Published in final edited form as:

Methods Mol Biol. 2019 ; 1999: 161–172. doi:10.1007/978-1-4939-9500-4_9.

Methods for studying DNA single-strand break repair and signaling in *Xenopus laevis* egg extracts

Yunfeng Lin, Anh Ha, and Shan Yan

Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, NC 28223, USA

Abstract

DNA single-strand breaks (SSBs) are the most common type of DNA lesions as they are generated approximately 10,000 times per mammalian cell each day. Unrepaired SSBs compromise DNA replication and transcription programs, leading to genome instability, and have been implicated in many diseases including cancer. In this chapter, we introduce methods to study the ATR-Chk1 DNA damage response (DDR) pathway and DNA repair pathway in response to a site-specific, defined SSB plasmid in *Xenopus laevis* egg extracts. This experimental system can be applied in future studies to reveal many aspects of the molecular mechanisms of SSB repair and signaling in eukaryotes.

Keywords

DNA damage response; SSB end resection; SSB repair; SSB signaling; *Xenopus* egg extracts

1 Introduction

DNA single-strand breaks (SSBs) are derived from oxidative stress, intermediate DNA repair products, and abortive cellular enzymes [1,2]. It has been estimated that approximately 10,000 times of SSBs are generated per mammalian cell each day; therefore, SSBs are considered as the most frequent type of DNA lesions [3,4]. If not repaired appropriately or promptly, SSBs will compromise DNA replication and transcription programs within cells, leading to genome instability [1,5]. Notably, accumulation of unrepaired SSBs has been implicated in cancer, aging, and neurodegenerative diseases [1,2,6]. However, the molecular mechanisms of SSB repair and signaling remain incomplete, largely because of the lack of a tractable experimental system [5].

Xenopus laevis egg extract systems have been developed and widely utilized in various studies of DNA metabolisms, such as DNA replication, DNA repair, and DNA damage response (DDR) pathways [7–14]. In particular, mechanistic studies using *Xenopus* system have provided critical insights into the molecular details of DNA repair and DDR pathways in response to DNA double-strand breaks (DSBs) and inter-strand crosslinks (ICLs) [15–19].

*Corresponding author: Shan Yan, Department of Biological Sciences, University of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC 28223, USA, Telephone: +1-704-687-8528, shan.yan@uncc.edu.

There are several different types of *Xenopus* egg extracts: low-speed supernatant (LSS), high-speed supernatant (HSS), and nucleoplasmic extracts (NPE) [20,21]. The detailed approaches to prepare these *Xenopus* egg extracts have been described previously [7,20,22].

In this chapter, taking advantage of *Xenopus* HSS system, we introduce methods to study SSB repair, SSB-induced DDR pathway, and the innovative 3'–5' SSB end resection process [5]. In particular, we will describe step-by-step methods including: generating a defined SSB plasmid, analyzing DNA repair of the defined SSB plasmid in the HSS system, elucidating activation and regulation of SSB signaling in the HSS system, isolating DNA-bound fractions from HSS to examine protein recruitment onto SSB site and dissecting the 3'–5' SSB end resection in the HSS system. Future studies using these methods will help to dissect molecular aspects of SSB repair and signaling pathways in eukaryotes.

2 Materials

2.1 Preparation of SSB plasmid and FAM-SSB

1. pS plasmid: mutant pUC19 by QuikChange II XL site-directed mutagenesis kit
2. *Nt.Bst**NI* site specific endonuclease
3. 10× NEB Buffer 3.1
4. 10× NEB CutSmart Buffer
5. TE: 1 mM EDTA, 10 mM Tris, pH 8.0
6. Alkaline Phosphatase, Calf Intestine (CIP)
7. Plasmid midi or maxi kit
8. Gel extraction kit
9. KOD hot start DNA polymerase kit
10. Forward primer: 6-FAM-5'-TCGGTACCCGGGGATCCTCTAG-3'
11. Reverse primer: 5'-ACAGCTATGACCATGATTACG-3'
12. M1-F primer: 5'-
GGTAACTATCGTCTTGAATCCAACCCGGTAAGACACG-3'
13. M1-R primer: 5'-CGTGTCTTACCGGGTTGGATTCAAGACGATAGTTACC-3'
14. M2-F primer: 5'-
CGTTCATCCATAGTTGCCTGGCTCCCCGTCGTGTAGATAAC-3'
15. M2-R primer: 5'-
GTTATCTACACGACGGGAGCCAGGCAACTATGGATGAACG-3'
16. M3-F primer: 5'-
CCGCTTCCTCGCTCACTGGCTCGCTGCGCTCGGTCGTTC-3'
17. M3-R primer: 5'-
GAACGACCGAGCGCAGCGAGCCAGTGAGCGAGGAAGCGG-3'

