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Phosphorylation-regulated binding of Ctp1 to Nbs1 is critical for repair of DNA double-strand breaks

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Abstract

Repair of DNA double-strand breaks (DSBs) is critical for cell survival and for maintaining genome stability in eukaryotes. In *Schizosaccharomyces pombe*, the Mre11-Rad50-Nbs1 (MRN) complex and Ctp1 cooperate to perform the initial steps that process and repair these DNA lesions via homologous recombination (HR). While Ctp1 is recruited to DSBs in an MRN-dependent manner, the specific mechanism of this process remained unclear. We recently found that Ctp1 is phosphorylated on a domain rich in putative Casein kinase 2 (CK2) phosphoacceptor sites that resembles the SDTD repeats of Mdc1. Furthermore, phosphorylation of this motif is required for interaction with the FHA domain of Nbs1 that localizes Ctp1 to DSB sites. Here, we review and discuss these findings, and we present new data that further characterize the cellular consequences of mutating CK2 phosphorylation motifs of Ctp1, including data showing that these sites are critical for meiosis.

The integrity of the genome is constantly threatened by a variety of genotoxic insults, with one of the most detrimental being DNA double-strand breaks (DSBs). The heterotrimeric Mre11-Rad50-Nbs1 (MRN) complex is a critical element in the detection, signaling, and homologous recombination (HR) repair of DSBs.[1] The MRN complex consists of Mre11 nuclease, Rad50 scaffolding, and Nbs1 regulatory subunits. The N-terminus of Nbs1 contains a forkhead-associated (FHA) domain and proximal tandem Brca1 C-terminal (BRCT) repeats while the C-terminus of Nbs1 harbors interaction motifs for Mre11 and ATM. Both the FHA domain and BRCT repeats are phosphopeptide-binding motifs, often involved in regulation of the cell cycle and DNA damage response by participating in phosphorylation-dependent protein-protein interactions.[2, 3] While Mre11 and Rad50 have been thoroughly characterized structurally and functionally, the activities of Nbs1, especially those tied to the N-terminus, are not well understood. While it was initially reported that the N-terminal FHA-BRCT domains of Nbs1 enabled phosphorylation-dependent interaction with γH2AX at DSBs,[4] this association was later shown to require mediator of the DNA-damage checkpoint 1 (Mdc1).[5] Multiple groups then demonstrated in mammalian cells that the FHA-BRCT domains of Nbs1 interact with diphosphorylated SDTD repeats of Mdc1 and that this binding facilitates a positive feedback loop of ATM signaling from DSBs.[6-9] Interestingly, this process is likely initiated through phosphorylation of Mdc1 SDTD sites by Casein kinase 2 (CK2). CK2 is a highly conserved serine/threonine protein kinase that is constitutively active, ubiquitously distributed in eukaryotes, and participates in an abundance of cellular processes.[10] In particular, CK2 has recently been implicated in multiple DNA damage-response pathways, primarily through

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generating phosphorylation-dependent binding interfaces at sites of DNA damage that are recognized by FHA domain-containing DNA repair factors.[11-18]

The MRN protein complex performs its roles in HR through collaboration with the DNA end-processing factor Ctp1. Ctp1 is an ortholog of the budding yeast Sae2 and human CtIP proteins,[19] and is phosphorylated both basally and in a DNA damage dependent manner.[20] In *S. pombe*, Ctp1 and Nbs1 are genetically epistatic,[19] and Ctp1 functions as a multi-copy suppressor of the DNA damage sensitivity caused by *nbs1-s10*, an FHA domain point mutant.[20] Furthermore, in human cells, $CtIP^{Ctp1}$ interacts with the MRN complex,[21] and coprecipitates specifically with Nbs1 when both proteins are overexpressed.[22] From these data and the fact that fungi lack an Mdc1 homolog, we surmised that Nbs1 might associate with Ctp1 through a mechanism similar to the Nbs1-Mdc1 interaction. In support of this idea, our group and others identified a motif spanning amino acids 74-94 of Ctp1 that resembles the SDTD repeats of Mdc1, which we termed the SXT domain.[23, 24] We observed that a *ctp1-5A* (*ctp1-S77A T78A T79A S87A T89A*) mutant, in which the five potential CK2 phosphoacceptor sites of the SXT domain were changed to alanine (Fig. 1A), exhibited slow growth and extensive DNA damage sensitivity. However, this mutation caused no alteration of Ctp1 protein stability or subcellular localization. Most importantly, we found that this mutation altered phosphorylation of Ctp1 and inhibited recruitment of Ctp1 to a defined DSB as observed by chromatin immunoprecipitation (ChIP).

