Caf1 regulates translocation of ribonucleotide reductase by releasing nucleoplasmic Spd1–Suc22 assembly

Shinya Takahashi, Kenji Kontani, Yasuhiro Araki and Toshiaki Katada*

Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan

ABSTRACT

Appropriate supply of deoxyribonucleotides by the ribonucleotide reductase (RNR) complex is essential for DNA replication and repair. One recent model for the RNR activation in *Schizosaccharomyces pombe* is translocation of the regulatory subunit Suc22 from the nucleoplasm to the cytoplasm. The RNR inhibitory protein Spd1, which retains Suc22 in the nucleoplasm, is rapidly degraded upon DNA-replication stress, resulting in release of Suc22 to form the active RNR complex in the cytoplasm. Here, we show that Caf1, a component of the Ccr4–Not complex, is responsible for resistance of the replication stress and control of the Suc22 translocation. Caf1 is required not only for the stress-induced translocation of Suc22 from nucleoplasm to cytoplasm but also for the degradation of nucleoplasmic Spd1. DNA-replication stress appears to allow Caf1 to interact with Suc22, resulting in release of the nucleoplasmic Spd1–Suc22 assembly. Taken together, these results suggest a novel function of Caf1 as a key regulator in the stress-induced RNR activation.

INTRODUCTION

The Ccr4–Not complex is known not only as the transcriptional factor but also as the major cytoplasmic deadenylase in *Saccharomyces cerevisiae* (1,2). The complex, which has been initially identified as a global regulator of transcription (3–5), consists of nine core subunits (Ccr4, Caf1/Pop2, Not1–5, Caf40 and Caf130) and additional components, such as Dbf2, Mob1, Caf4 and Caf16. Among these constituents, Ccr4 and Caf1/Pop2 have been well characterized. The Ccr4 or Caf1/Pop2 and the other proteins show distinct growth phenotypes and different binding partners (6). Ccr4 and Caf1/Pop2 also appear to function as cytoplasmic deadenylases (7). Their primary structure suggests that Ccr4 is a member of exo III family of nucleases, Mg^{2+}-dependent endonuclease and Caf1/Pop2 is categorized as a member of DEDDh family of RNases (8). Some residues, which are crucial for exonuclease activity, are missing in ScCaf1/Pop2, although the deadenylase activity of Caf1/Pop2 is detected *in vitro* (9). On the other hand, it has been reported in *S. cerevisiae* that the Ccr4–Not complex is responsible for the sensitivity to DNA-replication stress in large-scale studies (10,11). The sensitivity appears to be dependent on the deadenylase activity of Ccr4 (12) and the transcription of RNR genes by Ccr4, Caf1/Pop2 and Not1–5 (13). However, it remains unclear whether these activities in the Ccr4–Not complex are sufficient for the stress resistance.

In response to replication stress and DNA damage, stress-response and highly conserved checkpoint pathways are activated in order to prevent genome instability. The checkpoint pathway and the supplement of dNTPs are activated in response to chemical reagents that induce DNA-replication stress and DNA damage. The S-phase DNA-replication checkpoint pathway induces cell-cycle blockage (14–16). Proteins involved in the checkpoint pathway are categorized into three groups: damage sensors, adaptors and effector kinases. In *Schizosaccharomyces. pombe*, Rad3 and Rad26, which belong to the phosphoinositide 3-kinase family, have been found to sense DNA damage (17,18). These sensor proteins phosphorylate Serine/Threonine-kinase adaptors, and the activated adaptor kinases in turn phosphorylate effector kinases, such as Cds1, which control further downstream targets involved in the stress response (19).

In addition to the DNA-replication checkpoint pathway, the dNTP flow is precisely controlled under

DNA-replication stress and DNA damage. High-fidelity DNA replication requires an adequate supply of dNTPs (20–22). The dNTPs are synthesized from NTPs by the ribonucleotide reductase (RNR) complex, whose activity is elaborately controlled. In both *S. cerevisiae* and *S. pombe*, RNR is principally composed of two components; the large catalytic subunit Rnr1 or Rnr3 (SpCdc22) in the cytoplasm and the small regulatory subunit Rnr2–Rnr4 complex (SpSuc22) in the nucleoplasm. Multiple layers of regulation are imposed on the RNR activity. In *S. pombe*, Cid13 contributes to stabilize 1.9-kb Suc22 mRNA by its poly-adenylation, which proceeds only in response to DNA-replication stress (23). Furthermore, several proteins have been identified to regulate the activity and translocation of the RNR subunits. For example, Sml1 interacts with the catalytic subunit Rnr1 to inhibit the RNR activity in *S. cerevisiae* (24,25), and Spd1 (in *S. pombe*) is capable of inhibiting the RNR activity of Suc22–Cdc22 *in vitro* (26). In addition to the role in regulating activity of RNR, Spd1 captures the regulatory subunit Suc22 in the nucleoplasm and acts as a negative regulator for RNR in *S. pombe*. During S phase or DNA damage and replication stress, Spd1 is degraded by the Pcu4–Ddb1–Cop9 signalosome (CSN) complex; this releases Suc22 from the nucleoplasm to the cytoplasm, where it associates with Cdc22 to form the active complex (27). However, it is unclear how Spd1 degradation is initiated or regulated resulting in the dissociation of the nucleoplasmic Spd1–Suc22 complex in response to DNA damage or replication stress.

Here, we identified Caf1, a component of the Ccr4–Not complex, as a key regulator of Spd1 degradation in *S. pombe*. Caf1 is required for resistance to DNA-replication stress through the control of Suc22 translocation. Caf1 interacts with Suc22 in response to DNA-replication stress and promotes the degradation of nucleoplasmic Spd1 and the cytoplasmic translocation of Suc22. We propose that Caf1 plays an important role in regulating the RNR activity through releasing the nucleoplasmic Spd1–Suc22 assembly.

MATERIALS AND METHODS

Yeast strains

Schizosaccharomyces pombe strains were grown on YE3S (0.5% yeast extract, 2% glucose, 225 μg/ml each of adenine, leucine and uracil) for vegetative growth, or Edinburgh minimal medium (EMM) (28). DNA constructs for chromosomal disruptions and epitope tagging were made by PCR-using method and integrated by homologous recombination into the desired loci using the method described previously (29). When the kanMX6 and hygromycin cassette were used for a disruption marker, transformants were grown on YE3S plate for 1 day for integration and resistance gene expression, before plating on YE3S containing G418 or hygromycin. Some of the yeast strains used in this study are shown in Table 1.

