

# Degradation of Mrc1 promotes recombination-mediated restart of stalled replication forks

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## ABSTRACT

The DNA replication or S-phase checkpoint monitors the integrity of DNA synthesis. Replication stress or DNA damage triggers fork stalling and checkpoint signaling to activate repair pathways. Recovery from checkpoint activation is critical for cell survival following DNA damage. Recovery from the S-phase checkpoint includes inactivation of checkpoint signaling and restart of stalled replication forks. Previous studies demonstrated that degradation of Mrc1, the *Saccharomyces cerevisiae* ortholog of human Claspin, is facilitated by the SCF<sup>Dia2</sup> ubiquitin ligase and is important for cell cycle re-entry after DNA damage-induced S-phase checkpoint activation. Here, we show that degradation of Mrc1 facilitated by the SCF<sup>Dia2</sup> complex is critical to restart stalled replication forks during checkpoint recovery. Using DNA fiber analysis, we showed that Dia2 functions with the Sgs1 and Mph1 helicases (orthologs of human BLM and FANCM, respectively) in the recombination-mediated fork restart pathway. In addition, Dia2 physically interacts with Sgs1 upon checkpoint activation. Importantly, failure to target Mrc1 for degradation during recovery inhibits Sgs1 chromatin association, but this can be alleviated by induced proteolysis of Mrc1 after checkpoint activation. Together, these studies provide new mechanistic insights into how cells recover from activation of the S-phase checkpoint.

## INTRODUCTION

Checkpoint recovery is central to accurate cell division. Checkpoint pathways monitor progress during cell division so that in the event of an error, the checkpoint is activated to block the cell cycle and activate repair pathways. Checkpoint recovery mechanisms contribute to the suc-

cessful completion of a checkpoint. Checkpoint recovery encompasses the pathways responsible for inactivation of checkpoint signaling and cell cycle re-entry after the initial stress has been alleviated.

The DNA replication or S-phase checkpoint monitors the integrity of DNA synthesis. Replication stress such as limited free nucleotides or DNA damage in the form of DNA breaks or adducts leads to replication fork stalling and the initiation of the checkpoint-signaling pathway (1–4). In the budding yeast *Saccharomyces cerevisiae*, initiation of the S-phase checkpoint leads to activation and recruitment of the Mec1/ATR kinase (5–7). Mec1 activates the kinase Rad53 via mediator proteins that include Mrc1, Rad9, Tof1 and Csm3 (7–15). Rad53 activation results in replication fork stabilization and inhibition of further origin firing (2,4,16–18).

Recovery from the S-phase checkpoint requires inactivation of checkpoint signaling and restart of stalled replication forks, although it is not clear whether there is coordination between the pathways that inhibit checkpoint signaling and those that facilitate fork restart. In yeast, inactivation of the signaling kinase Rad53 by phosphatases Pph3 and Ptc2 contributes to fork restart during recovery from a methyl methane sulfonate (MMS)-induced checkpoint (19,20). In addition, fork restart during recovery from a hydroxyurea (HU)-induced checkpoint is dependent on the chromatin remodeling proteins Ino80 and Isw2 (21–23).

Protein degradation of checkpoint adaptor proteins is also important for recovery from the S-phase checkpoint. Our previous work showed that the degradation of the checkpoint adaptor and replication fork protein Mrc1, controlled by the SCF<sup>Dia2</sup> ubiquitin ligase complex, is important for cell cycle re-entry after cells are exposed to the alkylating agent MMS. Rad53 phosphorylation levels persist longer in *dia2*  $\Delta$  cells during recovery from an MMS-induced checkpoint and *dia2*  $\Delta$  cells exhibit greater recovery delays when the Rad53 phosphatase Pph3 is also absent, suggesting that Mrc1 degradation contributes to checkpoint signaling inactivation (24). Further, degradation of Claspin, the human homolog of Mrc1, is linked to inhibi-

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tion of Chk1 kinase signaling, after an HU-induced S-phase checkpoint (25–27).

Multiple mechanisms can lead to restart of stalled replication forks. In some cases, direct restart of a stable, stalled fork is possible, although little is known about direct restart mechanisms (28). Alternatively, there is evidence that compromised forks can be restarted after remodeling by fork reversal or nucleolytic processing of nascent DNA strands or recombination-mediated mechanisms (28–30). The Sgs1 helicase, a member of the RecQ helicase family and yeast ortholog of the Bloom Syndrome protein BLM, is important for recombination-mediated fork restart (31,32). Mph1, a member of the SF2 helicase family and the yeast homolog of FANCM, plays a role in fork maintenance and fork reversal (33,34), which is important for recombination-mediated fork restart.

In these studies, we reveal a mechanistic link between degradation of a checkpoint protein and recombination-mediated restart of stalled replication forks. We show that Dia2-dependent degradation of Mrc1 is required for replication fork restart in a pathway that includes Sgs1 and Mph1. Moreover, we find that Dia2 interacts with Sgs1 and that the recruitment of Sgs1 to chromatin during checkpoint recovery is dependent on Mrc1 degradation.

## MATERIALS AND METHODS

### Plasmids and strains

Tables 1 and 2 contain a list of strains and plasmids used in this study.

### Yeast cell culture and treatments

Yeast strains were cultured by standard methods (35). For  $\alpha$ -factor arrest, yeast cultures were grown in either liquid minimal medium or yeast extract-peptone-dextrose (YPD) and pulsed with 2  $\mu$ g/ml  $\alpha$ -factor peptide (Alpha Aesar) every hour for 2 h. HU or MMS treated cells were grown in minimal or YPD medium. For strains harboring galactose-inducible plasmids, cells were first treated with 2% raffinose for overnight followed by treatment with 2% galactose at 30°C.

### Immunoblotting

Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Pall Corporation, USA). After blocking in 5% milk, membranes were incubated with the following primary antibodies: 9E11 (Covance), anti-HA (Covance), anti-Sgs1 (Santa Cruz Biotech) and anti-Pgk1 (Invitrogen). Horseradish peroxidase conjugated secondary antibodies were used (anti-mouse, Jackson Immuno-Research or anti-goat, Santa Cruz Biotech). Protein bands were detected using a Pierce ECL kit (Thermo Fisher).

### Dual-labeling DNA fiber assay

Yeast strains capable of incorporating thymidine analogs were generated by transforming with linearized HSV-TK

plasmids (gift of O. Aparicio). Yeast strains were synchronized in G1 with  $\alpha$ -factor. Cells were released from G1 arrest in fresh media containing 400  $\mu$ g/ml IdU for 15 min. For replication fork restart and fork stability assays, after IdU treatment, cells were either untreated or treated with 0.05% MMS for 60 min followed by release in fresh media containing 400  $\mu$ g/ml CldU for 15 min. For measurement of replication fork velocity, no MMS treatment was done and cells were immediately transferred to CldU containing media for 15 min after 15 min IdU treatment. DNA fibers were stretched on silanized glass slides and IdU (red fibers) and CldU (green fibers) incorporation was detected by using mouse anti-BrdU clone B44 antibody (Becton Dickinson, cat # 347580) and Rat anti-BrdU clone BU1/75 antibody (AbD Serotec, cat # OBT0030G), respectively. Images were captured on a Deltavision microscope (Applied Precision) and analyzed using Deltavision softWoRx 5.5 software. All DNA fiber results shown are the means of three independent experiments (100 DNA fibers/experiment). Error bars show standard error of the mean. *P*-values for restart experiments were derived from the Student's *t*-test. *P*-values for fork velocities and nascent strand degradation were determined using the Mann–Whitney test.

