair but also cell cycle arrest and apoptosis (Marechal and Zou, 2013).

ATM signaling can be activated in *Xenopus* egg extracts with DNA substrates that mimic double-strand breaks, such as DNA plasmids cut with restriction enzymes (Costanzo et al., 2000; You et al., 2005) or annealed oligos (Yoo et al., 2004). Double stranded linear DNA fragments were also used to study the role of MRN in ATM activation. MRN was reported to activate ATM by a direct interaction between its NBS1 subunit and ATM (You et al., 2005; Dupré et al., 2006) but also by tethering DNA and thereby increasing the local DNA concentration (Dupré et al., 2006). Consistent with this, it was demonstrated that ATM activation depends on the concentration of DNA ends and the DNA length (You et al., 2007). It was suggested that the exonuclease activity of the MRN complex is required for ATM activation (Dupré et al., 2008) and that oligonucleotides generated at DSBs further activate ATM (Jazayeri et al., 2008).

*Xenopus* egg extracts have also been used to screen chemicals for ATM activation. The readout was the phosphorylation of histone H2AX in response to linearized plasmids and this screen identified the small molecule mirin as an inhibitor of ATM activation (Dupré et al., 2008). Mirin directly inhibits the endonuclease activity of the MRN complex required for homologous recombination but also MRN-dependent ATM activation.

In addition to activation of ATM, DSBs can also activate ATR. Using annealed oligos and sperm DNA digested by *Eco*RI, Yoo et al. demonstrated that ATM phosphorylates TopBP1 leading to enhanced ATR interaction and activation (Yoo et al., 2009). The interaction between ATM and TopBP1 also seems to require CtIP and the MRN complex (Yoo et al., 2009; Ramírez-Lugo et al., 2011).

## 7. Replication fork stalling

Stalling of DNA replication can occur when the replication fork encounters a physical impediment, such as unrepaired DNA lesions or stable secondary DNA structures. Extensive fork stalling can induce ATR checkpoint activation, which can promote fork stabilization and recovery. This process is not fully understood but involves many ATR target proteins, including homologous recombination factors, nucleases and helicases, and may involve a regressed replication fork as an intermediate (Cortez, 2015; Yeeles et al., 2013). Malfunctioning of the ATR checkpoint response, or failure to resolve the blocked replication fork, results in replication fork collapse. This causes a double-strand break and can lead to chromosomal rearrangements and genome instability.

In human cells, stalled replication forks are most often induced by addition of hydroxyurea, which depletes dNTP pools. In *Xenopus* egg extract, fork stalling can be accomplished by the addition of aphidicolin. Using LSS egg extract in combination with aphidicolin-treated sperm chromatin, it was established that ATR and ATM signaling, as well as the proteins Tipin and MRE11, are required for the recovery after fork stalling (Errico et al., 2007; Trenz et al., 2006). Furthermore, it was shown that phosphorylation of the DNA translocase SMARCAL1 by ATR plays an important role in limiting fork processing to ensure stabilization of a stalled fork (Couch et al., 2013). In addition to fork stalling, fork collapse was mimicked in egg extract by adding a nicking enzyme to the reaction, resulting in a DSB once the replication fork encountered the nick. This work indicated that some of the replisome components, such as GINS and polymerase ε, unload after fork collapse, and their reloading is mediated by Rad51 and MRE11 (Hashimoto et al., 2011).

Repetitive DNA sequences, or sequences that contain secondary structures, can also induce replication fork stalling. *Xenopus* egg extract (LSS) was recently used to study replication of human chromosomal segments containing repetitive sequences (Aze et al., 2016). While the repetitive sequences caused mild reduction in replication fork progression, indicative of fork stalling, surprisingly,

this was not accompanied by ATR activation. Electron microscopy showed dense DNA structures that likely prevent RPA loading and ATR activation at these repetitive centromeric sequences to facilitate their replication (Aze et al., 2016). Another study used single stranded DNA templates to investigate how the DNA replication machinery bypasses stable secondary DNA structures, called G-quadruplexes, formed in G-rich sequences (Castillo Bosch et al., 2014). In this study, DNA replication starts from a primer on the G-quadruplex containing ssDNA templates in HSS. Replication stalls transiently at the site of the G-quadruplex structure and resumes quickly after the secondary structure has been resolved. Unwinding of the G-quadruplex structure is in part mediated by the FANCJ helicase. This study suggests that there are several different mechanisms present in egg extract that can unwind these secondary structures. Consistent with this, several helicases have been shown to be able to unwind G-quadruplexes in reconstitution systems (Mendoza et al., 2016). In the future, the *Xenopus* egg extract system can be used to gain insight into the relative roles of these helicases in G-quadruplex unwinding, and to study how these unwinding mechanisms are regulated.

## 8. Discussion and future directions

The *Xenopus* egg extract system has made major contributions to our knowledge of several important genome maintenance pathways. The ability of these extracts to support DNA replication and repair, and the use of cleverly designed DNA templates (see Table 1), has provided unique opportunities to determine important mechanistic details of DNA repair pathways. Immunodepletion of specific proteins, in combination with rescue experiments using wildtype and mutant proteins, has proven to be a valuable method to determine biochemical function of the proteins acting in these pathways. However, the dependence on immunodepletions to remove specific proteins can also be a limitation. It is time consuming and expensive to generate antibodies capable of depletion, and co-depletion of interacting factors can complicate the results of the experiments. Yet, the latter can also be used as an advantage to gain insights into the composition of protein complexes in a physiological setting.

In the future, the development of additional sequence specific chemical modifications to DNA templates, such as various different DNA interstrand crosslinks, can create many additional opportunities to examine poorly understood DNA repair pathways. In addition, *Xenopus* egg extracts are efficient in nucleosome assembly, which enables the examination of chromatin remodeling during DNA repair in addition to damage dependent histone modifications. Furthermore, several recent studies have shown the great potential of combining the *Xenopus* egg extract system with mass spectrometry. This approach has been used to identify novel factors in checkpoint signaling and DSB repair (Duursma et al., 2013; Räschle et al., 2015). DNA templates, with repair factors bound to them, are isolated during these processes and the proteins are identified by mass spectrometry. The recent sequencing of the *Xenopus laevis* genome will further facilitate such approaches (Session et al., 2016). A similar technique that allows the isolation and identification of proteins on nascent DNA (iPond) from cells has recently been developed by the Cortez laboratory (Sirbu et al., 2011). While this is a very effective technique it depends on active DNA replication and it does not allow the use of exogenously modified DNA templates. Another promising development is the combination of *Xenopus* egg extract with single-molecule techniques that was recently established to study NHEJ. If this approach is expanded to study additional DNA repair pathways it could make major contributions in our knowledge of the biochemistry and kinetics of these pathways.

Recently, the Diffley laboratory succeeded in the in vitro reconstruction of budding yeast DNA replication initiation and elongation from purified components (Yeeles et al., 2015, 2017) This is a major accomplishment and will be invaluable in our biochemical understanding of DNA replication. Further development of this system will likely allow the study of several replication-linked processes such as DNA repair, chromatin remodeling and histone modifications. However, this system depends on the knowledge of all components required for these processes and this is not always available. In addition, there is no active signaling or cell cycle context in this system, which also limits the questions that can be addressed. Nonetheless, a combination of reconstitution, extract, and cellular systems will be required to start fully understanding the biochemical details of DNA repair mechanisms. This is of great importance for understanding how our genome is kept stable and can help the development of novel anti-cancer drugs.

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