

Fission yeast *Ase1*^{PRC1} is required for the G₂-microtubule damage response

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ABSTRACT

Schizosaccharomyces pombe delays entry into mitosis following G₂ microtubule damage. This pathway is dependent on Rad26^{ATRIP}, the regulatory subunit of the Rad26^{ATRIP}/Rad3^{ATR} DNA damage response (DDR) complex. However, this G₂ microtubule damage response pathway acts independently of the G₂ DNA damage checkpoint pathway. To identify other proteins in this G₂ microtubule damage pathway, we previously screened a cDNA overexpression library for genes that rescued the sensitivity of *rad26Δ* cells to the microtubule poison thiabendazole. A partial cDNA fragment encoding only the C-terminal regulatory region of the microtubule bundling protein *Ase1*^{PRC1} was isolated. This fragment lacks the *Ase1*^{PRC1} dimerization and microtubule binding domains and retains the conserved C-terminal unstructured regulatory region. Here, we report that *ase1Δ* cells fail to delay entry into mitosis following G₂ microtubule damage. Microscopy revealed that Rad26^{ATRIP} foci localized alongside *Ase1*^{PRC1} filaments, although we suggest that this is related to microtubule-dependent double strand break mobility that facilitates homologous recombination events. Indeed, we report that the DNA repair protein Rad52 co-localizes with Rad26^{ATRIP} at these foci, and that localization of Rad26^{ATRIP} to these foci depends on a Rad26^{ATRIP} N-terminal region containing a checkpoint recruitment domain. To our knowledge, this is the first report implicating *Ase1*^{PRC1} in regulation of the G₂/M transition.

Keywords: G₂-microtubule damage checkpoint Rad26^{ATRIP} *Ase1*^{PRC1}

INTRODUCTION

A diverse array of intrinsic and extrinsic insults threatens genomic integrity, and those that lead to double strand DNA breaks (DSBs) are particularly toxic. In humans, repair of DSBs occurs primarily via non-homologous end-joining (NHEJ) and homologous dependent repair (HDR) [1, 2]. NHEJ and HDR are stimulated by a trinity of synergistic phosphatidylinositol 3-kinase-related kinases (PIKKs): DNA-dependent protein kinase catalytic subunit (DNA-PKcs); ataxia-telangiectasia mutated (ATM); and ataxia-telangiectasia and Rad3-related (ATR) that initiate a number of other DDR responses as well, including cell cycle checkpoint control over G₁/S, G₂/M and S-phase [3–6]. ATM and ATR have also been implicated in pathways that respond to interphase microtubule (MT) damage. For example, MT toxins cause G₁ and G₂ cell

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cycle arrest in cultured cells by mechanisms that target downstream components of ATM and ATR signaling, including CHK1, CDC25C and CDC2 [7–11]. Exactly why eukaryotic cells keep the integrity of interphase microtubules under surveillance, and how they detect compromised interphase MTs, are areas for exploration.

Yeast model systems provide a framework for dissecting human DDR pathways [12]. In fission yeast, the conserved Rad26^{ATRIP}/Rad3^{ATR} complex directs DDRs similar to those in humans. The Rad26^{ATRIP}/Rad3^{ATR} complex also participates in a pathway that delays entry into mitosis following G₂-MT damage [13]. Rad26^{ATRIP} plays a cytoplasmic role during this response, as disruption of its C-terminal nuclear export signal prevents both cytoplasmic accumulation of Rad26^{ATRIP} and the G₂/M delay that follows treatment with MT poisons. Notably, this nuclear export allele of *rad26*^{ATRIP} does not compromise the DDR. Therefore, the checkpoint responses to DNA and G₂-MT damage are genetically separate pathways dependent upon Rad26^{ATRIP}. Also using fission yeast, Balestra and Jimenez [14] demonstrated that G₂-MT damage delays entry into mitosis through stabilization of Wee1^{WEE1}, a negative regulator of mitotic cyclin-dependent kinase (CDK) activity and target of the DDR [15–17]. We therefore suspect that the Rad26^{ATRIP}/Rad3^{ATR} complex initiates stabilization of Wee1^{WEE1} when G₂-MTs are damaged. Here, we present evidence that the microtubule bundling protein Ase1^{PRC1} is another component of this G₂-MT damage response in fission yeast.