18. 1% and 2% agarose gel

2.2 DNA purification

1. Agarose gel with ethidium bromide staining
2. Nuclease-free water
3. 3 M Sodium acetate (pH 5.0)
4. Phenol-Chloroform
5. 1 mg/mL Glycogen
6. Ethanol: 100%, and cold 70%

2.3 Isolation of DNA-bound fractions from the HSS system

1. High-speed supernatant (HSS)
2. 10× ELB (Egg lysis buffer) salt: 25 mM MgCl₂; 500 mM KCl; 100 mM HEPES-KOH, pH 7.7
3. 1 M Sucrose in water
4. ELB: 1× ELB salt, 0.25 M sucrose, pH 7.7 (*see Note 1*)
5. Sucrose cushion: 0.9 M sucrose in ELB
6. 2× Laemmli Sample Buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8

2.4 Fluorescence analysis

1. 10× TBE running buffer: Dissolve 108 g Tris base and 55 g Boric acid in 800 mL of water. Add 40 mL of 0.5 M EDTA (pH 8.0), and bring volume to 1 L (*see Note 2*).
2. 40% acrylamide/Bis solution: 19:1 (i.e., 5% crosslinker)
3. 10% Ammonium persulfate in water
4. TEMED (tetramethylethylenediamine)
5. 2 × TBE-Urea sample buffer: 90 mM Tris, 90 mM Boric acid, 2 mM EDTA, 12% Ficol, 7 M Urea, 0.03% Bromophenol blue, 0.03% Xylene cyanol
6. Typhoon 8600 imager
7. ImageJ software

The egg lysis buffer (ELB) should be freshly made, and can continue to be used for ~4 hours on ice. It is better to use the TBE running buffer within two weeks, or the boric acid will precipitate, which may or may not effect to the results. EDTA needs to be heated and the pH is adjusted to facilitate solubility.

3 Methods

3.1 Preparation of a defined site-specific SSB plasmid

We will introduce methods to prepare control (CTL) plasmid pS, SSB plasmid, and DSB plasmid in this section (Figure 1). To ensure the quality of SSB plasmid, steps or practices leading to unnecessary shearing of the DNA (e.g., vortex and frequent freeze and thaw) should be avoided or minimized, if possible.

1. To generate plasmid pS, mutant plasmid pUC19 on three sites (nt 1177–1181 on “+” strand, nt 706–710 on “-” strand, and nt 1694–1698 on “-” strand) sequentially with three pairs of primers (i.e., M1-F and M1-R primers, M2-F and M2-R primers, and M3-F and M3-R primers) using QuikChange II XL site-directed mutagenesis kit.
2. Sequence plasmid pS to verify mutations on the target sequence sites. Three point mutations (i.e., G1179A, A707G, A1695G) are created in pS. There is only one recognition site on pS for *Nt.BstNBI* (i.e., nt 427–431 on “+” strand).
3. Prepare a large amount of plasmid pS (e.g., several milligrams) using plasmid midi/maxi kit following vendor’s procedure. Utilize untreated pS as control (CTL) plasmid in experiments.
4. To generate a defined SSB plasmid between C435 and T436, treat the pS with *Nt.BstNBI* (10 U/μg) in 1× NEB Buffer 3.1 for 2 hours at 55 °C.
5. To generate the DSB plasmid, treat the pS with *SbfI-HF* (10 U/μg) with 1× NEB CutSmart Buffer for 2 hours at 37 °C.
6. Add CIP (10 U/μg) to the enzyme-treated pS mixture for 1 hour at 37 °C to catalyze the dephosphorylation at the 5’ side of SSB or DSB (Figure 1) (*see* Note 3).
7. Run samples on a 1% agarose gel and then gel purify the SSB plasmid via the gel extraction kit, followed by an optional Phenol-Chloroform extraction (*see* Note 4).
8. Dilute the SSB plasmid to a final concentration of 375 ng/μL with TE, and save at –20 °C or –80 °C in small aliquots (e.g., 20 μL) to avoid frequent freeze and thaw cycles.

