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Cytoplasmic localization of Hug1p, a negative regulator of the MEC1 pathway, coincides with the compartmentalization of Rnr2p–Rnr4p

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Abstract

The evolutionarily conserved MEC1 checkpoint pathway mediates cell cycle arrest and induction of genes including the RNR (ribonucleotide reductase) genes and HUG1 (Hydroxyurea, ultraviolet, and gamma radiation) in response to DNA damage and replication arrest. Rnr complex activity is in part controlled by cytoplasmic localization of the Rnr2p–Rnr4p subunits and inactivation of negative regulators Sml1p and Dif1p upon DNA damage and hydroxyurea (HU) treatment. We previously showed that a deletion of HUG1 rescues lethality of mec1Δ and suppresses dun1Δ strains. In this study, multiple approaches demonstrate the regulatory response of Hug1p to DNA damage and HU treatment and support its role as a negative effector of the MEC1 pathway. Consistent with our hypothesis, wild-type cells are sensitive to DNA damage and HU when HUG1 is overexpressed. A Hug1 polyclonal antiserum reveals that HUG1 encodes a protein in budding yeast and its MEC1-dependent expression is delayed compared to the rapid induction of Rnr3p in response to HU treatment. Cell biology and subcellular fractionation experiments show localization of Hug1p-GFP to the cytoplasm upon HU treatment. The cytoplasmic localization of Hug1p-GFP is dependent on MECP1 pathway genes and coincides with the cytoplasmic localization of Rnr2p–Rnr4p. Taken together, the genetic interactions, gene expression, and localization studies support a novel role for Hug1p as a negative regulator of the MEC1 checkpoint response through its compartmentalization with Rnr2p–Rnr4p.

1. Introduction

Cellular survival in response to DNA lesions and replication arrest requires the coordination of checkpoint-mediated mechanisms to ensure DNA damage repair, cell cycle arrest, and recovery for genome stability. Checkpoint pathways regulate the expression of protein kinases, which mediate a transcriptional response and cell cycle arrest through downstream effectors. In Saccharomyces cerevisiae, the evolutionarily conserved MEC1 (ortholog to the human ataxia telangiectasia mutated- and Rad3-related – ATR – protein) checkpoint pathway regulates origin firing, fork progression, and DNA repair and recovery (reviewed in [1]).

Mec1p and its effector kinases, Rad53p and Dun1p, activate both positive and negative effectors that regulate deoxyribonucleotide (dNTP) pools, cell cycle arrest, and recovery [1]. The activity of the positive effector RNPs (ribonucleotide reductases), which are responsible for the rate-limiting conversion step of ribonucleotides (rNDPs) to dNTPs, is tightly regulated. The homodimer Rnr1p and the heterodimers Rnr2p and Rnr4p, which compose the Rnr complex, are transcriptionally repressed by Crt1 [2,3] while Rnr1p contains binding sites for dATP allosteric inhibition [4]. In the absence of DNA damage, negative regulators such as Sml1p and Dif1p regulate Rnr complex activity through inhibition of the Rnr subunit, Rnr1p, and by subcellular compartmentalization of the Rnr2p–Rnr4p subunits to the nucleus [5,6]. However, in response to DNA damage or replication arrest, Sml1p and Dif1p are phosphorylated and degraded [5,7]. This, along with the transcriptional induction of RNPs and localization of the Rnr complex to the cytoplasm, serves to increase dNTP pools [5,7,8]. Checkpoint-mediated
response to DNA damage and replication arrest has been studied extensively, however we do not fully understand how cells recover from checkpoint arrest and downregulate Rnr activity to maintain genome stability.

In this paper, we examined the role of Hug1p (Hydroxyurea, Ultraviolet, Gamma) as a negative regulator of the MEC1 pathway. HUG1 is one of the most differentially expressed genes identified in a screen for gene expression in response to HU treatment [9]. Unlike SML1 and DIF1, the transcription of HUG1 is induced in cells treated with HU or exposed to ultraviolet or gamma radiation in a MEC1-dependent manner [2]. A deletion of HUG1 has been shown to rescue lethality due to a MEC1 null allele and suppress the HU sensitivity of dun1Δ mutants [2]. Studies with HUG1 have primarily focused on its transcriptional response to replication arrest and DNA damage [2,10–13]. Using a polyclonal serum to Hug1p, we have shown that HUG1 encodes for a protein. Our results for HUG1 overexpression phenotypes, a delayed induction pattern of Hug1p in response to HU treatment, and the MEC1-dependent compartmentalization of Hug1p in response to replication arrest define a novel role for Hug1p as a negative regulator of the MEC1–checkpoint response through its compartmentalization with Rnr2p–Rnr4p [14].