MATERIALS AND METHODS

PCR cloning of *ase1* and *rad26* into GFP-tagging expression vectors: PCR amplification of *ase1* and *rad26* cDNAs from a cDNA library (gift of M. Yamamoto) were performed using high-fidelity Phusion Master Mix (MO531S; New England Biolabs). Primer sequences appear in Table 1. Amplicons were cloned using the pJET blunt-end cloning kit (K1231; Thermo Scientific), transformed into DH5-Alpha Mix & Go! competent cells (T3007; ZYMO Research), and recombinant vectors were purified using a midi prep kit (12143; QIAGEN). cDNA inserts were then directionally cloned into the thiamine-repressible, amino-terminal pRep41 eGFP and LEU2 vector [18] as NdeI-BamHI fragments and transformed into yeast using the yeast transformation protocol described by Bähler et al [19].

Table 1: Primers used in this study

Primer Set Name	Sequence	Resulting protein Size, aa
Full <i>Ase1</i> gene	Forward: P-5' CAT ATG CAA ACA GTA ATG ATG 3' Reverse: P-5' GGA TCC TTA AAA GCC TTC TTC 3'	731
<i>Ase1</i> -C-term	Forward: P-5' CAT ATG GCT ATG ACG AGT CCA 3' Reverse: P-5' GGA TCC TTA AAA GCC TTC TTC 3'	218
1 Full <i>Rad26</i> gene	Forward: P-5' CAT ATG ATG GCT GAT GAA AGT 3' Reverse: P-5' GGA TCC CTA AAA ATT AGT GTA CAA 3'	615
2 <i>Rad26</i> Δ _{1-103aa}	Forward: P-5' CAT ATG TCC GAA GCT AAT AAT GCC 3' Reverse: P-5' GGA TCC CTA AAA ATT AGT GTA CAA 3'	512
3 <i>Rad26</i> Δ _{310-615aa}	Forward: P-5' AT ATG ATG ATG GCT GAT GAA AGT 3' Reverse: P-5' ACA TCA TCG TCG ATT TAG GAT CC 3'	309
4 <i>Rad26</i> Δ _{109-615aa}	Forward: P-5' AT ATG ATG ATG GCT GAT GAA AGT 3' Reverse: P-5' GGA TCC CTA TGA GGC TTG TGA GTT TAC GG 3'	108
5 <i>Rad26</i> Δ _{1-25aa} -Δ _{147-615aa}	Forward: P-5' CAT ATG GAA TTA GAG CAA CAA GCT CAA ACC 3' Reverse: P-5' GGA TCC CTA CTC ATG AAA TAG GGA TTT CGT 3'	121
6 <i>Rad26</i> Δ _{1-40aa} -Δ _{147-615aa}	Forward: P-5' CAT ATG GTA GTT GTA CCG AGT GAA AAG CAA 3' Reverse: P-5' GGA TCC CTA CTC ATG AAA TAG GGA TTT CGT 3'	106
7 <i>Rad26</i> Δ _{113-123aa}	Forward: P-5' CAA CGG AAA TTA GAG GAG CTT AAA AAA GAA 3' Reverse: P-5' TAA CGA TTC TAG GGC GGC ATT ATT AGC TTC 3'	604

Physiological methods: Spindle-pole-body (SPB) separation was monitored in different strains containing *cdc25.22* and the SPB marker *cut12-egfp* [20]. Cells of each strain were

cultured in YE5S liquid medium to optical density (OD) 0.3 at 30°C, then shifted to 37°C for 3 hours 5 minutes before 16 mg/ml Carbendzim (MBC) was added. Cultures were maintained in the presence of MBC at 37°C for another 25 minutes before downshifting to 20°C and releasing cells from the *cdc25.22* block. The percentage of cells containing two Cut12-EGFP foci was determined every 20 minutes following this downshift. Three trials were performed, and 200 cells were scored at every 20-minute time point. Strains used in this study are outlined in Table 2.