Remarkably, while the SXT domain of Ctp1 exhibits high conservation to the SDTD repeats of Mdc1, our additional mutational analyses revealed that a *ctp1-3A* (*ctp1-T78A T79A T89A*) mutant harboring combined alteration of SXT threonines alone (Fig. 1A) is sufficient for causing the severe DNA damage sensitivity observed in *ctp1-5A*. In addition, cells harboring individual or combined mutation of the SXT domain serines (Ser77/Ser87) exhibited no genotoxin sensitivity (data not shown). In contrast, the Mdc1-Nbs1 interaction depends on phosphorylation of both the serine and threonine of an individual SDTD motif.[6-9]

Mutation of SXT domain CK2 sites causes impedes basal phosphorylation of Ctp1 and causes DNA damage sensitivity

CK2 typically phosphorylates the minimum sequence S/T-X-X-D/E,[25] a consensus that fits each threonine in the SXT domain of Ctp1. Although another recent study made use of a phosphospecific antibody to show that Thr89 of Ctp1 is constitutively phosphorylated *in vivo*,[24] no specific evidence implicating CK2 in this process has been provided. To further investigate whether the basal phosphorylation of Ctp1 is likely catalyzed by CK2 or a protein kinase with a similar preferred phosphorylation site motif, we created a *ctp1-CK* (*ctp1-E81A D82A E92A*) mutant, in which the phosphoacceptor threonine residues of the SXT domain remain intact, while the acidic determinant (n+3) residue of each putative CK2 site was changed to alanine (Fig. 1A). This mutant exhibited slow growth and DNA damage sensitivity identical to the *ctp1-3A* mutant, suggesting a specific requirement for CK2 in the basal phosphorylation of Ctp1 (Fig. 1B). We previously demonstrated the importance of the SXT domain with regard to basal phosphorylation of Ctp1 through an increase in the gel mobility of Ctp1-5A protein. Here we show that this phenomenon is mirrored in *ctp1-3A* and *ctp1-CK*, as these mutants both run with identical faster mobility than wild-type (Fig. 1C). Since loss of the acidic determinant residues of each CK2 site alters gel mobility of Ctp1 to the same extent as loss of phosphoacceptor residues, this result suggests a specific requirement for CK2 in this process. Furthermore, these results corroborate loss of phosphorylation on the Ctp1 SXT domain with the extreme DNA damage sensitivity observed in the *ctp1-3A* and *ctp1-CK* strains (Fig. 1B). In addition, these mutants caused a dramatic increase in Ctp1 protein stability, which is known to occur in response to DNA damage.[26] We further attempted to verify the role of CK2 in basal phosphorylation of Ctp1

through employing the temperature sensitive CK2 mutant *orb5-19*.[27] However, we found that Ctp1 was extremely abundant at the restrictive temperature (data not shown), indicating that loss of multiple CK2-dependent processes leads to a large increase in spontaneous DNA damage.

We also generated a phospho-mimetic Ctp1 mutant, *ctp1-3D* (*ctp1-T78D T79D T89D*), in which the SXT domain threonines were exchanged for aspartic acid (Fig. 1A). Interestingly, this mutant showed enhanced DNA damage sensitivity similar to *ctp1-3A* (Fig. 1B), indicating that the significance of SXT domain phosphorylation is more extensive than increase in negative charge, such as providing a specific recognition platform for an FHA domain.