Table 1. Yeast strains used in this study

Name	Relevant genotype
YSP 001	Wild-type JY746
YSP 002	ccr4::kan^r
YSP 003	pan2::kan^r
YSP 004	parn::hygB^r
YSP 027	Wild-type pRep1-FLAG
YSP 066	caf1::hygB^r
YSP 069	cds1::kan^r
YSP 071	rad26::kan^r
YSP 072	ccr4::kan^r rad26::hygB^r
YSP 086	cid13::hygB^r
YSP 091	spd1-13myc::ura4^+
YSP 095	caf1::hygB^r cid13::kan^r
YSP 097	ccr4::kan^r cid13::hygB^r
YSP 102	caf1::hygB^r rad26::kan^r
YSP 103	caf1::hygB^r cds1::kan^r
YSP 104	caf1::hygB^r ccr4::kan^r
YSP 106	ccr4::kan^r cds1::hygB^r
YSP 112	spd1::hygB^r
YSP 131	not4::hygB^r
YSP 143	ski2::hygB^r
YSP 154	ccr4::hygB^r spd1-13myc::ura4^+
YSP 161	caf1::hygB^r spd1-13myc::ura4^+
YSP 162	not4::hygB^r spd1-13myc::ura4^+
YSP 172	xrn1::hygB^r
YSP 184	ccr4::kan^r pRep41-HA
YSP 185	ccr4::kan^r pRep41-HA-Ccr4
YSP 188	caf1::hygB^r pRep1-FLAG
YSP 189	caf1::hygB^r pRep1-FLAG-Caf1
YSP 190	caf1::hygB^r pRep1-FLAG-Caf1 D50A
YSP 195	caf1::hygB^r spd1-13myc::ura4^+ pRep1-FLAG
YSP 196	caf1::hygB^r spd1-13myc::ura4^+ pRep1-FLAG-Caf1
YSP 197	caf1::hygB^r spd1-13myc::ura4^+ pRep1-FLAG-Caf1 D50A
YSP 226	spd1-13myc::ura4^+ pRep1-FLAG

All strains are derivatives of JY746 with the following genotype: *h + ade6-M216 leu1-32 ura4-D18*.

HU-sensitivity analysis

For spot assays, 5 μl of 10-fold serial dilutions of logarithmically growing cells were spotted onto YE3S plates containing the indicated concentrations of HU and incubated for 2–3 days at 30°C. For complementation analysis, 5 μl of 10-fold serial dilutions of logarithmically growing cells in EMM-leu medium were spotted onto EMM-leu plate containing HU and incubated for 2–3 days at 30°C.

Construction of plasmid and yeast mutants

The plasmid pRep1-His-FLAG was constructed by inserting the double-stranded oligonucleotide, annealed OLI1 (5′-CA TGG ATG ACT GGT CAT CAC CAT CAC CAT CAC GGT GAC TAC AAG GAT GAC GAT GAC AAG GGT CA-3′) and OLI2 (5′-TAT GAC CCT TGT CAT CGT CAT CCT TGT AGT CAC CGT GAT GGT GAT GGT GAT GAC CAG TCA TC-3′) into the NcoI and NdeI site of pRep1. The *caf1* and *ccr4* ORF was amplified by PCR from an *S. pombe* cDNA library and inserted into pGEM-T EASY vector (Promega). The plasmid pRep1-His-FLAG-Caf1 was constructed by inserting a sequenced clone of Caf1 in NdeI and SalI site of pRep1-His-FLAG. The plasmid pRep41-HA-Ccr4 was also constructed by inserting a sequenced clone of

Ccr4 in SalI site of pRep41-HA. To isolate *caf1* mutants, an error-prone PCR method was performed using rTaq polymerase in the presence of 3.0 mM Mg^{2+}, 0.5 mM Mn^{2+}, 0.2 mM dATP, 0.2 mM dGTP, 1.0 mM dCTP, and 1.0 mM dTTP as previously reported (30).

Protein preparation, immunoprecipitation and western blot analyses

Logarithmically growing cells (5×10^8) in the EMM selective medium or YE3S were pelleted, washed once and resuspended in 400 µl of a lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40 and protease inhibitors. The cells were mixed with glass beads (1 g) and disrupted by 12 cycles of vortexing for 30 s followed by incubating on ice for 1 min. The cell extracts were obtained by two consecutive runs of centrifugation (15 000 *g* for 10 min). Immunoprecipitation of tagged proteins was performed as follows. The extracts (350 µl) were mixed with anti-FLAG antibody conjugated-beads (M2-Agarose-AFFINITY; Sigma) or anti-HA antibody conjugated-beads (Anti-HA Affinity Matrix; Roche) and further incubated on a rotator at 4°C for 2 h. The beads were spun down and washed extensively with the lysis buffer without protease inhibitors. Proteins binding to the beads were eluted with an SDS-PAGE sample buffer by boiling for 5 min. The cell extracts (12.5 µl) and one-third of the eluted fraction were subjected to SDS-PAGE and immuno-blotted with anti-Myc (9E10; 1:1000), anti-FLAG (M2; 1:1000), anti-HA (12CA5; 1:1000), anti-tubulin (gifted from K. Gull; 1:3000) monoclonal and anti-Suc22 (generous gift from Dr Masuda; 1:2000) polyclonal antibodies.

Northern blot analysis and the detection of poly(A) tails of total mRNAs

Logarithmically growing cells (5×10^8 cells) in the EMM selective medium or YE3S were removed, spun down and immediately frozen in liquid nitrogen. RNA prepara-tion and northern blot analysis were performed as previously described (31) with minor modification. RNA was separated by 1% agarose-gel electrophoresis and transferred to the Hybond XL (Amersham Pharmacia). *suc22* mRNA was detected by northern blotting using a PCR fraction of the ORF. The poly(A) length of total RNAs was analyzed as previously described (32) with some modifications. One microgram of purified total RNA was end-labeled with 5′-[^{32}P] pCp using T4 RNA ligase. The radiolabeled mRNA was digested with RNase A and separated on 12% polyacrylamide–7.5 M urea gel by electrophoresis. The membranes and gels were exposed to phosphorImager screens followed by quantitative analysis using Molecular Dynamics software.

Microscopic analysis

Spd1-13myc and Suc22 staining was performed by the method described previously (33) with some modifica-tions. Logarithmically growing cells (5×10^8 cells) were incubated in YE3S liquid in the presence or absence of 10 mM HU for 2 h. The cells were resuspended in 1 ml of PEM (100 mM PIPES, pH 6.9, 5 mM EGTA and 5 mM $MgCl_2$), fixed with 3.7% para-formaldehyde for 1 h at 30°C, washed and mixed with 10 mg/ml Zymolyase 100T in PEM containing 1.2 M sorbitol for 1 h at 37°C. Spd1-13myc was detected using anti-Myc (9E10) at 1:100 and Alexa488-conjugated anti-mouse antibody (Molecular Probes) at 1:100. Suc22 was detected using anti-Suc22 antibody at 1:100 and Alexa Fluor 488-conjugated anti-rabbit antibody (Molecular Probes). The cells were also counterstained with DAPI to visualize the DNA.