Table 2: Fission yeast strains used in this study

Strain	Genotype	Origin
TE236	<i>leu1-32 ura 4-d18 h-</i>	Kostrub <i>et al.</i> , (1998)
TE257	<i>rad26::ura4+ ade6-704 leu1-32 ura4-D18 h-</i>	Al-Khodairy <i>et al.</i> , (1994)
TW1197	<i>rad26-GFP::kanR leu1-32 ura 4-d18 h-</i>	Baschal <i>et al.</i> , (2006)
MCI728	<i>z::adh15:mcherry-atb2:natMX6 leu1-32 ura-D18 h+</i>	gift of Meredith Betterton
FY20056	<i>ase1-GFP::kanR leu1 h-</i>	Yeast Resource Center, yeast.nig.ac.jp
ST754	<i>ase1D::kanR leu1-32 ura4-D18 h+</i>	gift of Thibault Courtheoux
TW1341	<i>ase1D::kanR cdc25.22 cut12-gfp</i>	this study
TW1345	<i>ase1D::kanR rad26::ura+ cdc25.22 cut12-GFP</i>	this study
TW1300	<i>rad26::ura4+ cdc25.22 cut12-egfp::ura4+</i>	Herring <i>et al.</i> , (2010)
MKSP765	<i>rad26-mcherry::kanR leu1-32 ura4-D18 h+</i>	gift of Megan King
MKSP2074	<i>rad52-mcherry::kanr ura4-D18 leu1-32 his3-D1</i>	gift of Megan King
FY20720	<i>sad1-mcherry::KanR leu1 his2 h+</i>	Yeast Resource Center, yeast.nig.ac.jp

Microscopy: To visualize EGFP fusion proteins, 1 ml aliquots from cultures grown in liquid YE5S to OD 0.3 were centrifuged and resuspended in cold methanol for one minute, washed twice in 100 ml SlowFade Component C (SlowFade Antifade Kit, Molecular Probes) and air dried on coverglass. Once dried, 4.5 µl SlowFade Component A was dropped on the coverglass, which was then placed onto a slide. Achieving yeast monolayers that adhered tightly to the coverslips was crucial to observing fluorescence signals. To help ensure that such layers formed, the coverglass was soaked in acetone for one day, scrubbed with dishwashing soap, wiped with 70% ethanol and air dried prior to use. Images were acquired using a Leica DM5000 equipped with a Leica DFC350FX R2 digital camera and Leica FW4000 software.

RESULTS AND DISCUSSION

Previously, we screened a cDNA library for genes that, when overexpressed (OE), permit *rad26Δ* cells to grow on medium containing thiabendazole (TBZ) [21]. Of 10,000 transformants, four cDNAs representing Rad24, SPCC70.01, DASH complex subunit Dam1, and the C-terminal 218 amino acids of Ase1 (aa 513–731; Fig. 1A) were identified. Here, we investigate the role of Ase1 in the *rad26*-dependent G₂-MT damage response more closely, since both *ase1*⁺ and *rad26*⁺ are required for proper cell morphology and minichromosome stability [13, 22]. Ase1 is a conserved MT-bundling protein containing a dimerization domain, a MT-binding domain, and an unstructured C-term [22–26]. Below, we confirm that OE of the unstructured Ase1-C-term rescues the growth of *rad26Δ* cells on media containing a MT toxin, and report that Ase1 is required to delay mitotic entry following insult to G₂-MTs.

We observed *nmt*-promoter driven OE of full length Ase1-GFP to be toxic, presumably due to MT-hyper-bundling (Fig. 1B, C; [22]). Within these nonviable cells, we observed that OE Ase1-GFP assembled into bright linear structures (Fig. 1D), consistent with MT-hyper-bundling at overlapping microtubules [22]. Overexpression of the 218aa unstructured C-term of Ase1 was not toxic, and rescued *rad26Δ* growth on carbendazim (MBC; Fig. 1B, C). This C-term fragment localized diffusely throughout cells (Fig. 1D) and did not appear to integrate within the yeast MT cytoskeleton. Therefore, OE Ase1-C-term may rescue *rad26Δ* growth on MBC in a regulatory manner, as opposed to a structural one.