2. Materials and methods

2.1. Strains, plasmids, cell cycle arrest with HU and growth sensitivity to HU, MMS and BLM

Strains and plasmids are described in Supplementary Table 1. Transformations, cloning, and cell culture were performed using standard methods as previously described [2,15–17]. Additional strain and expression vector construction procedures are outlined in the supplementary materials and methods. Primer sequences are available upon request. Cell cycle arrest with 0.1 M HU (Fluka Chemika) was as described [2]. Cells (>90%) exhibited a large budded phenotype with S-phase DNA content, as determined by flow cytometry using an Accuri C6 flow cytometer (BD Accuri Cytometers) [18]. Serial dilutions of cells grown in medium selective for the plasmid were assayed for growth with dextrose (2%) or raffinose (2%) with 0.1 M HU, 0.01% MMS (Sigma–Aldrich), or 5 μM UBLM (Bristol-Myers Squibb) as described [18].

2.2. Western blots and subcellular fractionation

Western blots for Hug1p, Hug1p-GFP, Rnr3p-HA, Sir2p, Pgtk1p, Tub2p and subcellular fractionation were performed as described [18,19] using anti-HA (12CA5 Roche), -GFP (A11122 Invitrogen), -Sir2p (yN-19) (sc-6666 Santa Cruz), -Pgtk1p (459250 Invitrogen), -Hug1p and -Tub2p (antisera generated in Basrai Laboratory).

2.3. Localization of Hug1p-GFP

Hug1p-GFP expressing strains were grown to exponential phase in YPD and treated with 0.1 M HU for 3.5 h. For localization of GAL1-HUG1-GFP, cells were grown to exponential phase in synthetic medium with raffinose (2%) followed by growth in galactose (2%) medium for 2 h, shifted to dextrose (2%) medium with or without 0.1 M HU for 3.5 h. Harvested cells were fixed for microscopy as described [20] except that paraformaldehyde was the only fixative and Hoechst 33342 (Thermo Scientific) was used for nuclear staining. Images were acquired using a Zeiss Axio Observer Z1 microscope.

Image deconvolution and analysis were performed in ImageJ using plug-ins Diffraction PSF 3D to calculate the point-spread-function and Iterative Deconvolve 3D [21] for deconvolution. Localization analysis was performed as described [22]. Cytoplasmic localization was determined empirically to be a nuclear-to-cytoplasmic intensiometric ratio below 0.9, even distribution between 0.9 and 1.1, and nuclear localization above 1.1. For each strain, at least 100 large budding cells with a nucleus at the bud neck were counted.

Statistical analysis on the subcellular localization data was performed in SAS 9.3 using three-way factorial analysis of variance (ANOVA) with Tukey’s multiple comparison range test. Additional statistical analysis is provided in the supplementary materials and methods.

3. Results

3.1. Overexpression of HUG1 increases the sensitivity of wild-type strains to HU, MMS and BLM

We have previously shown that a deletion of HUG1 suppresses the viability of mec1Δ strains and HU sensitivity of dun1Δ mutants [2]. Similar results have been reported in the HUG1 paralogs, DIF1 and SML1, both of which are negative regulators of the Rnr complex and the checkpoint response [5,7,16]. To determine if Hug1p acts as a negative regulator of the MEC1 pathway, wild-type strains overexpressing HUG1 were assayed for growth on media containing HU and DNA damaging agents. GAL1-HUG1 was found to increase the sensitivity of wild-type strains to HU on medium containing galactose (GAL) and 0.15 M HU (Fig. 1A, Row 2). GAL1-HUG1 or vector (Fig. 1A, Rows 2 and 1, respectively) did not show growth defects on dextrose (DEX) plates with and without HU and GAL plates without HU. The phenotype was specifically due to expression of Hug1p, as a frame-shift mutation in the HUG1 open reading frame (GAL1-HUG1Δ) abolished the dosage lethality phenotype (data not shown).

Since the viability of mec1Δ strains is suppressed by sml1Δ-1 or sml1ΔΔ [5,7,16], the dosage lethality of GAL1-HUG1 in wild-type strains was examined for dependence on SML1. Similar to the wild-type strain, the sml1Δ strain with GAL1-HUG1 exhibited growth inhibition on HU containing medium (Fig. 1A, Row 6). Results verifying that SML1 is not required for the dosage lethality of strains containing GAL1-HUG1 are supported by recent work describing the ubiquitylation and subsequent degradation of Sml1p in response to DNA damage [7]. As expected, the mec1Δsml1Δ strain was sensitive to growth on HU containing plates with or without GAL1-HUG1 (Fig. 1A, Rows 3 and 4). mec1Δsml1Δ strains expressing GAL1-HUG1 also showed a slow growth phenotype even in the absence of HU (Fig. 1A, Row 4, center panel). These results are similar to the negative regulator, DIF1, which displays dosage lethality in mec1Δsml1Δ strains [5].