RESULTS

Caf1 and Ccr4 contribute to protect against DNA-replication stress by interacting with RNR-activation pathway

It has recently been reported that several components of mRNA-decay machinery are required for efficient cell-cycle progression after DNA damage and during replication stress in various species including *S. cerevisiae* (10,11,34). To clarify the importance of the decay machinery toward the stress responses in *S. pombe*, we initially investigated whether the disruption of genes encoding mRNA-specific exonucleases show high sensi-tivity to stress stimuli. For the analysis, hydroxyurea (HU), which impairs DNA replication by inhibiting RNR activity (35,36), was used as the stress stimulus. In accordance to previous work in *S. cerevisiae*, *ccr4Δ* and *caf1Δ* cells in fission yeast also exhibited high sensitivity to HU (Figure 1A). In contrast, the deletion of other possible deadenylases (Parn and Pan2) or a component of the 3′–5′ helicase (Ski2) did not impair the cell growth with HU, though cells lacking the 5′-exonuclease Xrn1 exhibited non-selective growth defect under the present conditions (37). Thus, among various mRNA-specific exonucleases, Caf1 and Ccr4 appear to have unique properties in terms of recovery from the DNA-replication stress.

Various pathways are activated in response to HU, including DNA-structure checkpoint and synthesis of dNTPs by RNR activation. To test whether Caf1 and Ccr4 are involved in these pathways, we carried out genetic interaction experiments. Double mutants lacking Caf1 or Ccr4 and well-characterized components of the above pathways were constructed and analyzed for their sensitivity to HU. As shown in Figure 1B, double mutants, *caf1Δ* with additional deletion of the checkpoint gene *CDS1* (*cds1Δ*) or *RAD26* (*rad26Δ*), showed marked increases in sensitivity to HU compared to either single mutant. Such synergistic phenotypes were also observed in double mutants of *ccr4Δ* and *cds1Δ* or *rad26Δ* (data not shown), suggesting that Caf1 and Ccr4 might act in a pathway(s) distinct from the DNA checkpoint. The possible involvement of Caf1 and Ccr4 in the dNTP-synthesis pathway was next investigated. We compared the sensitivity of *caf1Δ cid13Δ* or *ccr4Δ cid13Δ* double mutant with the respective single mutants, since HU inhibits RNR consisting of Suc22 and Cdc22 in the fission yeast and stimulates the poly-adenylation by the cytoplasmic poly(A) polymerase Cid13 of specific mRNAs coding stress-inducible genes, such as
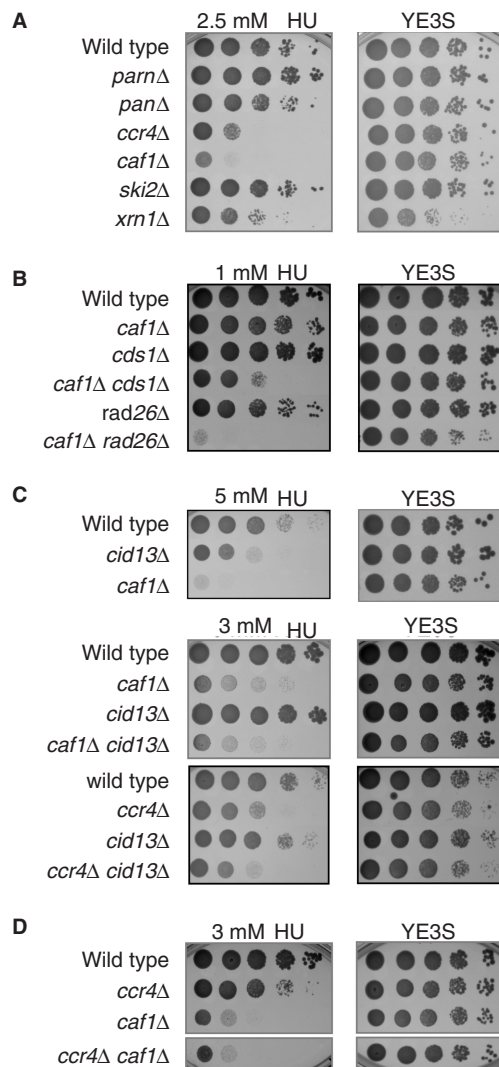
Figure 1. Caf1 and Ccr4 contribute to protect against DNA-replication stress by interacting with RNR-activation pathway. Ten-fold serial dilutions of logarithmically growing cells, wild type (YSP001) *parn*Δ (YSP004), *pan2*Δ (YSP003), *ccr4*Δ (YSP002), *caf1*Δ (YSP066), *ski2*Δ (YSP143) and *xrn1*Δ (YSP172) for (**A**), wild type (YSP001), *caf1*Δ (YSP066), *cds1*Δ (YSP069), *caf1*Δ *cds1*Δ (YSP103), rad26Δ (YSP071) and *caf1*Δ *rad26*Δ (YSP102) for (**B**), wild type (YSP001), *cid13*Δ (YSP086), *caf1*Δ (YSP066), *caf1*Δ *cid13*Δ (YSP095), *ccr4*Δ (YSP002) and *ccr4*Δ *cid13*Δ (YSP097) for (**C**) and *ccr4*Δ (YSP002), *caf1*Δ (YSP066) and *ccr4*Δ *caf1*Δ (YSP104) for (**D**) were spotted on YE3S plates containing the indicated concentrations of HU. The plates were photographed after 2 or 3 days for growth at 30°C.

1.9-kb *suc22* mRNA (23). As shown in Figure 1C, the sensitivity of these double mutant cells was equivalent to the single *caf1*Δ or *ccr4*Δ mutant, suggesting that Caf1 and Ccr4 act potentially in a step of the RNA activation pathway distal to Cid13. Although the phenotypes observed between *caf1*Δ and *ccr4*Δ cells were quite similar to each other, there was no synergistic enhancement of HU sensitivity in *caf1*Δ *ccr4*Δ double mutant cells (Figure 1D). Thus, both Caf1 and Ccr4 appear to contribute to protect against the HU-induced replication stress probably through the stabilization or translocation of a component(s) of the RNR complex.
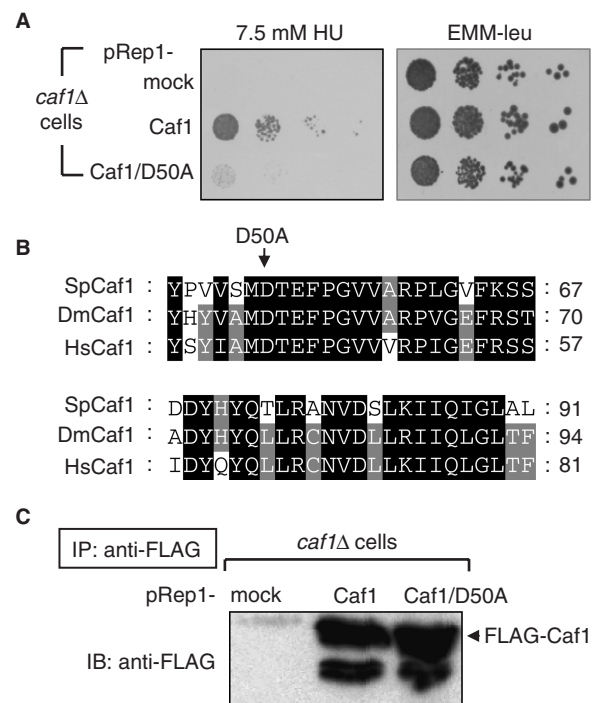