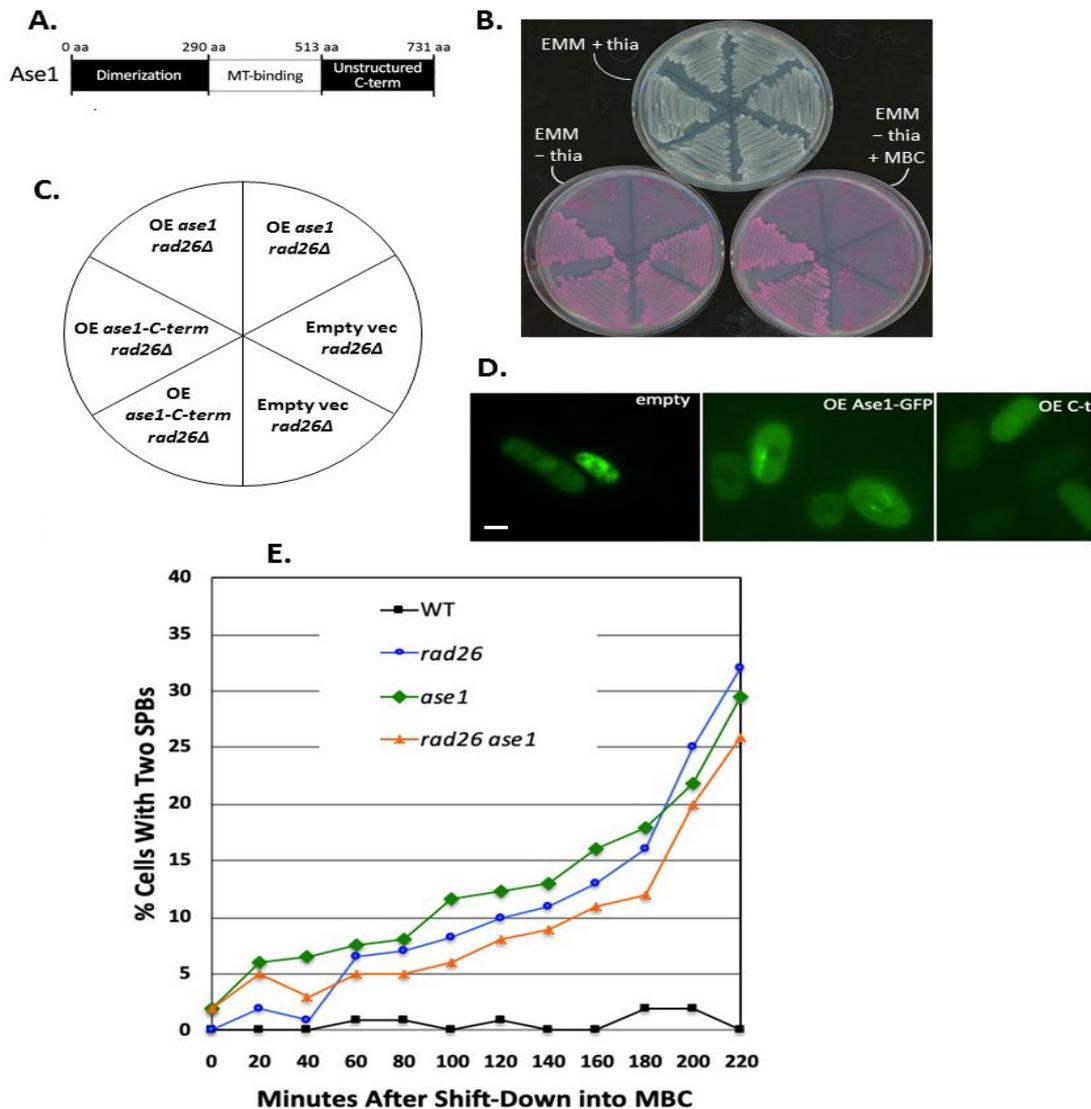


Figure 1: Ase1 is required to delay SPB separation following G₂-microtubule damage. **A.** Map of the dimerization domain, MT-binding domain and C-terminal region of Ase1. **B.** Overexpression of the C terminal (513–731) amino acids of Ase1 rescues the MBC sensitivity of *rad26Δ* cells. Thiamine (thia) represses transcription of vector inserts. In this experiment, a medium strength *nmt* thiamine-repressible promoter was fused to full length Ase1-GFP, Ase1-C-term-GFP, or empty GFP vector, and transformed into *rad26Δ* cells under repressive conditions (EMM + thiamine). Two transformants representing each condition were streaked to repressive conditions (EMM + thiamine) for two days before replica plating to media with inducing conditions and a pink vital dye. **C.** Map of the genotypic distributions on the EMM-thia replica plates shown in (B). **D.** Overexpressed full length Ase1-GFP assembled into bright linear structures within nonviable cells. Overexpressed Ase1-C-term was diffusely localized throughout viable cells. Bar = 5 μm. **E.** Ase1 is required to delay SPB separation following MBC treatment. Strains containing the *cdc25.22* and *cut12-gfp* alleles were arrested in G₂ before releasing into medium with or without 16 mg/ml MBC. At each time point, the percentage of cells containing two Cut12-GFP foci was calculated from 150 cells. WT refers to *rad26⁺ ase1⁺* cells with *cdc25.22 cut12-gfp* alleles in the background.

