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（要　旨）

**Introduction**

Double stranded breaks (DSBs) are a severe form of DNA damage that violates the integrity of the genome and may lead to diseases such as cancer. The most accurate pathway for the repair of DSBs is homologous recombination (HR). For HR to take place, DSB ends must be liberated from any occlusions and subsequently processed into 3' overhangs. This process is initiated by the Mre11-Rad50-Nbs1 (MRN) complex that nicks the 5' strand in the proximity of the break site. Such nuclease activity of the MRN complex requires phosphorylated CtIP/Ctp1/Sae2 through which it is regulated. However, the molecular mechanism of MRN regulation and activation by CtIP/Ctp1/Sae2 is largely unknown. In this work, I explore these mechanisms primarily by examination of an in vitro system using Schizosaccharomyces pombe MRN and Ctp1.

**Experimental Methods**

Recombinant S. pombe proteins were expressed in E. coli with the exception of Mre11-Rad50 (MR) complex and Nbs1 that were expressed in S. cerevisiae and S. frugiperda cells, respectively. Ctp1 was phosphorylated by Cka1 in the presence of ATP and re-purified. MRN-Ctp1 complex formation was demonstrated by co-immunoprecipitation of the His tagged Mre11/MR. Nuclease assays were generally conducted under 1:1 ratio of MRN subunits, Ctp1 and number of DNA ends. Synthetic peptides were ordered from Toray Research Center. Standard methodology was used for manipulation of S. pombe strains, sporulation and DNA damage assays.

**Results and Discussion**

***In vitro Reconstitution of S. pombe MRN-Ctp1 Complex***

I speculated that phosphorylation of Ctp1 by CK2 plays a critical role in MRN stimulation, possibly by causing the formation of MRN-Ctp1 complex. To test this, procedures for purification of S. pombe Mre11-Rad50 (MR) complex, Nbs1, Ctp1 and Cka1 (catalytic CK2 subunit) were established. First, I reconstituted the phosphorylation of Ctp1 in vitro, directly demonstrating that Cka1 phosphorylates Ctp1. When mixed with MRN complex subunits, phosphorylated Ctp1 strongly interacted with Nbs1. This interaction is essential for the formation of the Mre11-Rad50-Nbs1-Ctp1 (MRNC) complex. Phosphorylated Ctp1 was analyzed by mutational analysis as well as mass spectrometry to identify the N-terminally located SXT site as critical for interaction with Nbs1.

***Ctp1 Stimulation of MRN Complex Endonuclease Activities***

To determine if Ctp1 phosphorylation and MRNC complex formation play a role in stimulation of MRN nuclease, a DNA substrate with protein blocked ends was prepared. Phosphorylated Ctp1 strongly stimulated the MRN complex to cleave this substrate endonucleolytically, reaching maximal stimulation levels at 1:1 MRNC subunit ratio. Non-phosphorylated Ctp1 weakly stimulated MRN, with increasing amounts having a trend of overcoming the phosphorylation requirement. This strongly suggests that Ctp1 phosphorylation and MRNC complex formation does not cause the stimulation of endonuclease activity per se, but rather enhances the intrinsic potential of Ctp1 to stimulate MRN.

***Ctp1 C-terminal 15 Amino Acids (CT15) Site as the Critical Activator of MR Endonuclease Activities***

Ctp1 was systematically interrogated for the minimal functional motif of MR stimulation by truncation constructs. Remarkably, the minimal motif was found to be encircled within the last 15 C-terminal amino acids of Ctp1. This newly discovered site (CT15) is conserved between species. Mutation of conserved

CT15 aromatic residues caused DNA damage sensitivity similar to deletion of Ctp1 in vivo, supporting that CT15 is required for MR stimulation. Remarkably, synthetic peptide containing the CT15 sequence was as proficient as Ctp1 in stimulating the MR endonuclease. This strongly supports that the role of MRNC complex formation is the amplification of the MR activation by CT15.

***Activation of Human MRN Nuclease Activity by Peptide Derived from CtIP C-terminus***

I attempted to design a synthetic peptide for the stimulation of the human MRN complex based on my findings. Upon detailed inspection of conserved sequences, two CtIP phosphorylation sites were noticed within the immediate proximity of the CT15 site. Remarkably, synthetic peptide based on the CtIP derived CT15 site including these phosphorylated sites were proficient in stimulation of the human MRN complex. The identified conserved aromatic residues within such peptide were essential for its functionality, therefore strongly suggesting a conserved mechanism of MRN stimulation.

**Summary of Findings**

In this work I reconstituted the MRN and Ctp1 endonuclease reaction in vitro using purified Schizosaccharomyces pombe proteins. Using this system, I identified a conserved 15 amino-acid C-terminal sequence within Ctp1 (CT15) that is critical for MRN stimulation. Importantly, synthetic peptide activators of both S. pombe and human MRN complexes were derived based on this sequence. Furthermore, I reconstituted the phosphorylation of Ctp1 by CK2 in vitro and showed that phosphorylated Ctp1 is robustly recruited to the MRN complex via Nbs1. The MRN-Ctp1 complex formation drastically enhanced the endonuclease activation by CT15. I propose a mechanism of MRN stimulation and regulation, where phosphorylation dependent recruitment serves to amplify the constitutive activity of the C-terminal peptide activator of Ctp1. This core mechanism is likely conserved.