

DNA Replication Fork Restart and Homology-Based DNA Double-Strand Break Repair

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Cells are under constant attack from numerous exogenous and endogenous DNA damaging agents. Most of these lesions are repaired, but some escape repair and might stall the progression of replication forks. Stalled replication forks, if not properly restarted, might be processed into DSBs by various repair proteins, in particular those involved in homologous recombination. It has been estimated that up to 50 DSBs are produced during each cell cycle, mostly during the phase of DNA replication or S phase. DSBs can lead to chromosome breakage and chromosome rearrangements, which are hallmarks of most tumors.

The research in our laboratory focuses on understanding the biochemical mechanisms for DSB repair and replication fork restart. Currently, three major pathways have been identified to repair DSBs: non-homologous end joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA). NHEJ is an error-prone pathway that re-ligates DNA ends after they are polished. HR uses homologous sequence on the sister chromatid or homologous chromosome as the template to repair the missing information and it is error-free. SSA occurs when the break is between two direct repeat sequences and the final result is the deletion of one of the two repeats. Like HR, SSA is also homology dependent and both pathways are initiated by the processing of broken DNA ends into single-stranded tails. Moreover, recent genetic studies in yeast and *Drosophila* have suggested that HR and SSA share additional mechanistic similarities. Whereas meiotic HR proceeds via Holliday junction formation and resolution, mitotic HR appears to use synthesis-dependent DNA annealing (SDSA) during which the invading strand is extended but then released from the D-loop before the formation of Holliday junction. This extended strand is now complementary to the single strand from the other side of the break and the break is then repaired in a reaction essentially identical to SSA.

NHEJ has been well studied by a combination of genetic analysis *in vivo* and biochemical reconstitution *in vitro*. In contrast, our understanding of homology-dependent DSB repair has been more limited due to the lack of an *in vitro* system that can reconstitute these pathways. Similarly, proteins involved in homology-dependent DSB repair and cell cycle checkpoint response have been implicated in the restart of stalled replication forks, but the exact mechanism remains vague due to the lack of an *in vitro* biochemical system. Using extracts derived from the frog, *Xenopus laevis*, we have succeeded in establishing two powerful *in vitro* systems, one for replication of DNA containing a site-specific lesion and the other for homology-directed DSB repair. These two systems have allowed us to address some of the most fundamental mechanistic questions about homology-dependent DSB repair and replication fork restart.

Biochemical analysis of homology-directed double-stranded break repair. Toczylowski, McCane, Yan

Using nucleoplasmic extracts (NPE) derived from nuclei reconstituted in *Xenopus* egg extracts, we have succeeded in establishing an

in vitro biochemical system for single-strand annealing (SSA) repair of DNA double-strand breaks. The repair reaction depends on homologous sequences located at the break and is independent of RAD51, the protein that mediates strand invasion during HR. Using this



system, we have found that the *Xenopus* homolog of Werner syndrome protein (xWRN or FFA-1), plays an important role in SSA. Werner syndrome protein is a member of the well-conserved RecQ helicase family, which is critical for genome stability in all organisms studied. This protein has been implicated in replication fork restart and DSB repair, but its exact role is poorly understood. Our study provides the first evidence that directly links WRN to a particular DSB repair pathway. We will continue to use this system to analyze the role of other RecQ-type helicases (such as the Bloom syndrome protein), DNA polymerases, MRE11, RPA, and checkpoint response proteins in SSA.

The mechanistic similarity between SSA and HR also makes our *in vitro* system useful for studying biochemical reactions that are critical to both repair pathways. One of these reactions is DNA end processing, which is still poorly understood despite tremendous effort by many labs. We have found that DNA ends are rapidly processed into 3' single-stranded tails in NPE, which are the expected intermediates for both strand invasion (during HR) and annealing (during SSA or SDSA). This observation has prompted us to initiate a systematic characterization of the mechanism of end processing. We have found that end processing is ATP dependent and may involve a coupled unwinding/single-stranded degradation reaction. More importantly, we found that xWRN is important for DNA end processing by specifically acting at the unwinding step. These studies have greatly expanded our research into the broader field of homology-directed repair and our biochemical approach using an *in vitro* system would be extremely valuable for elucidating many important mechanistic questions in this field.