Next, we tested if *ase1⁺* is required for the *rad26⁺*-dependent checkpoint response to G₂-MT damage (Fig. 1E). The temperature-sensitive *cdc25.22* allele was used to reversibly block cells in G₂, and the spindle-pole-body (SPB) marker Cut12-EGFP was used to monitor SPB separation and mitotic entry [20,27,28]. After wildtype cells were released from the G₂ block into nutrient rich YE5S medium containing MBC, SPB separation was prevented for greater

than three hours (Fig. 1E). This result is consistent with those of Akera, Sato and Yamamoto [29], who observed that SPBs fail to separate during MBC treatment. Following release of *rad26Δ*, *ase1Δ*, and *rad26Δ ase1Δ* cells from the G₂ block into MBC media, precocious SPB separation began within the first hour in all three strains (Fig. 1E). That the kinetics of SPB separation were similar among the three strains suggests that Ase1 and Rad26 operate in the same pathway. We also tested if OE Ase1-C-term restored the G₂-delay to *rad26Δ* cells during treatment with MT-toxin. However, the *nmt*-promoter requires growth in nutrient-deficient, synthetic media to drive OE, and we observed that cells grown under these nutrient-limiting conditions fail to delay G₂/M following treatment with MT poisons (data not shown). This may be due to activation of a stress MAP kinase response that overrides this G₂-MT damage response in nutrient-depleted conditions [30]. In summary, we conclude that Ase1 participates in the Rad26-dependent G₂-MT damage response pathway.