### Viability and spotting assays

For viability assays, overnight cultures were first grown in YPD to log phase. Equal numbers of cells were plated on media containing the indicated amount of MMS or HU. Colony forming units (CFU) were counted after 4 days at 30°C. The percentage viability was calculated by dividing CFU on YPD with MMS or HU by CFU on YPD without MMS or HU. For spotting assays, 10-fold serial dilutions of  $2 \times 10^7$  to  $2 \times 10^3$  cells of the indicated strains were spotted on YPD, YPD + 0.007% MMS and YPD + 200 mM HU plates using replica plater and incubated at 30°C for 2–3 days.

### Immunoprecipitation experiments

Immunoprecipitation experiments were performed using protocols as previously described (36). Briefly, cell pellets were resuspended in NETN lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Igepal, 10 mM NaF, 25 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride and 1 mM pepstatin, as well as complete protease inhibitor cocktail (Roche Applied Science). After vortexing the cells for 10 min at 4°C with glass beads, lysates were microcentrifuged at 10 000 rpm at 4°C followed by transfer of clear supernatant to new tubes. One milligram of lysate was incubated with 1:200 anti-myc antibody. The samples were then incubated with protein A/G-agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. Beads were washed three times with same NETN lysis buffer, boiled in 1X Laemmli loading dye and loaded to SDS-PAGE for Western blotting.

### Chromatin fractionation experiment

Chromatin fractionation was performed as previously described (36). Briefly, spheroplasts were isolated and lysed

**Table 1.** Strains used in this study

Strain	Description	Reference
Y80	<i>ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 MATa</i>	(57)
DKY1032	As Y80 but <i>HIS3::BrdU-Inc</i>	This study
DKY194	As Y80 but <i>dia2Δ::kanMX</i>	(58)
DKY1033	As DKY194 but <i>HIS3::BrdU-Inc</i>	This study
DKY1042	As Y80 but <i>sgs1Δ::kanMX HIS3::BrdU-Inc</i>	This study
DKY1043	As Y80 but <i>mph1Δ::kanMX HIS3::BrdU-Inc</i>	This study
DKY1044	As Y80 but <i>dia2Δ::kanMX sgs1Δ::kanMX HIS3::BrdU-Inc</i>	This study
DKY1045	As Y80 but <i>dia2Δ::kanMX mph1Δ::kanMX HIS3::BrdU-Inc</i>	This study
AKY149	As DKY194 but <i>dia2Δ::kanMX::9MYC-DIA2 URA3</i>	(36)
AKY188	As AKY149 but <i>DIA2-ΔF</i>	(36)
AKY189	As AKY149 but <i>DIA2-ΔN200</i>	(36)
AKY190	As AKY149 but <i>DIA2-LRR</i>	(36)
AKY192	As AKY149 but <i>DIA2-TPR</i>	(36)
AKY193	As AKY149 but <i>DIA2-ΔN175</i>	(36)
AKY199	As AKY149 but <i>DIA2-ΔN134</i>	(36)
DKY1046	As AKY149 but <i>HIS3::BrdU-Inc</i>	This study
DKY1047	As AKY188 but <i>HIS3::BrdU-Inc</i>	This study
DKY702	<i>mrc1::HIS3::3HA-MRC1 LEU2 dia2::KanMX::9MYC-DIA2::URA3 his3 trp1 ade2 MATa</i>	(24)
DKY909	<i>dia2::KanMX mrc1::HIS3::3HA-AID-MRC1::LEU2 ura3 leu2 his3 trp1 ade2 MATa</i>	(24)
DKY1048	As DKY702 but <i>TRP1::BrdU-Inc</i>	This study
DKY1049	As DKY909 but <i>URA3::BrdU-Inc</i>	This study

**Table 2.** Plasmids used in this study

Plasmid	Relevant features	Reference
p403-BrdU-Inc	ADH1-hENTI, GPD-HSV-TK, HIS3 integration vector Amp <sup>r</sup>	(59)
p404-BrdU-Inc	ADH1-hENTI, GPD-HSV-TK, TRP1 integration vector Amp <sup>r</sup>	(59)
p306-BrdU-Inc	ADH1-hENTI, GPD-HSV-TK, URA3 integration vector Amp <sup>r</sup>	(59)

with 0.2% Triton-X100 and 150 mM NaCl. Whole cell extracts (WCE) were fractionated to supernatant (S) and pellet (P) using centrifugation at 15 000 ×g for 15 min at 4°C. Prior to centrifugation, WCEs were either untreated or treated with 150 kU DNaseI for 30 min. One volume of 2X Laemmli buffer was added in WCE/Input and supernatant (S) and for pellet (P) containing chromatin fraction, 2X Laemmli buffer to equal volume of S was added. Samples were boiled and then resolved by SDS-PAGE prior to immunoblotting.

### Auxin-induced degradation of Mrc1

Auxin induced Mrc1 degradation was performed as previously described (24). For chromatin fractionation and DNA fiber assays, either vehicle (100% ethanol) or 1.5 mM indole-3-acetic acid (IAA, Alfa Aesar) was added to cell cultures in YPD at 30°C.

### Flow cytometry

Flow cytometry was performed as previously described (24). Briefly, cells were harvested and fixed with 70% ethanol. Fixed cells were resuspended in 1x PBS, sonicated to break clumps and treated with 100 μg/ml RNase in Tris-EDTA overnight. Samples were stained with 50 μg/ml propidium iodide for 1 h and analyzed by flow cytometry using FACSCaliber (BD Biosciences) and FlowJo software (Tree Star). Cell cycle distribution graphs were prepared using Deltagraph software (Red Rock). 2C DNA content was quantitated using flow cytometry data gated for a standard

2C distribution developed from an asynchronous population and percentages were calculated by dividing the number of cells with 2C DNA content by the total number counted.

## RESULTS

We have previously demonstrated that Dia2-mediated degradation of Mrc1 is important for cell cycle re-entry from an MMS-induced S-phase checkpoint (24). The absence of Dia2 leads to increased persistence of Rad53 phosphorylation during checkpoint recovery, suggesting that Mrc1 degradation could contribute to checkpoint signaling inactivation. However, a key transition during checkpoint recovery is the restart of stalled replication forks. As *dia2Δ* exhibits enhanced phenotypes in combination with known replication fork restart mutants, including *sgs1Δ* and *pph3Δ* (37,38), we investigated the role of Dia2 and Mrc1 degradation in the restart of stalled replication forks.

### Dia2 is required for restart of stalled replication forks

Given the known genetic links between Sgs1 and Dia2 (37,38), we chose to compare the restart of stalled forks in *dia2Δ* cells to the *sgs1Δ* mutant and the functionally similar *mph1Δ* mutant. Sgs1 and Mph1 are yeast orthologs of human Bloom Syndrome protein BLM helicase and Fanconi anemia protein FANCM (32,39), respectively. Both Sgs1 and Mph1 protect stalled replication forks during replication stress and strains lacking *SGS1* or *MPH1* are hypersensitive to the replication inhibitors HU and MMS

(40–43). To determine if Dia2 is essential for stalled replication fork restart and if it works in the same pathway with Sgs1 and Mph1, we established wild type, *dia2Δ*, *sgs1Δ*, *mph1Δ*, *dia2Δsgs1Δ* and *dia2Δmph1Δ* strains bearing an integrated copy of HSV-TK (Herpes simplex virus-thymidine kinase) expression vector that allows efficient incorporation of thymidine analogs such as BrdU, IdU or CldU. We observed that *dia2Δ* cells exhibited reduced viability in combination with *sgs1Δ*, as expected (37,38), as well as with *mph1Δ* mutants when challenged with replication stress (Figure 1A and B).