The SXT domain is required for Ctp1 activity

The similar phenotypes of *ctp1Δ* and *ctp1-5A* mutants indicated that the SXT domain is critical for Ctp1 activity. To further characterize the cellular consequences of inhibiting phosphorylation of Ctp1, we counted $Rad22^{Rad52}$-YFP foci in *ctp1-5A* cells. Rad22 is essential for homologous recombination in fission yeast. We found that *ctp1-5A* cells accumulate a dramatically higher amount of spontaneous $Rad22^{Rad52}$-YFP foci (38%) than $ctp1^{+}$(11%), and that this is similar to what is observed in *ctp1Δ* (53%) (Fig. 2A). Since *ctp1Δ* cells are largely compromised for HR repair of DSBs (Limbo), a similar quantity of persistent $Rad22^{Rad52}$-YFP foci suggests a loss of HR repair activity in the *ctp1-5A* mutant as well.

Ctp1 plays an essential role in the repair of programmed DSBs during meiosis.[19] To examine the importance of the Ctp1 SXT domain in meiotic processes, we mated *ctp1-5A* haploids. Similar to what we observed in *ctp1Δ* × *ctp1Δ* crosses, the asci generated by a *ctp1-5A* × *ctp1-5A* mating are grossly defective in both spore size and number (Fig. 2B). Furthermore, the viability of the spores generated from the mating of *ctp1-5A* haploids is extremely poor (0.05%), similar to *ctp1Δ* (0.013%) (Fig. 2C). These results indicate that an intact SXT domain is essential for Ctp1 activity in both meiotic and mitotic cells. Taken together with our previous results, these data suggest that the *ctp1-5A* mutant essentially phenocopies the cellular defects observed in *ctp1Δ* cells.

Phosphorylation of Ctp1 mediates its association with the MRN complex through binding to the FHA domain of Nbs1

Although we were able to establish by ChIP that recruitment of Ctp1 to a defined DSB is Nbs1-dependent, isolation of Nbs1-Ctp1 complexes proved difficult, suggesting that Nbs1-Ctp1 binding might be short-lived or salt sensitive. By using an *mre11-H134S* nuclease-dead background,[28] Ctp1-Nbs1 complexes were stabilized *in vivo*, and could be observed by co-immunoprecipitation. Furthermore, this association is dependent on the phosphopeptide-binding pocket of the Nbs1 FHA domain. Here, we utilized the *ctp1-5A* mutant to examine the effect of SXT domain phosphorylation on association of Ctp1 with Nbs1. Despite the fact that *ctp1-5A* induces a dramatic increase in the amount of total Ctp1 protein, the amount of Nbs1 associated with Ctp1-5A following TAP-pulldown was quite low, resembling the background binding of Nbs1 to IgG Sepharose observed in the negative control (Fig. 3A). This implies that phosphorylation of the SXT domain is critical for association with Nbs1.

We also verified association of Ctp1 and Nbs1 by yeast two-hybrid analyses. Although we had previously been unable to observe interaction of Nbs1 with Ctp1 under high-stringency (-LWHA) conditions,[19] by titrating in increasing amounts of 3-AT, a competitive inhibitor of the *HIS3* gene product, we detected a specific Ctp1-Nbs1 interaction that was greater than

the autonomous activation of the *HIS3* reporter gene by the pGBKT7-Ctp1 plasmid (Fig. 3B). Consistent with our previous findings, this interaction was impeded by point mutations (R27A, K45A, RKAA) in the FHA phosphopeptide-binding pocket of Nbs1 (Fig. 3B). Since the association of Ctp1 with Nbs1 appears to be strictly phosphorylation dependent, the low level of specific binding observed by two-hybrid assay may reflect an inability of Ctp1 to be robustly phosphorylated in an *S. cerevisiae* background. Together these results further substantiate the conclusion that the primary function of Ctp1 SXT domain phosphorylation is to promote association with Nbs1.