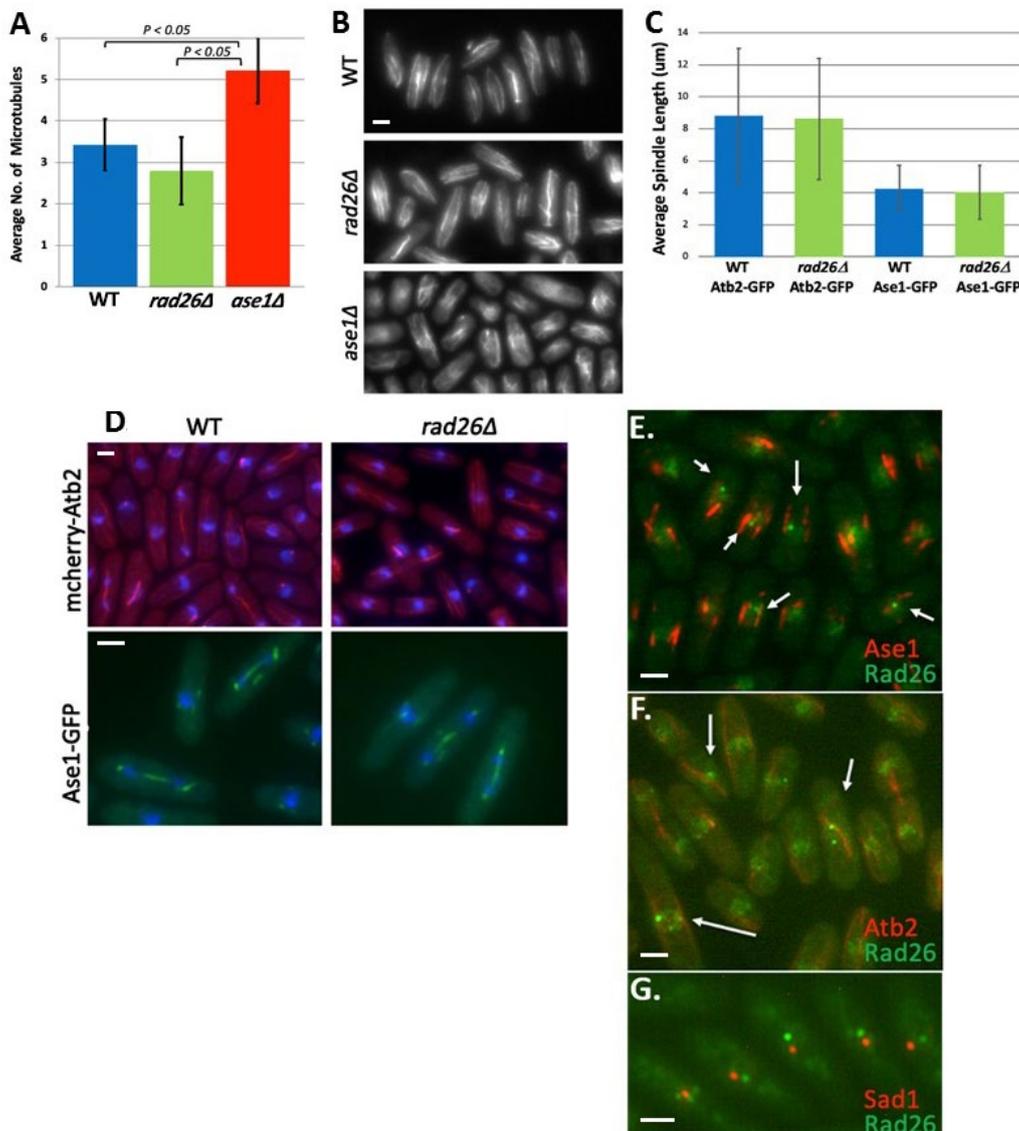


Figure 2: Rad26 does not influence MT structure, although it does tangentially localize with Ase1 and MTs. All strains were grown at 30°C in liquid YE5S to optical density 0.3 and fixed with cold methanol. The number of MT- bundles per cell (A, B) and spindle length (C, D) were normal in *rad26Δ* cells. (E, F and G) Rad26 tangentially localizes with Ase1 and MTs, but not the SPB. Strains expressing mCherry-Atb2 (MTs), Rad26-GFP, Rad26-mCherry, Ase1-GFP and Sad1-mCherry were used in these experiments (false colors are sometimes shown); DAPI (blue) was used to visualize nuclei in (D). All bars =5 μm.

Ase1 bundles MTs, and its loss results in an increased number of interphase MT bundles, as well as short mitotic spindles that are prone to collapse [22,31]. In agreement, we observed that the number of MT bundles in *ase1Δ* cells was significantly greater than the numbers in WT and *rad26Δ* cells, which were similar (Fig. 2A, B). In addition, the length of mitotic spindles decorated with either Atb2-GFP or Ase1-GFP was similar in WT and *rad26Δ* cells (Fig. 2C, D). Therefore, *rad26*⁺ does not appear to share *ase1*⁺-dependent functions related to MT bundling or spindle stability.