Figure 2. Isolation of Caf1 mutants that have defects in the HU-induced stress response. (**A**) Ten-fold serial dilutions of logarithmically growing cells, *caf1*Δ pRep1 (YSP188), *caf1*Δ pRep1-Caf1 (YSP189) and *caf1*Δ pRep1-Caf1/D50A (YSP190), were spotted on YE3S plates containing 7.5 mM HU and EMM-leu. (**B**) Sequence alignment of *CAF1* was performed with Clustal W: the most conserved amino acid residues are highlighted with a black background. Sp, *Schizosaccharomyces pombe*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*. (**C**) Lysates of logarithmically growing cells (YSP188, YSP189 and YSP190) were immunoprecipitated with anti-FLAG beads, and the precipitated fractions were subjected to SDS-PAGE and immuno-blotted with anti-FLAG for *caf1-FLAG*.

Isolation of Caf1 mutants that have a defect in HU-induced stress response

To better understand what biochemical properties of Caf1 and Ccr4 are relevant to the high sensitivity to HU stress, we focused on Caf1 showing more potent phenotype and isolated its point mutants by using the error-PCR method. One allele, which has a D50A mutation, was isolated as a mutant lacking the high sensitivity to the replication stress (Figure 2A), and this mutation is located in a region of Caf1 that is highly conserved from yeast to human (Figure 2B). Interestingly, the D50 residue is one of two residues known to be involved in the catalytic action of other members of the RNase D family and has been shown to be critical for both *S. cerevisiae* and mammalian Caf1 deadenylase activities *in vitro* (38–40). To examine the expression of this mutant protein, immunoprecipitation and western-blotting assays were performed. The protein level of FLAG-Caf1 under the *nmt1* promoter was almost the same between the mutant and wild-type cells, indicating that the D50A mutation does not disrupt the stability of Caf1 (Figure 2C). As will be described below, this Caf1 mutant exhibits unique biochemical properties in the HU-induced RNR pathway.
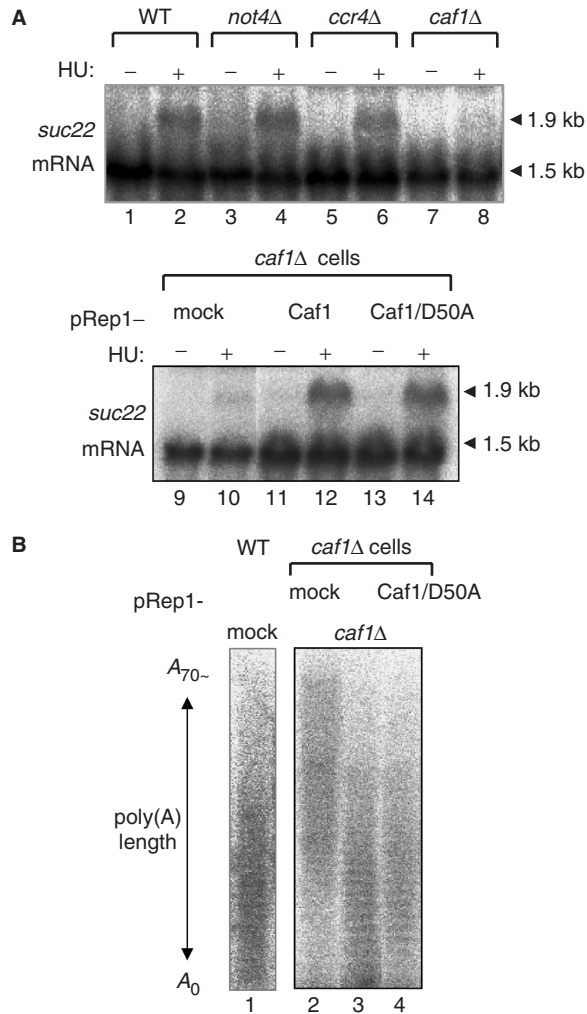
A

B

Figure 3. Neither increase of 1.9-kb *suc22* mRNA level nor dead-enylation of mRNA poly(A) tails by Caf1 contributes to the HU-induced stress-response pathway. (**A**) Logarithmically growing cells, wild type (YSP001), *not4Δ* (YSP131), *ccr4Δ* (YSP002), *caf1Δ* (YSP066), *caf1Δ* pRep1 (YSP188), *caf1Δ* pRep1-Caf1 (YSP189) and *caf1Δ* pRep1-Caf1/D50A (YSP190), were incubated in the presence (+) and absence (−) of 10 mM HU for 2 h at 30°C. RNA preparation and northern blotting analysis were performed as described in the Materials and Methods section. Membranes were exposed to phosphorImager screens and followed by the quantitative analysis of *suc22* mRNAs using Molecular Dynamics software. (**B**) Total RNA (1 μg) were purified from the above cells (YSP027, YSP188, YSP189 and YSP190) and end-labeled with 5′-[^{32}P] pCp using T4 RNA ligase. The radiolabeled mRNAs were digested with RNase A and separated with 12% polyacrylamide–7.5 M urea gel electrophoresis.

Neither increase of 1.9-kb *suc22* mRNA level nor deadenylation of mRNA poly(A) tails by Caf1 contributes to protect against the HU-induced replication stress

In *S. cerevisiae*, mutations of Ccr4–Not complex impair the transcription of mRNAs coding RNR genes in response to HU (13). In accordance to previous work (41), HU induced the 1.9-kb *suc22* mRNA in *S. pombe* wild-type cells (Figure 3A, lane 2). The increase of *suc22* mRNA level was still observed in *not4Δ* (lane 4) and *ccr4Δ* (lane 6) cells, but it was totally abolished in *caf1Δ* cells (lane 8). Moreover, the defect of *suc22* mRNA

increase in *caf1Δ* cells was compensated not only by the introduction of wild-type Caf1 (lane 12) but also by the Caf1/D50A mutant (lane 14). It thus appeared that Caf1/D50A mutation is still capable of inducing the *suc22* mRNA and that the ability of Caf1 to induce the 1.9-kb *suc22* mRNA is not responsible for the HU-high sensitivity observed in *caf1Δ* and *ccr4Δ* cells.