3.2 Analysis of DNA repair of the defined SSB plasmid in the HSS system

We have established an assay to analyze DNA repair of the defined SSB plasmid in the HSS system [5]. Here is the detailed method for SSB repair analysis.

After the treatment by *Nt.BstNBI*, the sample should be cooled down to 37 °C or below prior to the CIP treatment. CIP may lose its activity if added to the tube at 55 °C.

The recovery of purified plasmid from a gel extraction kit is around 50% in our hands. Loading the dissolved gel slices through the columns 1–2 more times can increase the binding of plasmid DNA to the columns, leading to higher overall yield using the gel extraction kit. Phenol–Chloroform extraction can increase the quality of the purified plasmid preparation but may decrease the overall yield of the plasmid.

1. Add 4 μL of the defined SSB plasmid (e.g., 375 ng/ μL) to 16 μL of HSS in microcentrifuge tube.
2. After incubation at room temperature for different timepoints (e.g., 1, 5, and 30 minutes), add nuclease-free water to each reaction of HSS that was incubated with SSB plasmid to a total volume of 300 μL (*see* Note 5).
3. Add equal volume of Phenol-Chloroform (i.e., 300 μL) to the reaction mixture and mix up and down at least 5 times.
4. Spin the mixture for 5 minutes at 16,000 rcf at room temperature.
5. Transfer the top aqueous layer to a new tube and repeat the Phenol–Chloroform extraction another 3 – 5 times until no obvious protein is left between the boundary of Phenol–Chloroform and water layers (*see* Note 6).
6. Supplement the final aqueous solution with sodium acetate (a final concentration of 0.3 M), glycogen (a final concentration of 1 $\mu\text{g}/\mu\text{L}$), and ethanol (100%, 2.5-fold volume/volume), followed by incubation at $-80\text{ }^{\circ}\text{C}$ for at least 30 minutes (*see* Note 7).
7. Spin the mixture for 15 minutes at 16,000 rcf at room temperature. Wash the pellet with 1 mL of cold 70% ethanol and air-dry for 30 minutes at room temperature before resuspension with 30 to 40 μL of nuclease-free water.
8. Quantify the purified DNA repair products via NanoDrop, and examine by 1% agarose gel electrophoresis stained with ethidium bromide (*see* Note 8). Gradual repair will occur in the HSS system (Figure 2).

3.3 Analysis of SSB signaling in the HSS system

The defined SSB plasmid but not CTL plasmid triggers Chk1 phosphorylation in the HSS [5]. As shown below, we have optimized the experimental conditions to establish the defined SSB induced ATR-Chk1 DDR pathway in the HSS and to analyze the recruitment of checkpoint proteins to SSB site in the HSS system.

3.3.1 Establishment of the defined SSB-induced DDR pathway in the HSS system

1. In microcentrifuge tube, add 2 μL of CTL or SSB plasmid to 8 μL of HSS to different final concentrations (e.g., 1, 50, 75, and 100 ng/ μL), followed by

Two reasons to bring up the mixture volume up to 300 μL with nuclease free water: one is to dilute the proteins in the HSS for better protein extraction and two is to increase the overall yield of DNA. If the overall DNA yield is low, consider the option of resuspending the mixture with nuclease free water to a total volume of 600 μL .