In addition to HU sensitivity, GAL1-HUG1 strains exhibited significant growth inhibition on MMS and BLM containing media (Fig. 1B, Row 3) when compared with empty-vector strains (Fig. 1B, Row 2). As expected, the mec1Δsml1Δ strain displayed growth inhibition on plates containing MMS and BLM (Fig. 1B, Row 1). Taken together, the synthetic dosage lethality of GAL1-HUG1 strains along with previous data support a role for Hug1p as a negative regulator of the MEC1-mediated checkpoint response to DNA damage and replication arrest.

3.2. Expression of Hug1p shows delayed induction to 0.1 M HU compared to Rnr3p, a positive effector of the MEC1 pathway

Using transcriptome profiling, we previously reported that HUG1 represents one of the most highly differentially expressed genes in the yeast genome [2,9]. Initial genome sequencing efforts annotated all ORFs of at least 100 contiguous codons, hence HUG1 was not
annotated as it encodes for a protein of 68 amino acids. To validate that HUG1 encodes for a protein, a rabbit polyclonal serum specific to Hug1p was generated. Results from Western blot analysis corroborated results of Northern blot analysis [2], as Hug1p expression was observed in a wild-type strain treated with HU (Fig. 2A, Lane 2). The control includes a hug1Δ strain that shows Hug1p expression when transformed with a plasmid expressing HUG1 from its own promoter (pHUG1; Fig. 2A, Lane 6). In agreement with previous results, tup1Δ and crt1Δ strains constitutively expressed Hug1p (Fig. 2A, Lanes 7–10). Crt1p, Tup1p and Ssn6p are transcriptional repressors that bind to X-box sequences in the promoter of HUG1 and RNRs in the absence of DNA damage and replication arrest [2]. The polyclonal serum also showed that, in agreement with previous Northern blot analysis, no HU induced expression of Hug1p was detected in mec1Δ sm1Δ strains (Fig. 2B, Lane 6) and SML1 was not required for the expression of Hug1p (Fig. 2B, Lane 4).

To gain further insight into the role of Hug1p, HU induced expression of Hug1p was compared with Rnr3p, a positive regulator of the MEC1 pathway. Hug1p expression was detected 1.5 h post-HU addition and increased until approximately 3.5 h post-HU addition after which no further induction was apparent (Fig. 2C). Consistent with previous reports [3], Rnr3p-HA was detected 30 min post-HU addition, increased until 90–120 min post-HU addition, and subsequently declined (Fig. 2C). The delayed induction of Hug1p with high levels present at 3.5–5 h post-HU addition resembles the profile of Crt1p [3], a negative regulator of RNRs and HUG1 gene expression.

3.3. Hug1p-GFP localizes to the cytoplasm in HU treated cells

The subcellular localization of Hug1p-GFP was analyzed by fusing GFP to the C-terminus of Hug1p expressed from its native promoter at the chromosomal locus in the genome. Western blot analysis showed expression of Hug1p-GFP in cells treated with HU (Fig. 3A). Fluorescence microscopy of Hug1p-GFP cells without HU treatment showed only background fluorescence (Fig. 3B, left column). However, upon treatment with HU, Hug1p-GFP was enriched in the cytoplasm and was notably excluded from the nucleus in 96.3 ± 3.1% of the cells (Fig. 3B, right column). DNA content measurement by FACs and nuclear morphology of the cells confirmed S-phase arrest of the HU treated cells (data not shown).

To rule out artifacts in localization due to GFP tagging of Hug1p, the data were corroborated by subcellular fractionation of cells expressing non-epitope tagged Hug1p from or without HU were analyzed by Western blot using anti-Hug1p, -HA (Rnr3p-HA) and -Tub2p (loading control).
expression of GAL1-HUG1-GFP. Western blot analysis showed that GAL1-HUG1-GFP was expressed in wild-type, mec1Δsml1Δ, and sml1Δ strains grown in galactose medium (Fig. 4A). Cells grown in the presence of galactose for 2 h, followed by growth in glucose medium with or without HU were examined for nuclear morphology and localization of GAL1-HUG1-GFP. Nuclear-to-cytoplasmic intensiometric ratios were quantified as described [22] to determine Hug1p-GFP subcellular compartmentalization for all strains (Fig. S1). In the absence of HU, Hug1p-GFP was primarily localized to the nucleus (88.2 ± 2.0%) whereas a majority of the HU treated cells (86.6 ± 3.3%) exhibited cytoplasmic localization (Fig. 4B, WT). The similar localization pattern of GAL1-HUG1-GFP to that of HUG1-GFP expressed under the native HUG1 promoter revealed that the cytoplasmic localization of Hug1p-GFP is independent of protein expression levels.