In *S. cerevisiae*, Caf1/Pop2 has been reported as a cofactor of Ccr4 deadenylase. To examine the deadenylase activity in *caf1Δ* cells, the poly(A)-tail regions of total mRNAs were isolated, and the length of poly(A) tails was examined. As shown in Figure 3B, longer poly(A) tails were observed in *caf1Δ* cells (lane 2) compared to wild-type cells (lane 1). The poly(A) length was much shortened by the introduction of wild-type Caf1 into the mutant cells (lane 3). Interestingly, the Caf1/D50A mutant was also capable of shortening the poly(A) length (lane 4), suggesting that the deadenylation-supportive activity of Caf1 is not involved in the HU-induced stress-response pathway. Altogether, these results raise the possibility that the Caf1/D50A mutation disrupts a cryptic biochemical activity of Caf1 that is distinct from the previously identified properties, increase of *suc22* mRNA level or deadenylation.

Caf1 is required for HU-induced cytoplasmic translocation of Suc22

The RNR activity is stimulated through the interaction of Suc22 with Cdc22 in the cytoplasm. However, translocation of Suc22 into cytoplasm is inhibited by nuclear Spd1 through the formation of a nucleoplasmic Spd1–Suc22 assembly. In S phase, degradation of Spd1 via the Pcu4–Ddb1–CSN complex leads Suc22 to be exported to cytoplasm (27). To explore the undescribed function of Caf1 in HU-induced stress response, we next investigated subcellular localization of the components of RNR pathway (Figure 4). In the absence of the replication stress, Suc22 was localized in the nucleoplasm of both wild-type and *caf1Δ* cells. In contrast, Suc22 dispersed into the cytoplasm in *spd1Δ* cells is probably due to the loss of the ability to retain the nucleoplasmic Suc22. After HU treatment, the cytoplasmic signal of Suc22 was detected in wild type cells. However, the localization of Suc22 was not altered in *caf1Δ* cells even after the HU treatment. These results indicate that Caf1 is required for HU-induced cytoplasmic translocation of Suc22.

Caf1 and Ccr4 are required for HU-induced nucleoplasmic reduction of Spd1

The data presented above predict that Caf1 is required for the cytoplasmic translocation of Suc22 in response to the replication stress by regulating the localization or the degradation of Spd1. To examine these possibilities, we investigated subcellular localization of Spd1 after HU treatment in wild-type and *caf1Δ* cells (Figure 5A). Under no-stress conditions, Spd1 was localized in the nucleoplasm of both cells. In response to HU, the signal of Spd1 disappeared from the nucleoplasm in wild-type cells, but was still present in *caf1Δ* cells.
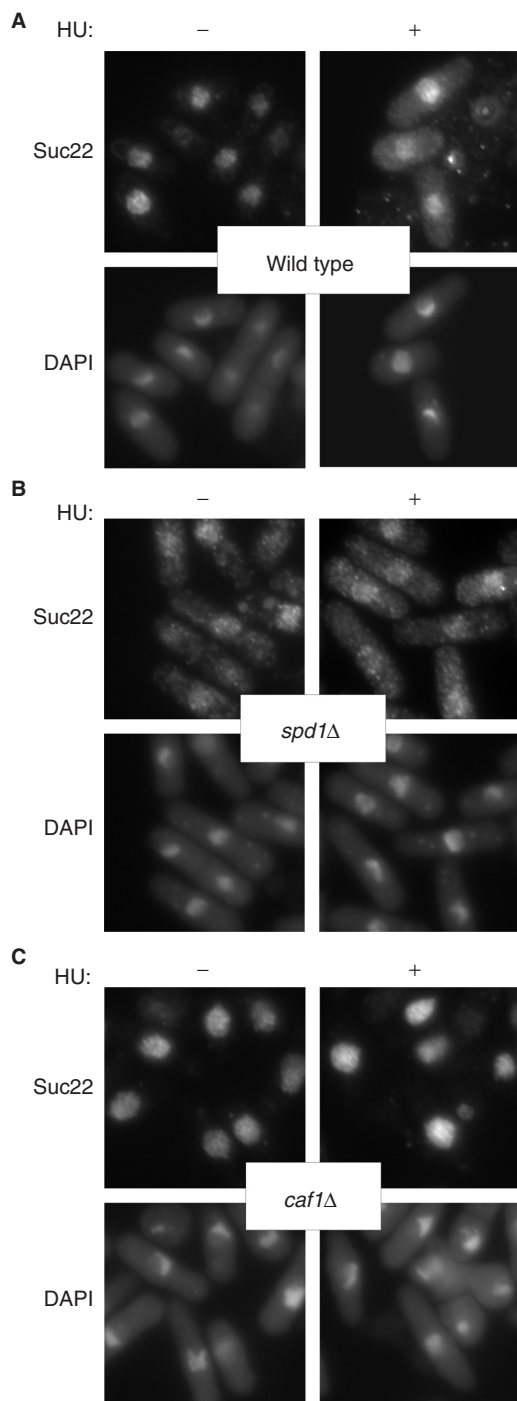
Figure 4. Caf1 is required for the cytoplasmic translocation of Suc22 in response to DNA-replication stress. Logarithmically growing cells, wild type (**A**, YSP001), *spd1Δ* (**B**, YSP112) and *caf1Δ* (**C**, YSP066), were incubated in the presence and absence of 10 mM HU for 2 h at 30°C. Immunofluorescence-staining images of Suc22 were obtained with an anti-Suc22 antibody.

Figure 5. Caf1 is required for HU-induced degradation of Spd1. (**A**) Logarithmically growing cells, *spd1-13Myc* (YSP091) and *caf1Δ spd1-13Myc* (YSP161) were incubated in the presence and absence of 10 mM HU for 2 h at 30°C. Immunofluorescence-staining images of Spd1 were obtained with an anti-Myc antibody. (**B**) Logarithmically growing cells, *spd1-13Myc* (YSP091), *ccr4Δ spd1-13Myc* (YSP154), *caf1Δ spd1-13Myc* (YSP161) and *not4Δ spd1-13Myc* (YSP162), were incubated in the presence (+) and absence (−) of 10 mM HU for 2 h. Extracts from the cells were blotted with an anti-tubulin antibody as an internal control. The extracts were also incubated with anti-Myc antibody, and the immunoprecipitated fractions were subjected to western blot analysis for the detection of Spd1. Three independent experiments were performed, and the HU-induced reduction of the Spd1 bands was quantitated by an imaging analyzer LAS1000. The results of one representative set are also shown in the inset. (**C**) Logarithmically growing cells, *spd1-13Myc* pRep1 (YSP226), *caf1Δ spd1-13Myc* pRep1 (YSP195), *caf1Δ spd1-13Myc* pRep1-Caf1 (YSP196) and *caf1Δ spd1-13Myc* pRep1-Caf1/D50A (YSP197), were incubated with the indicated concentrations of HU for 2 h at 30°C, and Spd1 was detected as described in (**B**).