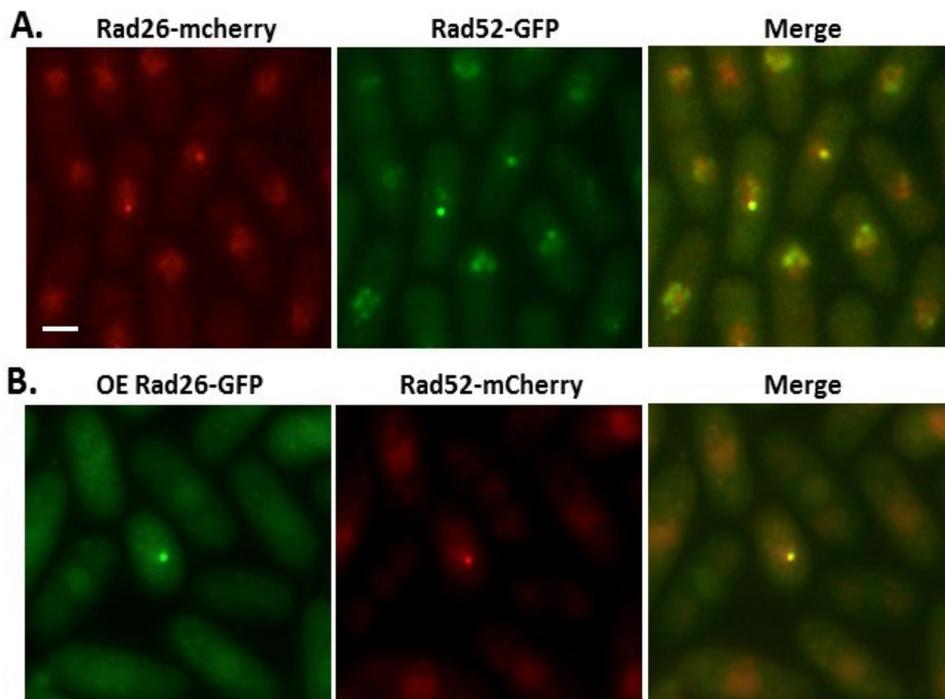


Figure 3: Rad26 and Rad52 colocalize. **A.** Colocalization of Rad26 and Rad52 was observed in cells grown at 30°C in liquid YE5S to optical density 0.3 and fixed with cold methanol. **B.** Overexpressed Rad26-GFP colocalizes with Rad52-mcherry. Cells were grown in promoter de-repressing liquid conditions (EMM – thiamine) for 20 hours and fixed with methanol. Bar=5 μm.

Next, we tested if Rad26^{ATRIP} and Ase1^{PRC1} colocalize with one another. Cycling fission yeast have approximately six faint nuclear Rad26 foci and, occasionally, one or two somewhat brighter foci [32]. We observed that these bright Rad26 foci tangentially localize with Ase1 (Fig. 2E) and MTs (Fig. 2F), but not to the spindle pole body (Fig. 2G). However, we believe that this association between Rad26 and Ase1 is related to the DDR, and not the G₂-MT damage response, because these bright Rad26 foci co-localized with Rad52 (Fig. 3A). Rad52 is a DNA recombination-promoting protein that displaces RPA-coated ssDNA to facilitate HDR [33]. At sites of DNA damage, Rad52 foci co-localize with DNA checkpoint proteins [34,35] and comigrate with the nuclear envelope-spanning linker of nucleoskeleton and cytoskeleton (LINC) complex [36]. Comigration of Rad52-foci with LINC is a microtubule- and Rad3-dependent process that directs HDR of damaged DNA. Therefore, we suggest that Ase1 tangential localization with Rad26/Rad52-foci is likely playing a role in this microtubule-dependent DDR pathway as opposed to the G₂-MT damage response.

To identify the region of Rad26^{ATRIP} that mediates co-localization with Rad52, we cloned the *rad26*⁺ cDNA in front of GFP in a medium strength thiamine-repressible expression vector. Rad26^{ATRIP} is 615 amino acids long and contains a coiled-coil motif in the N-terminus ([37]; Fig. 4A). Overexpression of the full length Rad26-GFP fusion protein resulted in production of bright nuclear foci that also co-localized with Rad52 (Fig. 3B). A large C-terminal truncation of Rad26^{ATRIP} did not affect localization to foci (Fig. 4A, construct 3), demonstrating that the N-

terminal half of the protein is sufficient for focus formation. A region near the N-terminal border of the coiled-coil was critical for focus formation, since neither 1–108aa (Fig. 4A, construct 4) or 104–615aa (Fig. 4A, construct 2) localized to foci. This led to identification of an N-terminal region within 26–146aa that directed focus formation (Fig. 4A, construct 5). The intact coiled-coil region of Rad26 was not required for focus formation, since an 11 amino acid deletion within the coiled-coil permitted focus formation (Fig. 4A, construct 7).