Discussion

Here we have provided additional evidence linking CK2 to the phospho-dependent interaction between Ctp1 and Nbs1 that is essential for HR repair of DSBs. and that this process represents overlapping functional conservation on at least two levels. First, CK2-dependent promotion of DNA damage response processes. Clear roles for CK2 in generating phosphorylation-dependent protein-protein interactions have been demonstrated in both non-homologous end joining (NHEJ) and single-strand break (SSB) repair.[11-18] Our data now suggests that CK2 may also promote HR repair of DSBs by promoting association of Ctp1 with the MRN complex. Second, phosphorylated SDTD-like motifs serve as preferred docking sites for the N-terminal phosphopeptide-binding domains of Nbs1. In addition to the aforementioned interactions with Ctp1 and Mdc1 that facilitate HR and signal amplification from DSBs, respectively, $Xrs2^{Nbs1}$ associates with phosphorylated $Lif1^{XRCC4}$ SETD sites in an FHA domain-dependent manner to facilitate NHEJ in budding yeast.[29] We infer from these observations that the N-terminus of Nbs1 likely performs a variety of signaling and DNA repair functions within the cellular DNA damage response.

In contrast to our results regarding N-terminal phosphorylation of Ctp1, recruitment of Sae2 and CtIP to DSBs requires CDK-mediated phosphorylation at a positionally conserved C-terminal site in budding yeast and humans, respectively.[30, 31] It is unknown how this phosphorylation regulates Sae2 or CtIP recruitment to damaged DNA, but it is still interesting that phosphorylation of Ctp1/Sae2/CtIP is critical for the targeting and activity of each protein, even if manner in which this occurs may have diverged. Although basal phosphorylation of Ctp1 appears to be at least partially performed by CDKs, combined mutation of the ST/P sites in Ctp1 yields no phenotype,[20] implying that CDK-mediated phosphorylation of Ctp1 has little or no role in its DNA repair-associated activities. Interestingly, while loss of CDK activity can be rescued using phosphomimetic Sae2/CtIP mutants,[30, 31] a phospho-mimetic Ctp1 has deleterious effects on genotoxin sensitivity (Fig. 1B). This suggests that although the C-terminal CDK-mediated phosphorylation of CtIP/Sae2 contributes to the targeting and activity of these proteins at a DSB, it may not do so in the same manner as N-terminal CK2-mediated phosphorylation of Ctp1 (through generating an interaction with Nbs1/Xrs2). In support of this notion, a recent report demonstrates that while both the N- and C-termini of CtIP interact with the MRN complex, it is the extreme N-terminus of CtIP that specifically enables binding to Nbs1.[32] A model that may unite these scenarios is one in which independent modifications of the N- and C-termini of CtIP/Sae2/Ctp1 could occur, each facilitating its own contact with the MRN complex.

Ctp1 is also phosphorylated in a DNA damage-dependent manner, a process that requires Mre11, $Rad3^{ATR}$, $Tel1^{ATM}$,[20] as well as Ctp1 SXT domain phosphorylation and the FHA domain of Nbs1[23, 24]. This suggests a mechanism in which localization of Ctp1 at a DSB is required for its DNA damage-induced phosphorylation by $Rad3^{ATR}$/$Tel1^{ATM}$. While PIKK-mediated DNA damage-induced phosphorylation of budding yeast $Sae2^{Ctp1}$ is important for its repair and recombination functions,[33] combined mutation of the PIKK-preferred ST/Q sites in Ctp1 fails to alter its phosphorylation or sensitivity in response to DNA damage.[20]

This suggests that either another kinase downstream of $Rad3^{ATR}$/$Tel1^{ATM}$ performs this modification, or that PIKK-induced phosphorylation of Ctp1 occurs at a deviant non-S/TQ site, the latter of which has been observed in a few substrates such as BRCA1 and ATM itself.[34, 35] Future studies will be necessary to map the specific site(s) where DNA damage-induced phosphorylation of Ctp1 occurs and to separate its functional significance from the initial priming phosphorylation event that is likely performed by CK2.