Thus, the stress-induced Spd1 degradation is impaired in *caf1Δ* cells. To confirm the role of Caf1 in the nucleoplasmic reduction of Spd1, we determined the quantity of Spd1 in various cells before and after HU treatment. The expression level of *spd1* mRNA was almost unc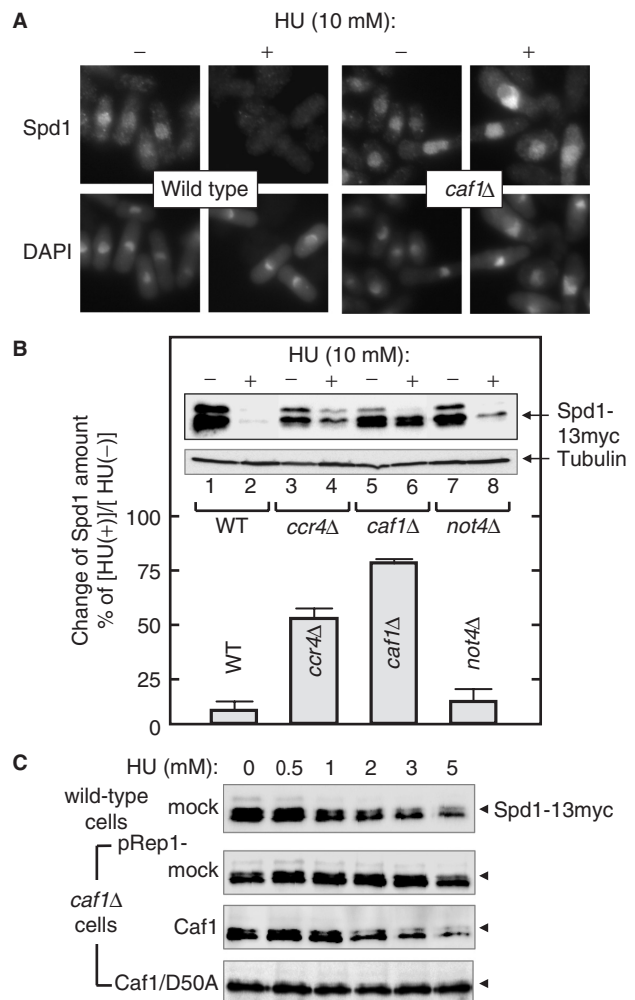hanged among these cells (data not shown). As shown in Figure 5B, Spd1 was detected as multiple bands in untreated wild-type cells (lane 1), and these bands disappeared almost completely after incubation of the cells with 10 mM HU (lane 2). Figure 5C (first panel) shows the concentration-dependent effect of HU: there was a progressive decrease in the amount of Spd1, as the concentration of HU was increased (0–5 mM). The HU treatment also reduced the amount of Spd1

in *not4Δ* cells (Figure 5B, lane 8). However, the HU-induced reduction of Spd1 was markedly inhibited in *caf1Δ* cells (lane 6). Such inhibition was also observed in *ccr4Δ* cells to a lesser extent (lane 4), and this may be related to the observation that *ccr4Δ* cells were less sensitive to HU (Figure 1A). The HU-induced reduction of Spd1 in wild-type or *not4Δ* cells and its diminishment in *caf1Δ* or *ccr4Δ* cells were still apparently observed in the presence of a protein synthesis inhibitor (50 μg/ml cycloheximide, data not shown). This excludes the possibility that the reduction of Spd1 might be due to changes in *de novo* protein synthesis of Spd1.

We next investigated whether the defect of Spd1 reduction in *caf1Δ* cells is compensated with the wild type and D50A mutant of Caf1 (Figure 5C). As expected, HU-induced Spd1 reduction was clearly restored by the expression of wild-type Caf1 under the nmt1-promoter (third panel). In contrast, the Caf1/D50A mutant failed to promote the reduction of Spd1 (fourth panel). Since the Caf1/D50A mutant still retains the ability to induce 1.9-kb *suc22* mRNA and shorten poly(A) tails (Figure 3), the additional biochemical activity of Caf1 is likely to be involved in the Spd1 turnover. Thus, this novel activity defective in Caf1/D50A appears to be responsible for the mutant phenotype that lacks the ability to complement HU-induced growth defect. Taken together, these observations further reinforce the notion that Caf1 and Ccr4 are involved in the HU-induced degradation of Spd1 leading to the activation of the RNR pathway.

HU stress allows Caf1 to interact with Suc22 in the RNR pathway

To further investigate the role of Caf1 and Ccr4 in the degradation of Spd1, we examined whether Caf1 and/or Ccr4 are capable of interacting physically with the RNR component Suc22. When FLAG-tagged Caf1 that had been expressed in *caf1Δ* cells was immunoprecipitated with an anti-FLAG antibody, no apparent band of Suc22 was observed in the Caf1-immunoprecipitated fraction (Figure 6A, 0-h time). However, Suc22 appeared to interact with Caf1 when the cells had been incubated with HU. The amount of Suc22 that interacted with Caf1 increased progressively with the incubation times and reached a plateau at 4-h incubation, though total protein levels of Suc22 and Caf1 in the cell lysates were constant during the incubation. The same experiments were performed with HA-tagged Ccr4 that had been expressed in *ccr4Δ* cells. However, Suc22 was not observed in the Ccr4-immunoprecipitated fraction after HU treatment (Figure 6B). The physical association observed between Suc22 and Caf1 was not due to an artifact resulting from aggregation of the immunoprecipitated materials, since the immunoprecipitated fractions exhibited essentially the same patterns of protein distribution upon SDS-PAGE analysis (data not shown). These results indicate that there is a separation of Suc22 and Caf1 in the absence of HU stress and that HU stress allows Caf1 to interact with Suc22. Interestingly, when the Caf1/D50A mutant had been expressed in *caf1Δ* cells, HU-dependent interaction of the mutant Caf1 with Suc22
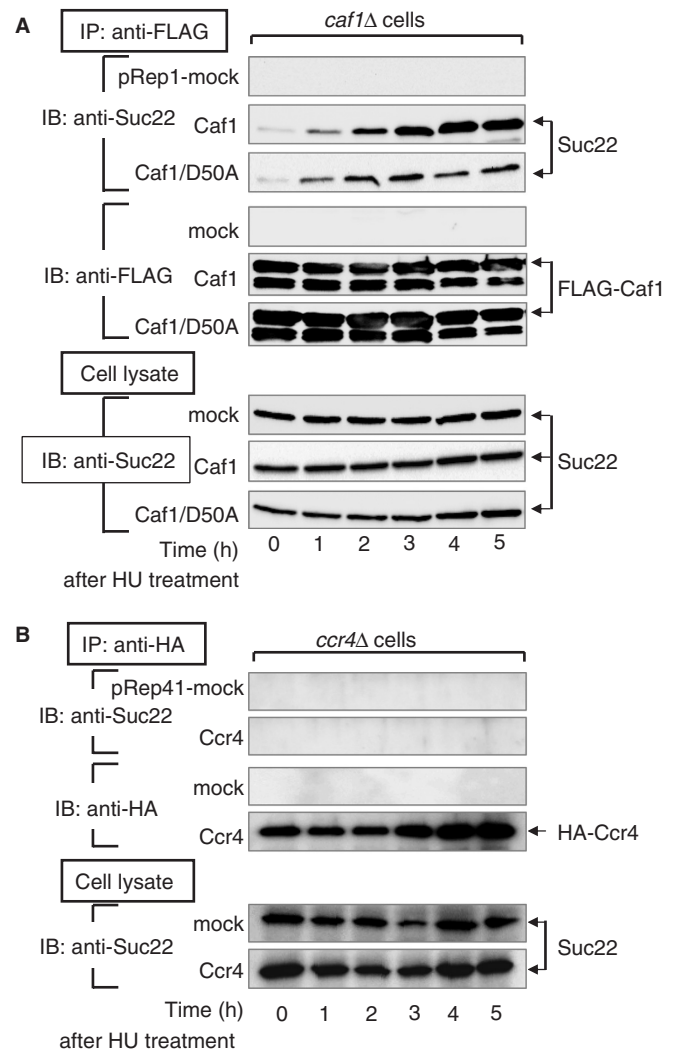