A lot of DNA binding proteins can be bound with plasmid from the HSS. The additional 3–5 repeats of Phenol-Chloroform extraction is quite important to get rid of DNA-bound proteins from the repair products. If proteins are not removed completely or still bound with DNA, it is difficult to analyze DNA repair products appropriately via subsequent agarose gel electrophoresis. Because additional Phenol-Chloroform extraction also means less recovery of DNA, a balance in the number of Phenol-Chloroform extractions and yield of DNA should be considered and optimized.

Longer incubation and lower temperature provide better recovery of DNA.

A lower percentage agarose gel will offer better separation of DNA repair products, but the gel is more easily broken. 1% agarose is a starting percentage, and can be optimized.

incubation for different time (e.g., 1, 5, 30, or 60 minutes) at room temperature with gentle inversion every 10 minutes (*see* Note 9).

2. Add 50 μL of 2 $\times$ Laemmli Sample Buffer to each 10 μL of reaction mixture followed by immunoblotting analysis for Chk1 phosphorylation at the Ser 344 (total Chk1 as loading control). Figure 3 shows example results. The dose-dependent experiment indicates that 75 ng/ μL of the defined SSB structure, but not CTL plasmid, triggered a robust Chk1 phosphorylation at S344 after 30-minute incubation in the HSS system (Figure 3a) (*see* Note 10, 11). The time-dependent experiment shows that the SSB-induced Chk1 phosphorylation appeared at 5 minutes, and peaked at 30 minutes after incubation in the HSS (Figure 3b) (*see* Note 12). This SSB signaling technology can be tailored to future mechanistic studies using immunodepletion approach and/or small molecule inhibitors (*see* Note 13).

3.3.2 Analysis of protein recruitment to SSB site in the HSS system

1. Incubate 10 μL of the SSB plasmid or CTL plasmid with 40 μL of HSS (to a final concentration of 75 ng/ μL) for 30 minutes at room temperature (*see* Note 14).
2. Then, dilute the 50 μL of reaction mixture with 200 μL of ELB buffer and spin through a 1 mL of sucrose cushion at 9,000 rcf for 15 minutes at 4 °C in a swinging bucket.
3. After centrifugation, remove the supernatant, and resuspend the DNA-bound protein fractions with 30 μL of 2 $\times$ Laemmli Sample Buffer. Then examine the DNA-bound fractions via immunoblotting analysis (Figure 4) (*see* Note 15).

3.4 SSB end resection in the 3'–5' direction in the HSS system

To detect the SSB end resection in the 3'–5' direction in the HSS system, we have developed a method to prepare the FAM-labeled SSB structure (designated as FAM-SSB, shown in Figure 5a). The purified FAM-SSB structure can be utilized in the HSS system to examine the 3'–5' SSB end resection (Figure 5b).

Different batches of HSS preparation may have different quality. Thus, it is a good practice to optimize the concentration of SSB plasmid and incubation times in the HSS system. Nevertheless, ensuring high quality of HSS is very important for the SSB signaling experiments.

Frequent freeze and thaw cycles may generate more unwanted DNA breaks in plasmid DNA. We typically make 375 ng/ μL of the defined SSB in stock. For every use, we can then just dilute the plasmid in HSS (i.e., 1 volume : 4 volume) to set up the reactions. The defined SSB structure (75 ng/ μL), but not CTL plasmid, triggered a robust Chk1 phosphorylation at S344 after 30-minute incubation in the HSS system (Figure 3a). Higher concentration of SSB plasmid than 75 ng/ μL in the HSS had no noticeable effect on Chk1 phosphorylation (Figure 3a). These observations suggest that the defined SSB structure induces a unique ATR-Chk1 DDR pathway in the HSS in a dose-dependent manner.

Incubation periods of longer than 30 minutes at room temperature had no noticeable effect on Chk1 phosphorylation (Figure 3b). These observations suggest that the SSB structure induces a unique checkpoint signaling in the HSS in a time-dependent manner. This SSB signaling system can also be applicable for studies testing whether other checkpoint proteins play a direct role for the SSB signaling, especially proteins required for DNA replication initiation and/or elongation. The protein of interest (e.g., APE2, [5]) can be removed via immunodepletion using antibodies against the target protein. Alternatively, small molecule inhibitor specific for target protein (e.g., ATR specific inhibitor VE-822, [5]) can be added to the HSS system to test whether such small molecule affects the SSB-induced ATR-Chk1 DDR pathway in the HSS system.