Biochemical analysis of eukaryotic replication fork restart. Liao, Yan

Replication forks appear to stall or collapse upon encountering roadblocks such as DNA base lesions and single-strand nicks. Restarting and repairing replication forks depends on a multitude of proteins, many of which are key players in homology-based recombinational repair of double-strand breaks. In addition, checkpoint response is activated when replication forks are stalled and this checkpoint increases the stability of replication forks by a mechanism yet to be understood. The conver-

gence of replication, repair, recombination, and checkpoint response at replication forks represents a major paradigm shift in our understanding of genome maintenance. Most of the studies in this area have so far been conducted in genetic systems like bacteria and yeast. Many models have been proposed to explain the myriad of genetic data on the interplay among replication, repair, recombination, and checkpoints. However, the intrinsic limitation of genetic methods makes it difficult to directly test these models. To address these important issues, we have devoted major effort over the past three years to develop an *in vitro* system for replication fork stall and restart so that the underlying mechanism can be rigorously investigated with biochemical methods.

The system we have succeeded in developing utilizes nucleoplasmic extracts (NPE) derived from nuclei reconstituted in *Xenopus* egg extracts. *Xenopus* egg extracts is the only eukaryotic replication system that relies solely on cellular replication proteins such as ORC, MCMs, CDK2, CDC45, etc. This is in sharp contrast to viral replication systems, such as SV40, which depend on viral proteins like T antigen for DNA replication. Compared to normal egg extracts, NPE has an advantage in that it can also support high efficiency replication of plasmid DNA. In collaboration with Y. Matsumoto⁸ we have prepared plasmid DNA containing a specific lesion (apurine/apyrimidine (AP) site) as the substrate for studying replication fork stall and restart. AP sites are the common intermediate of base excision repair of many DNA lesions *in vivo* with a steady state level as high as 50,000–200,000 per genome in human and rodent tissues. *In vitro* AP sites are strong stalling sites for many DNA polymerases. Previous studies in yeast have suggested that translesion synthesis (TLS) is a major mode of replication over AP sites. However, it remains in dispute which nucleotide is inserted opposite AP sites during TLS, with some studies showing A (A-rule) and others showing C (C-rule) being the dominant nucleotide inserted. Also in dispute are exactly which TLS polymerases are recruited to stalled replication forks and how they coordinate with regular replicative polymerases during the replication of AP sites. In addition, it has been postulated that AP sites are replicated not only by TLS but also by an error-free (presumably

homologous recombination based) pathway, yet definitive evidence for the existence of such a pathway is still lacking. By using AP-containing DNA as the substrate for replication in NPE, we have succeeded in developing the first biochemical system to study how a fork-stalling lesion is replicated in a truly eukaryotic cellular replication system. The use of a site-specific lesion makes it possible for us to rigorously analyze the stalling site in relation to the lesion and the nucleotide(s) inserted opposite of the AP site after replication. Our data, which are being prepared for publication, provide convinc-

ing evidence that AP sites do stall replication forks and that both TLS and error-free modes are used to replicate over AP sites. Using this system, we are currently analyzing the role of various important proteins, such as Werner syndrome protein, Bloom syndrome protein, checkpoint protein ATR, recombination protein Rad51, and translesion DNA polymerases zeta and REV1, in lesion DNA replication. We expect our biochemical studies to reveal the intricate network of how these proteins interact with each other to facilitate the replication of DNA lesions.

Publications

Yan, H., McCane, J., Toczylowski, T., Chen, C. Analysis of the Xenopus Werner syndrome protein in DNA double-strand break repair. *J. Cell Biol.* 171:217-227, 2005.

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Advanta Championships: Event co-chairs Lauren Hart (left) and Virginia and Harvey Kimmel