Figure 6. HU stress allows Caf1 to interact with Suc22 in the RNR pathway. Logarithmically growing cells, *caf1Δ* pRep1 (YSP188), *caf1Δ* pRep1-Caf1 (YSP189) and *caf1Δ* pRep1-Caf1/D50A (YSP190) for (**A**) and *ccr4Δ* pRep41 (YSP184) and *ccr4Δ* pRep41-Ccr4 (YSP185) for (**B**), were incubated with 10 mM HU for the indicated times at 30°C. Extracts from the cells were incubated with an anti-FLAG (A) or an anti-HA (B) antibody, and the immunoprecipitated fractions were subjected to western-blot analysis for the detection of Suc22 and Caf1 or Ccr4 as described in the Materials and Methods section. The whole cell lysates were also subjected to western blot analysis for the detection of Suc22.

was markedly reduced compared with wild-type Caf1 (Figure 6A). We thus conclude that Caf1, in concert with Ccr4, induces the Spd1–Suc22 disassembly to stimulate dNTP synthesis in the recovery from DNA-replication stress. Although more-detailed biochemical analysis is required, it is very likely that Caf1 tightly associates with Suc22 to release and/or degrade the inhibitory Spd1 in a manner dependent on Ccr4 and that this novel Caf1 function is impaired by D50A mutation.

DISCUSSION

We have shown that Caf1, a component of the Ccr4–Not complex, plays an important role in the mechanisms for

resistance to DNA-replication stress and in the proper supply of dNTPs to maintain genome stability and DNA replication in *S. pombe*. Although Caf1 has been characterized as a multi-functional component that is largely involved in the transcription of RNR genes and the regulation of Ccr4 deadenylase, the defects observed in the present *caf1Δ* cells are not solely explicable by either of the two major pathways. Caf1 deletion impairs not only the stress-induced translocation of Suc22 into cytoplasm but also the nucleoplasmic degradation of Spd1. The Caf1/D50A mutant isolated in the present study also reinforces the novel role of Caf1 in the RNR pathway. The Caf1 mutant, that fails to restore the growth-defect phenotype of *caf1Δ* cells, still has the ability to increase 1.9-kb *suc22* mRNA level and shorten poly(A) tails as the wild type. However, the Caf1 ability to support the stress-induced degradation of Spd1 is completely abolished by the D50A mutation. Taken together, interaction of Caf1 with Suc22 leading to Spd1–Suc22 disassembly is necessary for the protection against DNA-replication stress.

Caf1 as a multi-functional component of the Ccr4–Not complex

Each component of the Ccr4–Not complex has its own biochemical role(s) and seems to coordinate the activity of other components. In addition, some components confer distinct biochemical functions independently from the whole complex. For examples, Ccr4–Caf1 (Pop2) and Not4 have deadenylation and ubiquitination activities, respectively (42–44). This is also true in stress responses and in Spd1 degradation. We observed that *ccr4Δ* and *caf1Δ* cells show high sensitivity to HU (Figure 1A), though *not4Δ* cells do not (data not shown). The HU-induced degradation of Spd1 was markedly impaired in *ccr4Δ* and *caf1Δ* cells, though Spd1 was degraded normally in *not4Δ* cells (Figure 5B). However, Caf1 was required for the increase of 1.9-kb *suc22* level, while Ccr4 and Not4 were not (Figure 3A). Suc22 appeared to interact with Caf1, not with Ccr4 after HU treatment (Figure 6). It is interesting that distinct components in the complex are required for the respective biochemical reactions even though they are all triggered by the same reagent, HU. The Ccr4–Not complex has been found in several species as a large complex, 1 or 1.9 MDa, in addition to their constituent subunits (5,6). The spectrum of the Ccr4–Not components associated with the platform protein Not1 may be altered rapidly in response to external conditions as well as cell-cycle progression. The function of each member is possibly distinct and drastically dependent on their constituent members and/or the complex conformation. One possible model is that Caf1 in the context of the Ccr4–Not complex receives a signal following HU stress and Caf1 is then released to bind Suc22. Characterization of components in each form of the Ccr4–Not complex should help to explain their diverse biochemical versatility.

As described above, one of the outstanding features in *caf1Δ* cells is the lack of the HU-dependent increase of 1.9-kb *suc22* mRNA level (Figure 3A). It has been shown

that Cid13 stabilizes the *suc22* mRNA by its poly-adenylation upon DNA-replication stress and stimulates RNR activity (23). We have shown that *caf1Δ cid13Δ* double mutant cells are not synergistically sensitive to HU. This indicates that Caf1 and Cid13 act potentially in the same pathway initiated by DNA-replication stress. The key to the non-synthetic phenotype in *caf1Δ* and *cid13Δ* cells is the increase of 1.9-kb *suc22* mRNA level. Therefore, Suc22 mRNA may be first induced by Caf1, and its poly-adenylation subsequently takes place by Cid13. Alternatively, Caf1 may poly-adenylate 1.9-kb *suc22* mRNA cooperatively with Cid13. Since there are many other genes that are regulated by Caf1 upon DNA-replication stress, Caf1 may induce and stabilize stress-response genes in cooperation with Cid13. Thus, the stabilization of stress-induced mRNAs closely correlates with the absence of phenotypic enhancement in *caf1Δ cid13Δ* double mutant cells.