This is scaled up in comparison to the experimental setup described in section 3.3.1. for detectable yield of DNA-bound fractions. PCNA can be used as a loading control, since it binds to both CTL and SSB plasmid under these experimental conditions. The representative checkpoint proteins such as ATR, RPA32, and Rad9 are shown in Figure 4.

3.4.1 Preparation of FAM-SSB structure

1. First, generate a FAM-labeled dsDNA (i.e., 70 bp dsDNA with FAM-labeled at the 5'-side of plus strand) by PCR using the forward and reverse primers with the plasmid pS as template. PCR conditions include denaturation at 95 °C for 30 seconds, annealing at 60 °C for 10 seconds, and extension at 70 °C for 30 seconds for a total of 30 cycles.
2. Then purify the FAM-dsDNA PCR product via gel extraction.
3. Treat the purified FAM-dsDNA with *Nt.BstNBI* (10 U/μg) for 2 hours at 55 °C.
4. Then cool the tube down to ~37 °C, and supplement with CIP (10 U/μg) for 1 hour at 37 °C to dephosphorylate at the 5' side of the SSB structure.
5. Purify the FAM-labeled SSB structure on a 2% agarose gel. After gel extraction, save the FAM-SSB structure at -20 °C in small aliquots for *in vitro* exonuclease assay.

3.4.2 SSB end resection of FAM-SSB structure in the HSS system

1. To examine how SSB is resected, incubate 8 μL of the FAM-SSB substrate (50 nM) with 112 μL of HSS at room temperature.
2. After incubation at different time points (i.e., 0, 1, 5, 10, 15, or 30 min), take out 12 μL of sample and mix with 12 μL of 2× TBE-Urea sample buffer (i.e., equal volume) to quench the reactions. Then denature the samples at 95 °C for 5 minutes.
3. Without cooling down, load and resolve the hot samples on a 16% TBE-Urea denaturing polyacrylamide gel (*see* Note 16). Add 'Marker' including four FAM-labeled different-length of ssDNA to label the positions of DNA migration on TBE-Urea gel.
4. To prepare a 16% polyacrylamide gel, mix 4 mL of 10× TBE running buffer, 16 mL of 40% acrylamide mixture and 8 mL of water in 100 mL flask. Add 16.8 g urea into the flask and keep stirring by magnetic bar (*see* Note 17). Add 300 μL of 10% ammonium persulfate and 30 μL of TEMED and cast gel within an 18.3 cm × 20 cm × 3 mm gel cassette. Insert a 15-well gel comb immediately without introducing air bubbles. Once the gel is solidified, pre-run the TBE-Urea gel for at least 30 minutes at 20 W.
5. Once the samples are loaded, run the TBE-Urea gel in 1× TBE running buffer at 25 W for ~ 1 – 2 hours (*see* Note 18).
6. Scan the TBE-Urea gel using a Typhoon 8600 imager (PMT at 600 V, excitation at 532 nm and emission at 526 nm). Use ImageQuant 5.2 to visualize gels, and quantify DNA products with ImageJ, if needed. As shown in Figure 5b, the

The chosen percentage of gel is based on the size of the FAM-DNA structure, and can be optimized. It may take up to 10 minutes for Urea to dissolve. The final volume is around 40 mL. The running time for TBE-Urea gel can be optimized depending on the size of ssDNA products.

FAM-SSB can be resected in the 3' to 5' direction into Type I resected products in the HSS system (*see* Note 19).

Acknowledgement

The Yan lab was supported, in part, by funds from University of North Carolina at Charlotte (Duke Energy Endowment Special Initiatives Fund and Faculty Research Grants) and grants from the National Institute of General Medical Sciences (R15GM101571 and R15GM114713) and the National Cancer Institute (R01CA225637) in the National Institutes of Health.