We next monitored replication events in individual chromosomes during recovery from an MMS-induced checkpoint using a dual-labeling DNA fiber assay. Cells synchronized in G1 were allowed to enter S-phase and then were pulse-labeled with IdU (red label) for 15 min. Following IdU labeling, cells were either untreated or treated with 0.05% MMS for 1 h, followed by a second pulse-labeling with CldU (green label) for 15 min in the absence of MMS (Figure 1C). DNA fibers were isolated and incubated with anti-IdU and anti-CldU antibodies to visualize DNA replication. Wild type cells were able to restart the majority of MMS-induced stalled forks (76%), as shown by the green-labeled DNA tracts (Figure 1D and E). However, *dia2Δ* cells exhibited severe deficiency in stalled replication fork restart (37%), suggesting that Dia2 is important for efficient recovery of the MMS-stalled replication forks. Interestingly, depletion of Dia2 in *sgs1Δ* or *mph1Δ* strains did not exaggerate the phenotype observed in *sgs1Δ* or *mph1Δ* single-deficient strains (36% and 39% in *dia2Δsgs1Δ* and *dia2Δmph1Δ* strains versus 38% and 37% in *sgs1Δ* and *mph1Δ* strains) suggesting that Dia2, Sgs1 and Mph1 are epistatic for restart of MMS-stalled replication forks.

### Dia2 and Sgs1 are important for maintenance of normal replication fork velocity

In addition to its role in recombination-mediated replication fork restart, human BLM helicase is also essential for maintaining normal replication fork velocity. Thus, we investigated whether Sgs1, Dia2 and Mph1 also affect normal replication fork velocity. We pulse labeled G1-synchronized wild type, *dia2Δ*, *sgs1Δ*, *mph1Δ*, *dia2Δsgs1Δ* and *dia2Δmph1Δ* strains with IdU for 15 min after release into S phase, followed immediately by another pulse labeling with CldU for 15 min. To assess ongoing replication fork velocity, we measured CldU (green label) tract lengths in IdU-CldU double-labeled (red-green labels) tracts. The distribution pattern of tract lengths was similar between wild type and *mph1Δ* strains with median tract lengths being 13.25  $\mu\text{m}$  and 13.65  $\mu\text{m}$ , respectively. By contrast, the *dia2Δ* and *sgs1Δ* strains had shorter tract length distribution with median tract lengths of 11.15  $\mu\text{m}$  and 11.50  $\mu\text{m}$ , respectively, suggesting that Dia2 and Sgs1, but not Mph1, maintain normal replication fork velocity. The *dia2Δsgs1Δ* strain also had a similar tract length distribution (median tract length 11.75  $\mu\text{m}$ ) to *dia2Δ* and *sgs1Δ* strains, further suggesting that Dia2 and Sgs1 are epistatic for maintaining normal fork velocity (Figure 2A).

### The Dia2 F-box domain is essential for stalled replication fork restart and maintenance of replication velocity

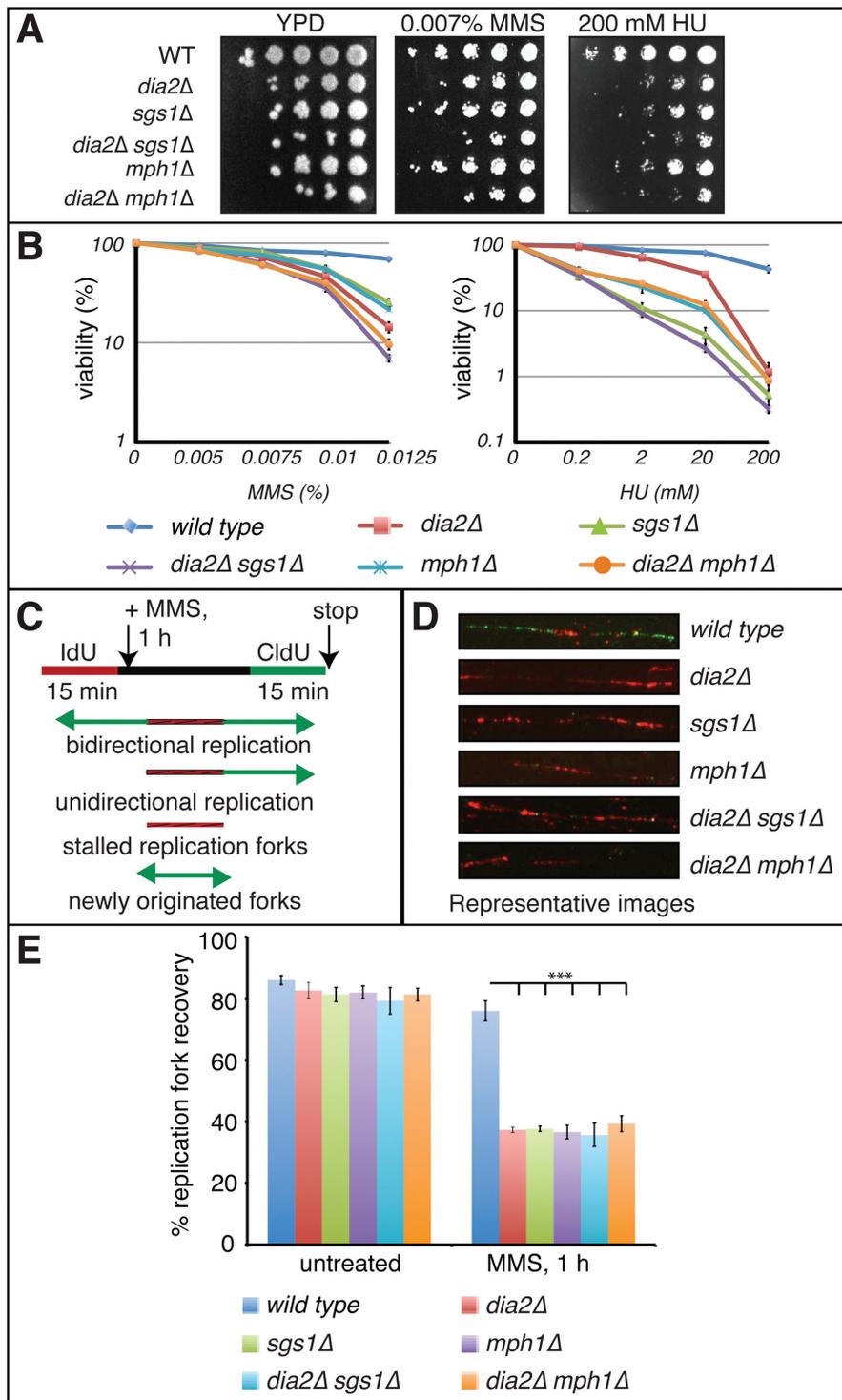
To determine whether the function of Dia2 in replication fork restart and fork velocity was due to its role as a part of a ubiquitin ligase, we examined a Dia2 mutant lacking the F-box domain using the same methods as described above. The absence of the F-box domain resulted in a severe deficiency of restart of MMS-stalled replication forks (Figure 2B) and reduced fork velocity (Figure 2C). The defects were comparable to the phenotypes in strains lacking Dia2, suggesting that Dia2's role as a ubiquitin ligase is essential to promote stalled fork restart as well as maintenance of unperturbed replication fork velocity.