Proper dNTP pools ensure genome stability and DNA replication

It has been shown that dNTP pools are elevated upon DNA damage, for example 7-fold in *S. cerevisiae* and 2-fold in *S. pombe* (26,45). However, excessive dNTP pools cause genomic mutation (46), and reduced dNTP pools affect genome stability (45,47). In mammalian cells, constitutive RNR activation through inhibition of the RB pathway transforms cells into carcinoma (48). Moreover, dNTP imbalances also induce increased frequency of replication frame-shift mutations to a significant extent (46,49). These data indicate that tight control of dNTP pools is necessary to maintain complete fidelity of DNA replication. Similarly, the increase of unavailable nucleotides or the intermediates are also harmful to the regulation of genome stability and induce aberrant DNA replication. Indeed, we have observed the accumulation of abnormal nucleotides in *caf1Δ* cells under logarithmic growing conditions and a slight defect in the cell proliferation (data not shown). Thus, Caf1 may have an important role in the maintenance of proper dNTP pools upon DNA damage via its interaction with Suc22.

Possible roles of Caf1 in promotion of Spd1–Suc22 disassembly

As discussed above, the RNR activity is regulated not only by the transcription of their constituent subunits but also by the subcellular translocation of the regulated subunits. Our present results address the mechanism of this translocation (Figure 7). There may be at least two possible mechanisms, by which Caf1 promotes the Spd1–Suc22 disassembly that involves the degradation of Spd1 (scheme 1) and the translocation of Suc22 from nucleoplasm to cytoplasm (scheme 2). First, Caf1 interacts with Suc22 in a manner competitive with Spd1, which facilitates the Pcu4–Ddb1–CSN-induced degradation of Spd1 and the release of Suc22 into cytoplasm. Thus, Caf1 may have a transporter role in the Suc22 translocation. Alternatively, Caf1 may function as an activator protein associating with a component(s) of the Pcu4–Ddb1–CSN complex to enhance its
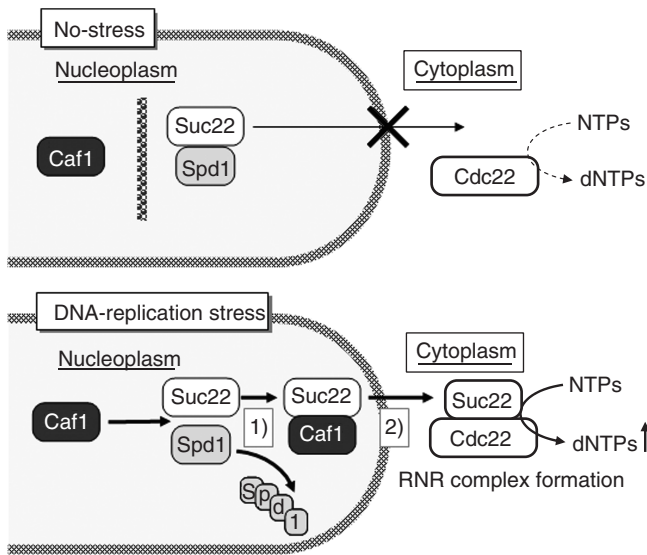
Figure 7. Possible models for the DNA-replication stress-induced translocation of Suc22. Under no-stress conditions, there is a compartment between Caf1 and the Suc22–Spd1 complex in the nucleoplasm. When cells sense DNA-replication stress, Caf1 interacts with Suc22 to facilitate the degradation of Spd1 (scheme 1) and the translocation of Suc22 from nucleoplasm to cytoplasm (scheme 2). See Discussion for further explanation.

degradation activity. These two models are not mutually exclusive. Our findings that Caf1 progressively associates with Suc22 after HU treatment and that the stress-induced Spd1 degradation requires Caf1 may be consistent with the first transporter model. On the other hand, a physical interaction analysis shows that Caf1 interacts with Csi1, a subunit of the CSN complex (50). Moreover, we detected a possible interaction between Caf1 and Csn1 by immunoprecipitation assay (data not shown). Immuno-fluorescence observations revealed that Caf1 localizes both in the nucleoplasm and in the cytoplasm and that HU exposure does not apparently alter the Caf1 localization. These observations may support the second activator model.

Each organism may have evolved distinct mechanisms to localize the regulatory subunits of RNR. In mammalian cells, the regulatory subunit p53R2 localizes in the cytoplasm under normal conditions and relocates to the nucleoplasm in response to DNA damage (51). However, in yeast cells, the regulatory subunits (Suc22 or Rnr2–Rnr4 complex) localize in the nucleoplasm under the standard conditions and enter the cytoplasm upon DNA damage. The apparent reason for the differential translocation mechanisms between yeast and mammals remains unknown. In this regard, Lee and Elledge point out that the different amounts of DNA have to be replicated between the two species (52). Consistently, the efficiency of replication rate is possibly important. The balance between the length of S phase (approximately 40 min and 8 h for yeast and mammal, respectively) and the DNA amounts to be replicated (mammalian genome is 250 times longer than yeast) suggests that mammalian cells replicate DNA about 20 times faster than yeast.

We speculate that the efficiency may explain the difference in translocation mechanism.

Mechanisms of nucleoplasmic anchoring of RNR regulatory subunits in yeast

It has been recently reported in *S. cerevisiae* that Wtm1, containing WD40 repeats, functions to anchor the regulatory subunit Rnr2–Rnr4 in the nucleoplasm (52,53). It is not known whether a WD40-dependent anchoring system is also conserved in *S. pombe*. In this regard, one of the Caf1-interacting proteins, Caf4, has a WD40 repeat, although Caf1 itself does not. It is thus tempting to speculate that Caf4 anchors Suc22 in the nucleoplasm to inhibit the cytoplasmic release of the regulatory subunit under no-stress conditions. Alternatively, such a WD40-containing protein may be involved in the cytoplasmic translocation of Suc22 and/or the recruitment of Spd1 to the Pcu4–Ddb1–CSN complex in *S. pombe*. A WD40 repeat-containing protein, Cdt2, which regulates the degradation of Spd1 via Pcu4–Ddb1–CSN ubiquitin ligase, might be one of the potential candidates (54). It would be interesting to determine whether WD40-dependent mechanisms are conserved across species, and whether there is a cytoplasmic anchoring protein for the regulatory subunit p53R2 in mammals. The understanding of RNR translocation mechanisms will help to explain the precise timing of the elevation of dNTP flows for DNA replication. These important issues are currently under investigation in our laboratory.

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