References

1. Caldecott KW (2008) Single-strand break repair and genetic disease. *Nat Rev Genet* 9 (8):619–631. doi:10.1038/nrg2380 [PubMed: 18626472]
2. Yan S, Sorrell M, Berman Z (2014) Functional interplay between ATM/ATR-mediated DNA damage response and DNA repair pathways in oxidative stress. *Cell Mol Life Sci* 71 (20):3951–3967. doi:10.1007/s00018-014-1666-4 [PubMed: 24947324]
3. Ciccia A, Elledge SJ (2010) The DNA damage response: making it safe to play with knives. *Mol Cell* 40 (2):179–204. doi:10.1016/j.molcel.2010.09.019 [PubMed: 20965415]
4. Tubbs A, Nussenzweig A (2017) Endogenous DNA Damage as a Source of Genomic Instability in Cancer. *Cell* 168 (4):644–656. doi:10.1016/j.cell.2017.01.002 [PubMed: 28187286]
5. Lin Y, Bai L, Cupello S, Hossain MA, Deem B, McLeod M, Raj J, Yan S (2018) APE2 promotes DNA damage response pathway from a single-strand break. *Nucleic Acids Res* 46 (5):2479–2494. doi:10.1093/nar/gky020 [PubMed: 29361157]
6. Nassour J, Martien S, Martin N, Deruy E, Tomellini E, Malaquin N, Bouali F, Sabatier L, Wernert N, Pinte S, Gilson E, Pourtier A, Pluquet O, Abbadie C (2016) Defective DNA single-strand break repair is responsible for senescence and neoplastic escape of epithelial cells. *Nat Commun* 7:10399. doi:10.1038/ncomms10399 [PubMed: 26822533]
7. Walter J, Sun L, Newport J (1998) Regulated chromosomal DNA replication in the absence of a nucleus. *Mol Cell* 1 (4):519–529 [PubMed: 9660936]
8. Van C, Yan S, Michael WM, Waga S, Cimprich KA (2010) Continued primer synthesis at stalled replication forks contributes to checkpoint activation. *J Cell Biol* 189 (2):233–246. doi:10.1083/jcb.200909105 [PubMed: 20385778]
9. Kumagai A, Shevchenko A, Shevchenko A, Dunphy WG (2010) Treslin collaborates with TopBP1 in triggering the initiation of DNA replication. *Cell* 140 (3):349–359. doi:10.1016/j.cell.2009.12.049 [PubMed: 20116089]
10. Wallace BD, Berman Z, Mueller GA, Lin Y, Chang T, Andres SN, Wojtaszek JL, DeRose EF, Appel CD, London RE, Yan S, Williams RS (2017) APE2 Zf-GRF facilitates 3'–5' resection of DNA damage following oxidative stress. *Proc Natl Acad Sci USA* 114 (2):304–309. doi:10.1073/pnas.1610011114 [PubMed: 28028224]
11. DeStephanis D, McLeod M, Yan S (2015) REV1 is important for the ATR-Chk1 DNA damage response pathway in *Xenopus* egg extracts. *Biochem Biophys Res Commun* 460 (3):609–615. doi:10.1016/j.bbrc.2015.03.077 [PubMed: 25800873]
12. Yan S, Willis J (2013) WD40-repeat protein WDR18 collaborates with TopBP1 to facilitate DNA damage checkpoint signaling. *Biochem Biophys Res Commun* 431 (3):466–471. doi:10.1016/j.bbrc.2012.12.144 [PubMed: 23333389]
13. Bai L, Michael WM, Yan S (2014) Importin beta-dependent nuclear import of TopBP1 in ATR-Chk1 checkpoint in *Xenopus* egg extracts. *Cell Signal* 26 (5):857–867. doi:10.1016/j.cellsig.2014.01.006 [PubMed: 24440306]
14. Yan S (2015) Teaching and learning in a *Xenopus* research lab. *Lab Anim (NY)* 44 (8):327. doi:10.1038/labani.817 [PubMed: 26200089]

Because the resected products are arranged from ~4 nt to ~12 nt, it is estimated that the SSB structure is resected ~18 nt to ~26 nt in the 3' to 5' direction in the HSS system.