### Mph1 but not Dia2 or Sgs1 protects nascent DNA strands from degradation after fork stalling

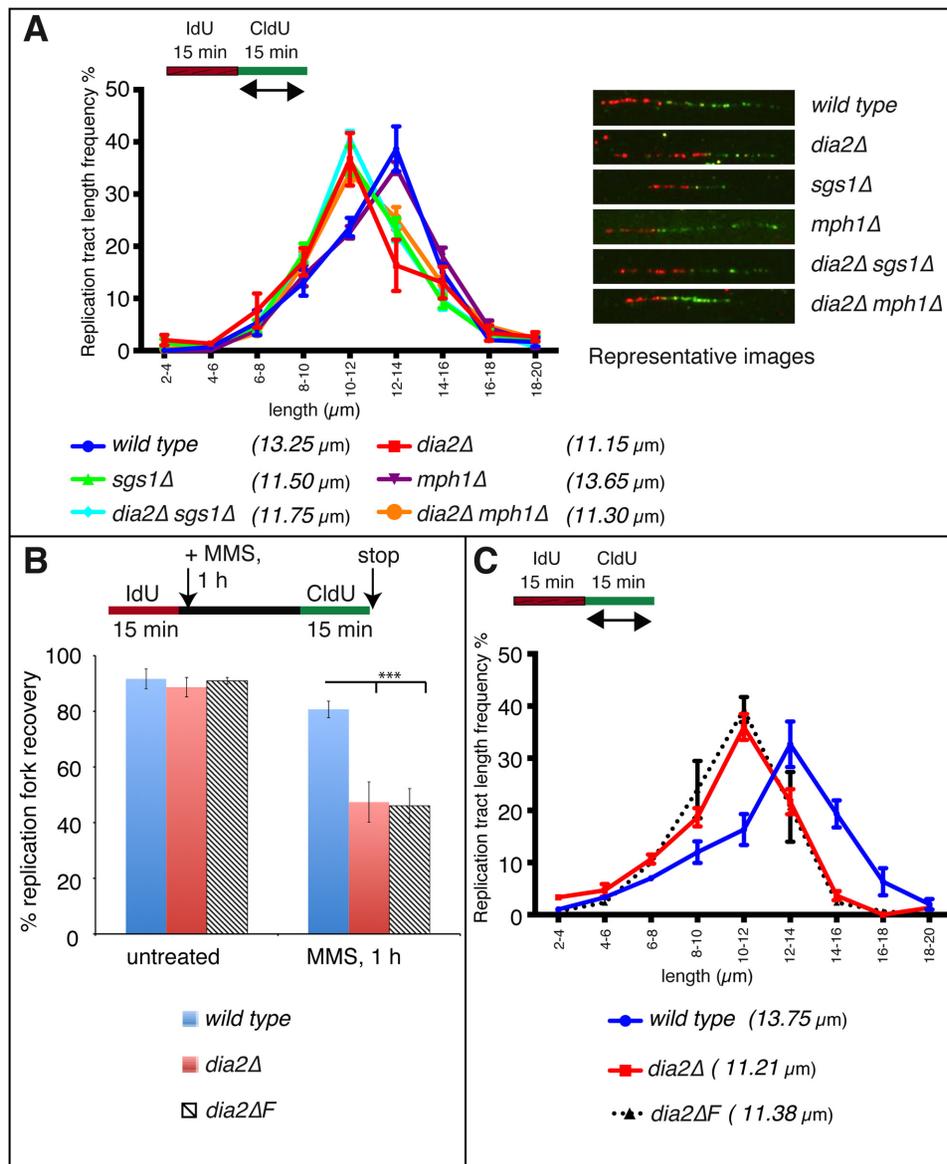
In human cells, Fanconi Anemia proteins have dual roles in replication fork restart and protection of DNA strands from degradation after fork stalling whereas Human BLM is dispensable for nascent DNA strand stability at stalled forks (44,45). As we observed replication fork restart defects in *dia2Δ*, *sgs1Δ* and *mph1Δ* cells, we asked if Dia2, Sgs1 or Mph1 have any role in nascent DNA strand protection after MMS-induced fork stalling. For this, cells were labeled with IdU (red label) for 15 min followed by 1 hour 0.05% MMS treatment. DNA tract length distributions were analyzed in wild type, *dia2Δ*, *sgs1Δ*, *mph1Δ*, *dia2Δsgs1Δ* and *dia2Δmph1Δ* strains. As Dia2 and Sgs1 maintain normal replication fork velocity (Figure 2A), *dia2Δ* and *sgs1Δ* strains exhibited slightly shorter tract length distribution patterns (median of 11.48  $\mu\text{m}$  and 11.77  $\mu\text{m}$ , respectively) compared to the wild type (median of 13.66  $\mu\text{m}$ ; Figure 3). Strikingly, *mph1Δ* strains showed significantly shorter tract length frequency (median of 7.55  $\mu\text{m}$ ) suggesting that Mph1, but not Dia2 or Sgs1, is important for protection of nascent DNA strands from degradation after MMS-induced fork stalling. The *dia2Δmph1Δ* strain showed tract length patterns similar to *mph1Δ* strains (median of 7.60  $\mu\text{m}$  compared to 7.55  $\mu\text{m}$ ) further suggesting that the phenotype observed in the double-deficient strain is due to the absence of Mph1 alone (Figure 3).

### Dia2 physically interacts with Sgs1 during replication stress

We next asked if Dia2 physically interacts with Sgs1. To determine this, we used a strain expressing full-length Myc-tagged Dia2 (Dia2<sup>Myc</sup>) at the endogenous locus. Cells were either untreated or treated with MMS or HU to induce replication fork stalling. Immunoprecipitation of Dia2<sup>Myc</sup> protein was carried out by incubating whole cell lysates with anti-Myc antibody. As shown in Figure 4A, Dia2<sup>Myc</sup> co-precipitated with Sgs1 only during MMS or HU treatment. Moreover, Sgs1 did not co-precipitate with the anti-Myc antibody in wild-type cells, indicating that the Dia2–Sgs1 interaction is specific. To determine if this interaction was direct or via DNA-bridges, we repeated the immunoprecipitation with and without DNase I. Interaction between Dia2 and Sgs1 was similar in both untreated or DNase I-treated cell lysates suggesting that the Dia2–Sgs1 interaction is not mediated by DNA-bridges (Figure 4B).



**Figure 1.** Dia2 is essential for restart of stalled replication forks. (A) Ten fold serial dilutions of the indicated strains were spotted on yeast extract-peptone-dextrose (YPD), YPD + 0.007% methyl methane sulfonate (MMS) or 200 mM hydroxyurea (HU) and incubated at 30°C. (B) Equal number of cells were plated on media containing the indicated amounts of MMS or HU and colony-forming units were counted after 4 days at 30°C. Error bars represent standard deviations from three independent experiments. (C) Schematic of DNA fiber assay depicting sites of replication. Red tracts, IdU; green tracts, CldU. (D) Representative figure showing inability to restart replication after 0.05% MMS treatment in absence of Dia2, Sgs1 or Mph1. (E) Dia2, Sgs1 and Mph1 work in concert to mediate replication fork restart after MMS-induced fork stalling. The replication restart efficiencies of wild type, *dia2Δ*, *sgs1Δ*, *mph1Δ*, *dia2Δsgs1Δ* and *dia2Δmph1Δ* strains were measured as the number of restarted replication forks (IdU–CldU tracts) compared with the total number of IdU-labeled tracts (IdU only tracts plus IdU–CldU tracts). \*\*\* $P < 0.001$ .