Materials and Methods

General Methods

Strains used in this study are listed in Table 1. Spore viability assays were performed exactly as previously described.[19] Survival assays in response to chronic genotoxin exposure were performed by resuspending midlog phase cultures to 1×10^7 cells/ml, serially diluting the cultures fivefold, and spotting the dilutions onto YES agar plates containing the indicated amounts of CPT or HU.

Ctp1 immunoprecipitation and Western blotting

Following preparation of whole-cell extracts in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 2.5 mM EDTA, 0.002% NP-40, 50 mM NaF, protease inhibitor tablet [Roche, complete Mini]), Ctp1-FLAG was immunoprecipitated with α-FLAG antibody (Sigma) and then subjected to western blotting as previously described.[20]

Microscopy

Flourescent microscopy of Rad22-YFP expressing strains was performed as previously described.[36] For meiosis experiments, cells of opposite mating types were mixed and incubated for 3 days at 25°C on SSA plates to obtain zygotic asci. Images were taken with a Nikon Eclipse E800 microscope, Photometrics Quantix CCD camera, and IPlab Spectrum software.

Nbs1-Ctp1 Coimmunoprecipitation

Soluble whole-cell extracts were made from exponentially growing cells by disrupting in low-salt lysis buffer (50 mM Tris [pH 8.0], 50 mM NaCl, 2.5 mM EDTA, 0.002% NP-40, 50 mM NaF, protease inhibitor tablet [Roche, complete Mini]) with a bead beater. Ctp1-TAP and associated proteins were precipitated from extracts with a 50% suspension of IgG Sepharose (GE Healthcare), washed three times with low-salt lysis buffer, resolved on 8% SDS-PAGE, and examined by western blotting.

Yeast Two-Hybrid Analysis

Indicated proteins were fused to Gal4 activating domain and DNA binding domain in pGADT7 and pGBKT7, respectively, and expressed in *S. cerevisiae* strain AH109 (Clontech Matchmaker system). Strains positive for cotransformation with the indicated pair of plasmids were assayed by growth on Dex-WL plates (minimal glucose media lacking tryptophan and leucine). Positive interactions were assayed by growth on medium-stringency Dex-WLH plates (minimal glucose media lacking tryptophan, leucine, and histidine) and high-stringency Dex-WLHA (minimal glucose media lacking tryptophan, leucine, histidine, and adenine). The stringency of Dex-WLH plates was increased by addition of 3-AT.

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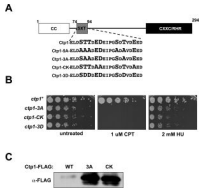

Figure 1.

Basal phosphorylation of Ctp1 is carried out by Casein kinase 2. (A) Schematic of Ctp1 depicting conserved N-terminal coiled coil domain (CC), C-terminal core homology region (CXXC/RHR), and a CK2 phosphoacceptor motif (SXT). Motif enlargement shows residue substitutions in the *ctp1-5A*, *ctp1-3A*, *ctp1-CK*, and *ctp1-3D* strains. (B) *ctp1-CK* and *ctp1-3D* cells are sensitive to exogenous DNA damaging agents, similar to *ctp1-3A* cells. (C) Basal phosphorylation is equally altered in Ctp1-3A and Ctp1-CK relative to wild-type.

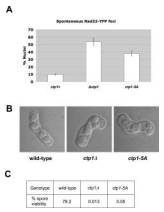

Figure 2.
The *ctp1-5A* mutant phenocopies cellular defects observed in *ctp1Δ*. (A) *ctp1-5A* cells accumulate increased levels of spontaneous Rad22-YFP foci. (B) Asci from a *ctp1-5A* × *ctp1 5A* mating are abnormal. (C) Spore viability in *ctp1-5A* × *ctp1-5A* is very low.