15. Costanzo V, Robertson K, Bibikova M, Kim E, Grieco D, Gottesman M, Carroll D, Gautier J (2001) Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. *Mol Cell* 8 (1):137–147 [PubMed: 11511367]
16. Dupre A, Boyer-Chatenet L, Gautier J (2006) Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex. *Nat Struct Mol Biol* 13 (5):451–457. doi:10.1038/nsmb1090 [PubMed: 16622404]
17. You Z, Shi LZ, Zhu Q, Wu P, Zhang YW, Basilio A, Tonnu N, Verma IM, Berns MW, Hunter T (2009) CtIP links DNA double-strand break sensing to resection. *Mol Cell* 36 (6):954–969. doi:10.1016/j.molcel.2009.12.002 [PubMed: 20064462]
18. Raschle M, Knipscheer P, Enoiu M, Angelov T, Sun J, Griffith JD, Ellenberger TE, Scharer OD, Walter JC (2008) Mechanism of replication-coupled DNA interstrand crosslink repair. *Cell* 134 (6):969–980. doi:10.1016/j.cell.2008.08.030 [PubMed: 18805090]
19. Ben-Yehoyada M, Wang LC, Kozekov ID, Rizzo CJ, Gottesman ME, Gautier J (2009) Checkpoint signaling from a single DNA interstrand crosslink. *Mol Cell* 35 (5):704–715. doi:10.1016/j.molcel.2009.08.014 [PubMed: 19748363]
20. Lebofsky R, Takahashi T, Walter JC (2009) DNA replication in nucleus-free *Xenopus* egg extracts. *Methods Mol Biol* 521:229–252. doi:10.1007/978-1-60327-815-7_13 [PubMed: 19563110]
21. Cupello S, Richardson C, Yan S (2016) Cell-free *Xenopus* egg extracts for studying DNA damage response pathways. *Int J Dev Biol* 60 (7–8-9):229–236. doi:10.1387/ijdb.160113sy [PubMed: 27160070]
22. Willis J, DeStephanis D, Patel Y, Gowda V, Yan S (2012) Study of the DNA damage checkpoint using *Xenopus* egg extracts. *J Vis Exp* (69):e4449. doi:10.3791/4449

Figure 1. Schematic diagram of the generation of defined SSB and DSB structures. This figure is adapted from Lin et al., 2018, *Nucleic Acids Research* [5].

Figure 2.

The defined SSB structure is gradually repaired in HSS system after incubation at different times (0, 1, 5, or 30 minutes) in the HSS system. Marker represents the 1kb DNA ladder. The result from this figure is consistent with Fig. 1B from Lin et al., 2018, *Nucleic Acids Research* [5].

Figure 3.

SSB structure induces a novel ATR-Chk1 DDR pathway in the HSS system in a dose- and time-dependent manner. **(a)** CTL or SSB plasmid was added to HSS at different concentration as indicated. After in a 30-min incubation, extracts were examined for Chk1 phosphorylation via immunoblotting analysis. **(b)** CTL or SSB plasmid was added to HSS at a final concentration of 75 ng/ μ L for indicated time. Extracts were examined for Chk1 phosphorylation via immunoblotting analysis. The result from this figure is consistent with Fig. 2A and 2B from Lin et al., 2018, *Nucleic Acids Research* [5].

Figure 4.

Checkpoint proteins are recruited to the SSB site in the HSS system. CTL or SSB plasmid was added to HSS followed by incubation at room temperature. Then the DNA-bound fractions from HSS were isolated, and examined via immunoblotting analysis.

Figure 5.

The FAM-SSB structure is resected in the 3'–5' direction in the HSS system. (a) Schematic presentation of the defined FAM labeled SSB structure (i.e., FAM-SSB). (b) FAM-SSB structure was added to HSS for different time as indicated. Then samples were examined via 16% TBE-Urea denaturing gel and visualized via Typhoon imager. The result from this figure is consistent with Fig. 6B in Lin et al., 2018, *Nucleic Acids Research* [5].