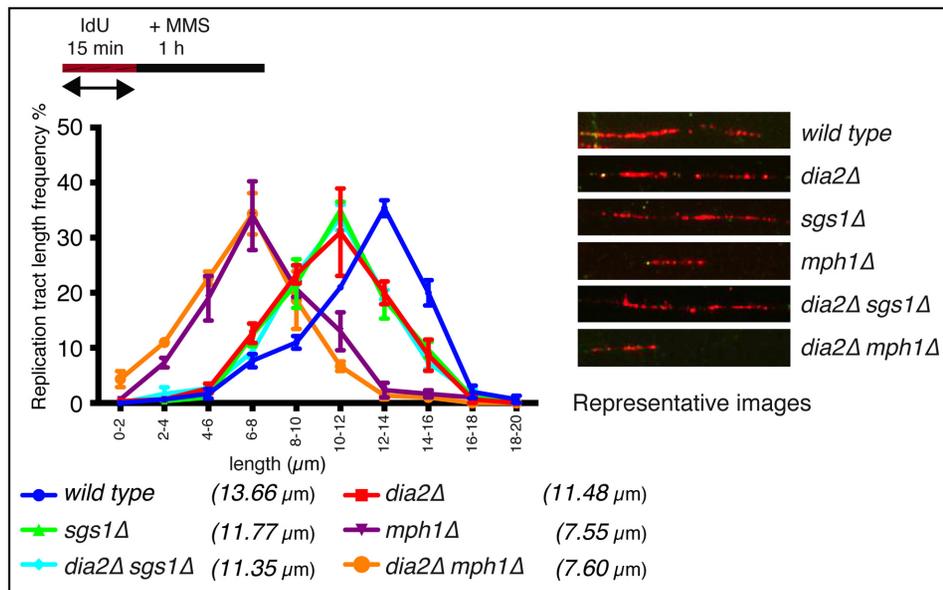
**Figure 2.** Dia2 and Sgs1 maintains normal replication velocity and F-Box domain of Dia2 is important for both fork restart as well as fork speed. (A) Distributions of CldU tract lengths were determined on IdU-CldU double-labeled DNA fibers isolated from untreated wild type, *dia2* $\Delta$ , *sgs1* $\Delta$ , *mph1* $\Delta$ , *dia2* $\Delta$ *sgs1* $\Delta$  and *dia2* $\Delta$ *mph1* $\Delta$  strains.  $P < 0.05$  for wild type versus *dia2* $\Delta$ , *sgs1* $\Delta$ , *dia2* $\Delta$ *sgs1* $\Delta$  or *dia2* $\Delta$ *mph1* $\Delta$  strains and  $P$ -value is not significant for wild type versus *mph1* $\Delta$  strain. (B) Replication fork restart efficiencies of wild type, *dia2* $\Delta$  and the *dia2* F-Box domain deletion strains (*dia2* $\Delta$ F) were measured as the number of restarted replication forks (IdU-CldU tracts) compared with the total number of IdU-labeled tracts (IdU only and IdU-CldU tracts). \*\*\* $P < 0.001$ . (C) CldU tract length distributions were determined in wild type, *dia2* $\Delta$  and *dia2* F-Box domain deletion strains (*dia2* $\Delta$ F).  $P < 0.05$  for wild type versus *dia2* $\Delta$  or *dia2* $\Delta$ F.

### Dia2 interacts with Sgs1 via an N-terminal domain

Next, we mapped the interaction site of Sgs1 on Dia2. For this we used strains containing full-length as well as truncated versions of Myc-tagged Dia2, expressed from the endogenous locus, using co-immunoprecipitation conditions as previously described ((36) and Figure 4C(i)). As shown, full-length Dia2 coimmunoprecipitated Sgs1 (Figure 4C(ii)). The Dia2 mutant lacking the F-box domain ( $\Delta$ F) and an N-terminal fragment (amino acids 1–209), which contains the TPR domain and NLS, also co-precipitated with Sgs1. By contrast, the  $\Delta$ N mutant, which lacks the first 200 amino

acids of the protein, including the TPR domain, did not co-precipitate with Sgs1. Likewise, a C-terminal Dia2 fragment containing the LRR domain but lacking other known domains did not co-precipitate with Sgs1. Taken together, these results indicate that the N-terminal portion of Dia2 containing the TPR domain and NLS interacts with Sgs1 (Figure 4C(ii)).

Given that the N-terminal domain of Dia2 interacts with both Mrc1 (46,47) and Sgs1, we asked whether this domain was important for both MMS sensitivity and recovery from an MMS-induced checkpoint. We have previously generated two N-terminal truncations of Dia2,  $\Delta$ N134 and



**Figure 3.** Mph1 protects nascent DNA strands from nucleolytic degradation after fork stalling. Nascent replication fork tract lengths (labeled with IdU only) were measured after 1 h of 0.05% MMS treatment in *dia2Δ*, *sgs1Δ*, *mph1Δ*, *dia2Δsgs1Δ* and *dia2Δmph1Δ* strains.  $P < 0.05$  for wild type versus *dia2Δ*, *sgs1Δ* or *dia2Δsgs1Δ* and  $P < 0.001$  for wild type versus *mph1Δ* or *dia2Δmph1Δ* strains.

$\Delta$ N175 (36) (note that the numbering has been updated to reflect recognition of verified *Dia2* start site (46)). The  $\Delta$ N175 mutant lacks the entire TPR repeat region (amino acids 48–144) plus a small section of the protein prior to the F-box domain (residues 145–175) and is MMS-sensitive like *dia2Δ* cells, whereas the  $\Delta$ N134 mutant is missing most but not all of the TPR domain and is not MMS sensitive (36) (Figure 5). We tested whether these mutants can re-enter the cell cycle efficiently during recovery from an MMS-induced checkpoint. Cells were arrested in G1, released into MMS-containing media for 40 min to induce fork stalling and then allowed to re-enter the cell cycle in the absence of MMS. Cell cycle progression was monitored by flow cytometry. We observed that the  $\Delta$ N175 mutant exhibited delayed cell cycle re-entry kinetics with 44% of cells in G2/M at the 40-minute timepoint, which was similar to the *dia2Δ* strain with 39% of cells at G2/M at the same time. By contrast, the  $\Delta$ N134 mutant finished S phase with kinetics similar to wildtype cells, with 69% and 72%, respectively, in G2/M phase at the 40 min timepoint (Figure 5). Together, these data suggest that the MMS-sensitivity of *dia2Δ* cells is linked, at least in part, to the function of *Dia2* in checkpoint recovery. Further, the majority of the TPR domain is not required for checkpoint recovery, but a section of the protein between residues 134 and 175 is required.

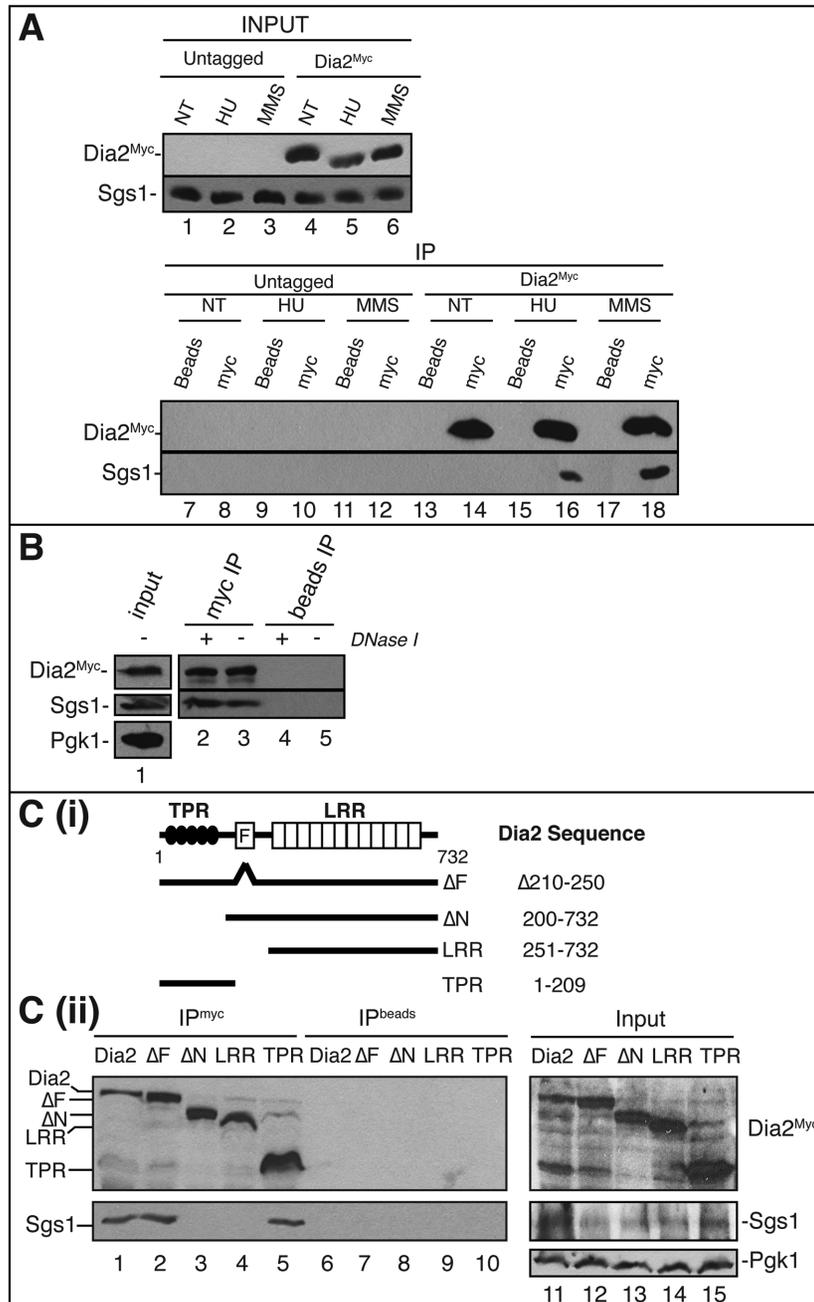
#### Upon stalled fork recovery, *Dia2* dissociates from *Sgs1*