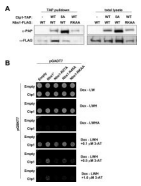

Figure 3.

CK2-mediated phosphorylation of Ctp1 facilitates interaction with Nbs1. (A) Ctp1 and Nbs1 associate in a manner dependent on phosphorylation of the Ctp1 SXT motif and the Nbs1 FHA domain. Strains were generated that express TAP-tagged Ctp1 and FLAG-tagged Nbs1 in an *mre11-H134S* background. IgG Sepharose beads were used to precipitate TAP-tagged Ctp1 and any associated proteins. (B) Yeast-two hybrid assays show that Ctp1 associates with Nbs1. Titrating increasing amounts of 3-AT reveals a specific interation between Ctp1 and Nbs1 that is FHA domain-dependent.

S. pombe strains used in this study

Strain	Genotype	Source or reference
PR109	*h- leu1-32 ura4-D18*	Lab stock
PR110	*h^+ leu1-32 ura4-D18*	Lab stock
OL4121	*h- leu1-32 ura4-D18 ctp1::kanM×6*	Limbo, et al., 2007
OL4122	*h^+ leu1-32 ura4-D18 ctp1::kanM×6*	Limbo, et al., 2007
YYY4181	*$h^?$ leu1-32 ura4-D18 his3-D1 arg3::HO site(kanM×6) ars1:nmt-(HO endonuclease):ampR:his3$^+$:ars1 mre11-H134S-13myc:kanM×6 nbs1-5flag:kanM×6 ctp1-TAP:hph*	Williams, et al., 2008
YYY4182	*$h^?$ leu1-32 ura4-D18 his3-D1 arg3::HO site(kanM×6) ars1:nmt-(HO endonuclease):ampR:his3$^+$:ars1 mre11-H134S-13myc:kanM×6 nbs1-5flag:kanM×6*	Williams, et al., 2008
YYY4447	*$h^?$ leu1-32 ura4-D18 his3-D1 arg3::HO site(kanM×6) ars1:nmt-(HO endonuclease):ampR:his3$^+$:ars1 mre11-H134S-13myc:kanM×6 nbs1-R27A K45A-5flag:kanM×6 ctp1-TAP:hph*	Williams, et al., 2009
GD4588	*h^+ leu1-32 ura4-D18 ctp1-S77A T78A T79A S87A T89A-TAP:kanM×6*	Williams, et al., 2009
OL4589	*h- leu1-32::ctp1-3flag:leu+ ura4-D18 ctp1::hphM×6*	Limbo, et al., 2007
GD 4834	*h- leu1-32 ura4-D18 ctp1-S77A T78A T79A S87A T89A-TAP:kanM×6*	This study
GD 4835	*$h^?$ leu1-32 ura4-D18 his3-D1 arg3::HO site(kanM×6) ars1:nmt-(HO endonuclease):ampR:his3$^+$:ars1 mre11-H134S-13myc:kanM×6 nbs1-5flag:kanM×6 ctp1-S77A T78A T79A S87A T89A-TAP:kanM×6*	This study
GD 4836	*h- leu1-32 ura4-D18 ctp1-T78D T79D T89D-TAP:kanM×6*	This study
GD 4837	*$h^?$ leu1-32 ura4-D18 ctp1-S77A T78A T79A S87A T89A-TAP:kanM×6 rad22-YFP:kanM×6*	This study
GD 4838	*h- leu1-32::ctp1-E81A D82A E92A-3flag:leu+ ura4-D18 ctp1::hphM×6*	This study
GD 4839	*h- leu1-32::ctp1-T78A T79A T89A-3flag:leu+ ura4-D18 ctp1::hphM×6*	This study
GD 4840	*$h^?$ leu1-32 ura4-D18 ctp1::hphM×6 rad22-YFP:kanM×6*	This study
GD 4841	*$h^?$ leu1-32 ura4-D18 ctp1-TAP:kanM×6 rad22-YFP:kanM×6*	This study