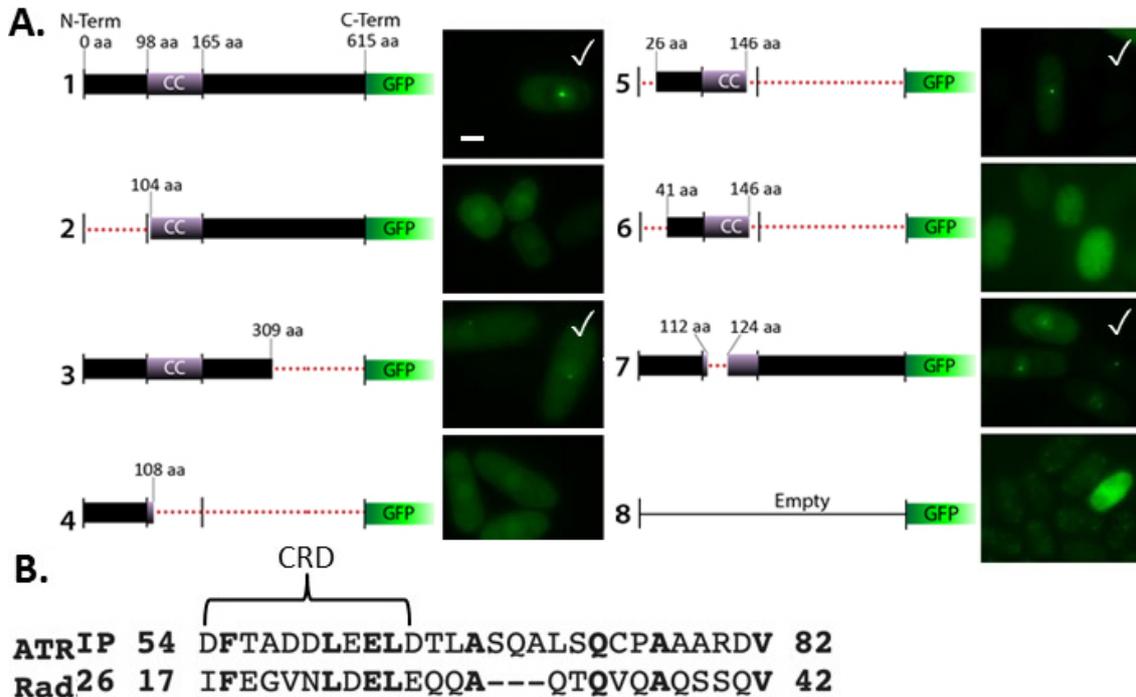


Figure 4: Rad26 contains an N-terminal Checkpoint Recruitment Domain Required for Focus Formation. A. Summary of Rad26-GFP truncations. CC = coiled-coil region; ... = DNA removed; ✓ = positive for foci. Bar = 5 μm. B. An area that resembles the Checkpoint Recruitment Domain (CRD) of ATRIP [39] lies within the N-terminal region of Rad26 that directs focus formation (25–146aa).

Within ATRIP, an N-terminal region between 43–108aa contains a major RPA-ssDNA interacting domain [38]. An acidic *checkpoint recruitment domain* (CRD) exists within this N-terminal region (54–68aa) that mediates ATRIP focus formation and interaction with a basic cleft of RPA subunit RPA70N [39,40]. This CRD is conserved in budding yeast Ddc2^{ATRIP} where it also mediates focus formation and interaction with RPA. While destruction of this motif impairs ATRIP localization to nuclear foci, it does not completely impair ATR signaling. Here, we identify a region resembling the CRD that exists within the 25–146aa region of Rad26 that directed focus formation (Fig. 4A, construct 5; Fig. 4B). Experiments to determine if it is required for Rad3^{ATR} DNA checkpoint signaling in *S. pombe* are planned.

To our knowledge, this is the first report implicating Ase1^{PRC1} in regulation of the G₂/M transition. During G₂, Ase1^{PRC1} bundles and stabilizes interphase MTs [31]. During the transition from G₂ to M, Ase1^{PRC1} localizes to the duplicated SPBs as they separate, yet Ase1^{PRC1} is not required for this event in wildtype cells [41–43]. Later, Ase1^{PRC1} is required to stabilize the pre-anaphase spindle and then the spindle midzone, where it influences the future site of division [22,23,31,44–47]. Ase1^{PRC1} plays two regulatory roles following entry into mitosis. Prior to metaphase, Cdc2^{CDK1} phosphorylates target sites in the Ase1-C-term to prevent release of the kinesin-like motor Klp9 from Ase1^{PRC1} [48]. After metaphase, the Clp1/Flp1^{CDC14} phosphatase removes these inhibitory phosphate groups to permit release of Klp9 from Ase1^{PRC1} in order to initiate anaphase B microtubule sliding. Ase1^{PRC1} is also required for the Clp1/Flp1^{CDC14}- and septation initiation network (SIN)-dependent cytokinesis checkpoint that

inhibits successive nuclear divisions following perturbation of actomyosin ring components [22,49-52]. In summary, Ase1^{PRC1} has well established structural and regulatory roles during later mitotic events, and this report here appears to be the first to identify an earlier G₂/M role for Ase1^{PRC1}.

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Conflict of Interest: We declare there is no conflict of interest to report.

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