To determine the association dynamics between *Dia2* and *Sgs1* during checkpoint recovery from MMS-induced fork stalling, we used wild-type cells expressing Myc-tagged *Dia2* at the endogenous locus. Cells were either untreated or treated with 0.05% MMS for 20 or 40 min and released to fresh media for 20, 40 or 60 min. Co-immunoprecipitation with Myc-*Dia2* showed that *Dia2* interacts with *Sgs1* after

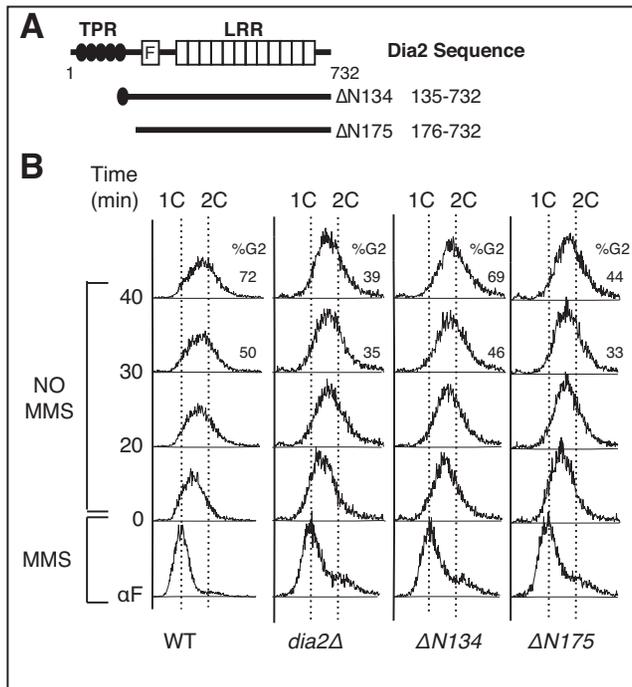
MMS treatment through at least the first 20 min of recovery from MMS. At later time points during recovery, we were unable to detect any co-precipitation between *Dia2* and *Sgs1* (Figure 6). Together, these results suggest that the interaction of *Dia2* with *Sgs1* is important after checkpoint activation through the beginning of recovery from an MMS-induced checkpoint, consistent with their roles in replication fork restart.

#### Failure to degrade *Mrc1* inhibits replication fork restart

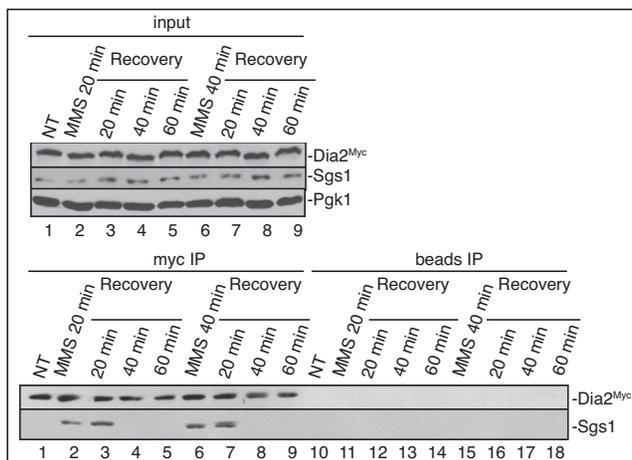
Our previous studies have shown that *Dia2* mediated degradation of *Mrc1* is important for cell cycle re-entry during recovery from an MMS-induced checkpoint (24). However, it is not clear whether *Mrc1* contributes to restart of MMS-stalled replication forks. To address this question, we performed DNA fiber assays using wild type and a *dia2Δ*-*Mrc1*-IAA-inducible degron strain, which allows for temporal control of *Mrc1* degradation. Cells synchronized in G1 were allowed to enter S phase and then were pulse-labeled with IdU (red label) for 15 min. Following IdU labeling, cells were either untreated or treated with 0.05% MMS for 1 h, followed by a second pulse-labeling with CldU (green label) for 15 min in the absence of MMS. Auxin (IAA) was added 15 min prior to CldU labeling to induce *Mrc1* protein degradation. DNA fibers were isolated and incubated with anti-IdU and anti-CldU antibodies to visualize DNA replication. Strikingly, IAA-induced degradation of *Mrc1* in *dia2Δ* cells completely rescued the deficiency of replication fork restart during recovery from an MMS-induced checkpoint (Figure 7A) and replication velocity (Figure 7B) phenotypes observed in the *dia2Δ* strain. Thus, the failure to appropriately target *Mrc1* for degradation in the absence of *Dia2* is the key constraint to restart of MMS-stalled replication forks in *dia2Δ* cells.



**Figure 4.** Dia2-Sgs1 interaction is induced by stalled replication forks. (A) *Dia2* physically interacts with *Sgs1*. Untreated (NT), 0.05% MMS treated or 200 mM HU treated cell lysates prepared from wild type as well as 9myc-tagged *Dia2* strains were used in anti-myc immunoprecipitations (lanes 8, 10, 12, 14, 16 and 18) or incubated with protein A/G beads without antibody as a negative control (lanes 7, 9, 11, 13, 15 and 17). Immunoblots were probed with anti-myc or anti-Sgs1 antibodies. (B) *Dia2* interaction with *Sgs1* is not via DNA bridges. Cell lysates from wild-type cells (lane 1) were either untreated or treated with DNaseI prior to immunoprecipitation with anti-Myc antibody. (C) *Sgs1* interacts with the TPR domain in *Dia2*. (i) Schematics showing *Dia2* protein domains and mutants used in the immunoprecipitation study. TPR, tetratricopeptide repeats; F, F-Box domain; LRR, leucine-rich repeats. (ii) Strains expressing Myc-tagged *Dia2* protein segments were treated with 0.05% MMS. Cell lysates (lanes 11–15) were prepared for immunoprecipitations with an anti-myc antibody (lanes 1–5) or protein A/G beads without antibody (lanes 6–10). Immunoblots were probed with anti-myc and anti-Sgs1 antibodies.



**Figure 5.** An N-terminal domain of Dia2 is required for checkpoint recovery. **(A)** Schematic showing N-terminal deletion mutants of Dia2. **(B)** Cells were arrested in G1 phase of cell cycle by  $\alpha$ -factor ( $\alpha$ F), released into YPD + 0.033% MMS for 40 min followed by release into YPD without MMS for indicated time points to allow checkpoint recovery. Samples were analyzed by flow cytometry. 1C and 2C indicate DNA content and percentage of cells with 2C DNA content is shown on the right side of the selected profiles.



**Figure 6.** Upon stalled fork recovery, Dia2 dissociates from Sgs1. Wild-type cells were either untreated or treated with 0.05% MMS for 20 or 40 min and allowed to recover in the fresh media for 20, 40 and 60 min. Whole cell lysates were prepared and used in immunoprecipitations with an anti-myc antibody (lanes 1–9) or protein A/G beads without antibody (lanes 10–18). Immunoblots were probed with anti-myc or anti-Sgs1 antibodies.

### Mrc1 degradation during checkpoint recovery promotes Sgs1 chromatin association

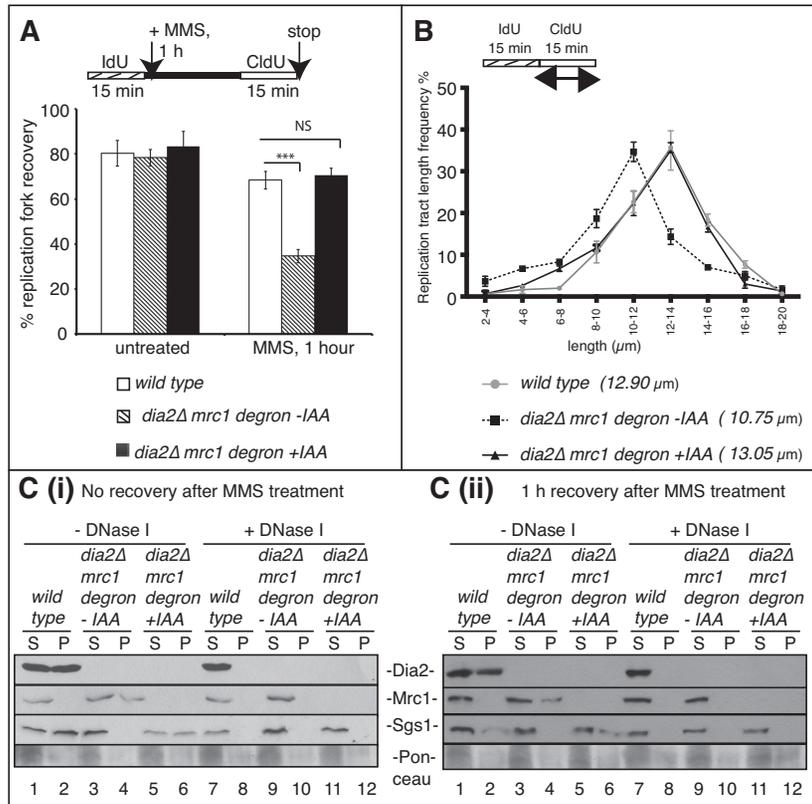
Given that both Mrc1 and Sgs1 interact with Dia2 (46,47), we sought to determine how Dia2-mediated Mrc1 degra-

ation might affect Sgs1 function. As Mrc1 and Sgs1 interact with the N-terminal TPR-containing region in Dia2 and this region is required for Dia2 chromatin association, we initially asked if Mrc1 degradation affected the dynamics of Sgs1 or Dia2 recruitment to chromatin. For this, we used the same wild type and *dia2* $\Delta$ -Mrc1-IAA-inducible degenon strains, synchronized to examine chromatin association during recovery from an MMS-induced checkpoint, in the presence or absence of auxin (IAA) to induce Mrc1 degradation. Cell lysates were either untreated or treated with DNase I and then pellets containing chromatin fractionations were separated from soluble cytoplasmic lysates. In wild-type cells, both Dia2 and Sgs1 were detected on chromatin during checkpoint activation (Figure 7C(i)) and after 1 h of checkpoint recovery (Figure 7C(ii)). By contrast, Mrc1 was not detected in the chromatin fraction at these times (Figure 7C(i) and (ii)). Strikingly, in the absence of Dia2, Mrc1 was detected in the chromatin fraction at both time points, suggesting that Dia2-dependent degradation of Mrc1 prevents large-scale chromatin association of Mrc1. Interestingly, Sgs1 chromatin association was severely inhibited in the absence of Dia2. However, IAA-induced degradation of Mrc1 in *dia2* $\Delta$  cells completely rescued Sgs1 chromatin association. Together, these results suggest that Dia2-mediated degradation of Mrc1 promotes Sgs1 chromatin association during recovery from an MMS-induced checkpoint (Figure 7C(i) and (ii)).

### DISCUSSION

Our previous work demonstrated that Dia2-dependent degradation of Mrc1 was important for cell cycle re-entry during recovery from an MMS-induced S-phase checkpoint, but the molecular impact of this degradation remained unclear. In these studies, we find that degradation of Mrc1 facilitated by the SCF<sup>Dia2</sup> complex is critical to restart of MMS-stalled replication forks (Figure 8). Moreover, Dia2 functions in conjunction with the Sgs1 and Mph1 recombination-mediated fork restart pathway and physiologically interacts with Sgs1 specifically after checkpoint activation. Although the human homologs of Sgs1 and Mph1 have been shown to be required for fork restart (44,48,49), our DNA fiber studies are the first direct demonstration that these roles are conserved in budding yeast. Importantly, we find that failure to target Mrc1 for degradation during recovery from an MMS-induced checkpoint inhibits Sgs1 chromatin association but this can be alleviated by induced proteolysis of Mrc1 after checkpoint activation.

Successful recovery from the S-phase checkpoint requires both inactivation of the checkpoint signaling pathway and restart of stalled or collapsed forks to complete DNA replication. Degradation of Mrc1 likely contributes to signaling inactivation as phosphorylated Rad53 levels persist in *dia2* $\Delta$  cells and the checkpoint recovery defect in *dia2* $\Delta$  cells is exacerbated by the absence of the Rad53 phosphatase Pph3 (24). Here we demonstrate that degradation of Mrc1 is also required for a recombination-mediated fork restart pathway, suggesting that there may be coordination between signaling inactivation and fork restart pathways. One straightforward explanation is that signaling inactivation might lead to rearrangement of protein complexes at the



**Figure 7.** Depletion of Mrc1 in *dia2Δ* strains rescues deficiencies in fork restart and maintenance of replication velocity by recruiting Sgs1 to chromatin. (A) Replication fork restart efficiencies of wild-type and a *dia2Δmrc1 degron* strain with or without IAA were measured as number of restarted forks (IdU-CldU tracts) compared with the total number of IdU-labeled tracts (IdU only plus IdU-CldU tracts). \*\*\*  $P < 0.001$ , NS = not significant. (B) CldU tract length distributions were determined in wild type, and a *dia2Δmrc1 degron* strain with or without IAA.  $P < 0.05$  for wild type versus *dia2Δmrc1 degron - IAA*, and  $P$ -value is not significant for wild type versus *dia2Δmrc1 degron + IAA*. (C (i) and (ii)) Crude pellet containing chromatin fractions were separated from soluble cytoplasmic fractions from the untreated (lanes 1–6) or DNaseI-treated (lanes 7–12) whole cell lysates of wild type and a *dia2Δmrc1 degron* strain with or without IAA. Samples were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted for Dia2<sup>Myc</sup>, Mrc1<sup>HA</sup> and Sgs1 using anti-Myc, anti-HA or anti-Sgs1 antibodies, respectively. S, soluble cytoplasmic fraction; P, pellet containing chromatin fraction.

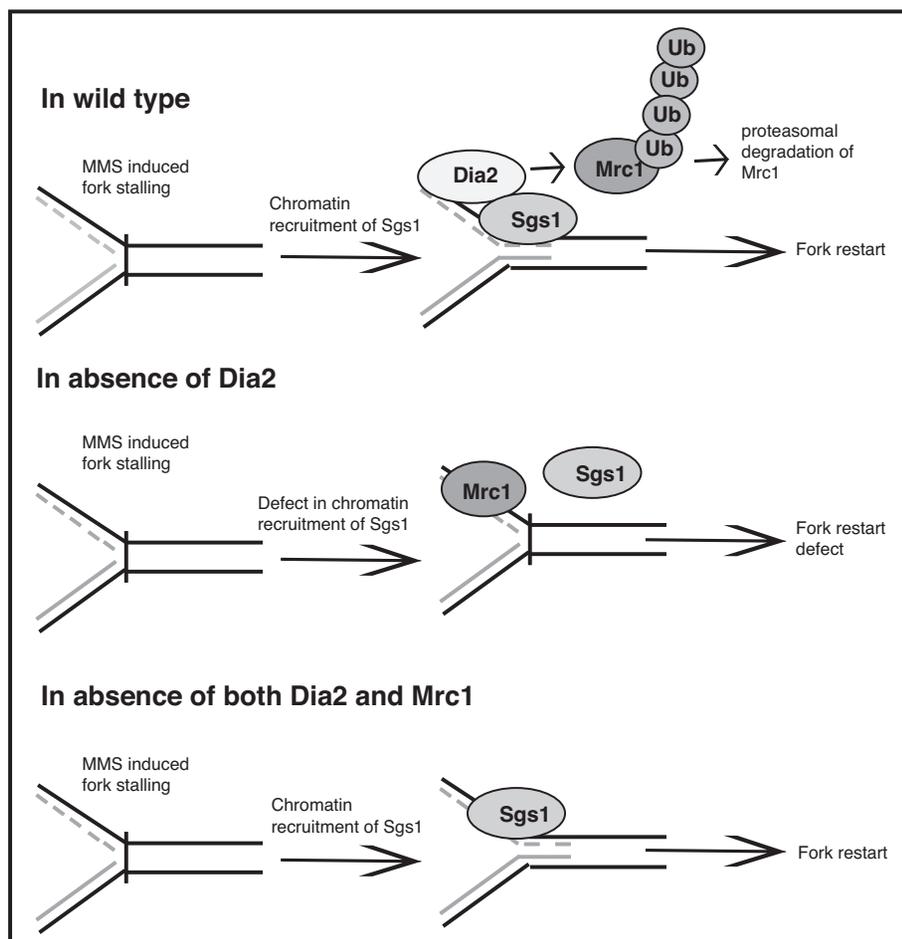
fork that facilitate the transition from a stalled or damaged fork configuration to an active fork. This is consistent with previous studies that have demonstrated that inactivation of the Rad53 kinase is required for fork restart (19,20,50).

Dia2 associates specifically with the helicase Sgs1 upon S-phase checkpoint activation and both proteins function in the same recombination-mediated fork restart pathway after an MMS-induced checkpoint. Sgs1 also has a role in S-phase checkpoint signaling by promoting Rad53 activation (41,51–54). It is unlikely that Dia2 is required for this aspect of Sgs1 function, as Rad53 is constitutively activated in *dia2Δ* cells (37,38) and *dia2Δ* exhibits no enhanced phenotype with a *sgs1* mutant that cannot activate Rad53, but does with a *sgs1Δ* strain (54). We anticipate that Dia2 interacts with Sgs1 on chromatin, as Dia2's N-terminal fragment containing the TPR domain is required for Dia2 chromatin association and this section of the protein is also required for interaction with Sgs1, as well as Mrc1. The coordination of Dia2 interaction with both Mrc1 and Sgs1 remains to be determined in future studies.

Our data suggest that MMS sensitivity can be linked to defects in recovery from the S-phase checkpoint. We find that *dia2* mutants that fail to efficiently re-enter the cell cy-

cle during recovery from an MMS-induced checkpoint also exhibit growth defects in the presence of MMS. A segment of the N-terminal section of Dia2 is required for both viability in the presence of MMS and recovery from an MMS-induced checkpoint. It seems likely that the TPR domain is not the main domain responsible for the MMS sensitivity and checkpoint recovery, as the  $\Delta$ N134 mutant, which rescues MMS sensitivity and checkpoint recovery, lacks all but the last 10 amino acids of the TPR domain. Likewise, a  $\Delta$ N144 mutant that lacks the entire TPR domain still exhibits growth in the presence of MMS (46), as does a  $\Delta$ N159 mutant, albeit when overexpressed (47). We think that there may be an as yet undetermined domain following the TPR section but prior to the F-box domain that is specifically linked to the checkpoint recovery pathway, although future studies will be needed to verify this idea.

There are conflicting reports about whether Mrc1 associated with the replisome is targeted for degradation during DNA replication (46,47,55). Our studies do not directly address this question in a normal S phase. However, during recovery from an S-phase arrest induced by MMS, we do observe that Mrc1 accumulates on chromatin in *dia2Δ* cells when fork restart is defective and substantial depletion of



**Figure 8.** Model depicting role of Dia2 in replication fork restart. Dia2 mediates stalled replication fork recovery by promoting recruitment of Sgs1 and degradation of Mrc1. In response to MMS induced replication fork stalling, Dia2 interacts with Sgs1. Dia2 and Sgs1 work in concert to restart stalled fork. Dia2 mediates ubiquitination of Mrc1 to facilitate Mrc1 degradation via the proteasome. In the absence of Dia2, Mrc1 degradation, Sgs1 chromatin association and replication fork recovery are inhibited. These defects can be reversed by induced degradation of Mrc1 in *dia2Δ* cells.

Mrc1 is necessary to rescue fork restart in these cells. The depletion of Mrc1, including the chromatin-associated fraction, coincides with the accumulation of the helicase Sgs1 on chromatin. Thus, we favor a model in which degradation of chromatin-associated Mrc1 leads to rearrangements at the fork that are critical to recruitment of Sgs1 to chromatin to achieve restart of a substantial fraction of MMS-stalled replication forks. However, it is possible that a small fraction of Mrc1 remains associated with the replisome under these conditions, but that we are unable to detect it in our assay. Future studies will be necessary to comprehensively determine protein complex composition and changes at individual forks during recovery from an MMS-induced checkpoint.

We found that both Sgs1 and Dia2, but not Mph1, are also important for the maintenance of replication speed in an unperturbed replication fork during S-phase. This is consistent with work showing that the BLM helicase is important for maintaining replication speed (44,56). By contrast, Mph1, but neither Dia2 nor Sgs1, protects nascent DNA strands from nucleolytic degradation after MMS treatment. In human cells, Fanconi Anemia (FA) proteins

FANCD2 and FANCD1 (also known as BRCA2) but not BLM maintain stability of the nascent DNA strands after replication stress (44,45,57). The eight FA proteins FANCA/B/C/E/F/G/L/M) form a core complex that mediates monoubiquitination of the central FA effector proteins FANCD2 and FANCI, which then contribute to the repair of damaged DNA. Mph1 is the only known ortholog of the FA core complex protein FANCM, and there are no known homologs of other FA core complex proteins or effector proteins FANCD2 and FANCI in budding yeast (39). Thus, it appears that the functional separation of Sgs1 and Mph1 roles in fork restart and protection of nascent DNA strands is similar to what is observed in human cells between Fanconi Anemia and Bloom Syndrome pathways.

Together, these studies expand our understanding of how cells recover from genotoxin-induced checkpoints. As chemotherapy and radiotherapy induce genotoxic stress to trigger cell death in cancer cells, checkpoint recovery components may serve as alternative targets of treatment to sensitize cancer cells to antitumor therapies. With a link between checkpoint signaling inactivation and recombination-mediated replication fork restart, we have

the tools to begin to delineate the molecular mechanisms required for recovery from agents that damage DNA or cause replication stress.

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