3.4. Cytoplasmic localization of Hug1p in response to HU treatment is not merely due to overexpression of the protein

Since HUG1 expression is induced in response to DNA damage and replication arrest in a checkpoint dependent manner [2], we examined if the cytoplasmic localization of Hug1p under these conditions may reflect its high level of expression using cells expressing GAL1-HUG1-GFP. Western blot analysis showed that GAL1-HUG1-GFP was expressed in wild-type, mec1Δsml1Δ, and sml1Δ strains grown in galactose medium (Fig. 4A). Cells grown in the presence of galactose for 2 h, followed by growth in glucose medium with or without HU were examined for nuclear morphology and localization of GAL1-HUG1-GFP. Nuclear-to-cytoplasmic intensiometric ratios were quantified as described [22] to determine Hug1p-GFP subcellular compartmentalization for all strains (Fig. S1). In the absence of HU, Hug1p-GFP was primarily localized to the nucleus (88.2 ± 2.0%) whereas a majority of the HU treated cells (86.6 ± 3.3%) exhibited cytoplasmic localization (Fig. 4B, WT). The similar localization pattern of GAL1-HUG1-GFP to that of HUG1-GFP expressed under the native HUG1 promoter revealed that the cytoplasmic localization of Hug1p-GFP is independent of protein expression levels.

3.5. Cytoplasmic localization of Hug1p-GFP is MEC1-dependent and coincides with the compartmentalization of Rnr2p–Rnr4p to the cytoplasm

Since genes in the MEC1 pathway are required for the DNA damage and replication arrest induced expression of Hug1p [2], we examined whether the cytoplasmic localization of Hug1p is dependent on the MEC1 effector kinases, MEC1, RAD53, and DUN1. In the absence of HU, Hug1p-GFP mainly localized to the nucleus in mec1Δ sml1Δ strains (92.1 ± 8.5%), similar to that observed in wild-type strain (88.2 ± 2.0%). However, contrary to the cytoplasmic localization of Hug1p-GFP in wild-type cells (86.6 ± 3.3%), very few of the mec1Δsml1Δ cells showed localization to the cytoplasm (8.5 ± 1.1%) in response to HU treatment. The majority of Hug1p-GFP in the mec1Δsml1Δ cells was nuclear (64.0 ± 7.3%) or was evenly distributed throughout the cell (27.5 ± 7.0%). The localization pattern observed in mec1Δsml1Δ strains was independent of SML1 as sml1Δ strains exhibited a localization pattern more closely resembling wild-type strains. In the sml1Δ strain, Hug1p-GFP localized to the nucleus (97.1 ± 1.0%) in the absence of HU and to the cytoplasm (85.5 ± 2.3%) in the presence HU (Fig. 4B).

We next examined the localization of GAL1-HUG1-GFP in rad53Δ and dun1Δ strains. In the absence of HU, both rad53Δ (74.8 ± 11.2%) and dun1Δ (83.9 ± 0.5%) strains showed nuclear localization of Hug1p-GFP similar to that observed in the wild-type strain (Fig. 4B). However, unlike the wild-type cells, in the presence of HU, only a small fraction of rad53Δ cells localized to the cytoplasm (15.7 ± 4.9%) with a majority of the cells exhibiting an even distribution (46.3 ± 4.5%) or nuclear localization (37.9 ± 9.3%) of Hug1p-GFP signal. In the presence of HU, the dun1Δ strains exhibited a cytoplasmic localization profile of Hug1p-GFP that was intermediate to the pattern in mec1Δsml1Δ and wild-type strains. Cytoplasmic localization was observed in approximately half the population (52.5 ± 5.0%) of dun1Δ cells whereas the remaining cells had either an even distribution (45.3 ± 4.1%) or nuclear localization (1.9 ± 0.9%) of Hug1p-GFP. Taken together, these data indicate that Hug1p-GFP localizes to the cytoplasm in response to HU treatment and this localization is dependent on MEC1, RAD53, and DUN1 and is independent of SML1.

4. Discussion

Checkpoint mediated recovery from DNA damage and replication arrest is in part mediated by stringent regulation of Rnr activity. Negative effectors of the MEC1 pathway, namely SML1 and DIF1, interact with Rnr complex subunits and regulate its activity and subcellular compartmentalization. The downregulation of Dif1p and Sml1p in response to DNA damage or replication arrest increases dNTP pools [5–7]. However, after recovery from check-
Fig. 4. Cytoplasmic localization of Hug1p-GFP depends on MEC1 pathway genes and is coincident with compartmentalization of Rnr2p–Rnr4p. (A) Western blot analysis of wild-type (WT, W1588-4A), sml1Δ (U952-3B), and mec1Δsml1Δ (U953-61A) strains expressing GAL1-HUG1-GFP (pMB830) grown with or without galactose (GAL) in the absence or in the presence of 0.1 M HU for 3.5 h probed with anti-GFP or -Tub1p (loading control). (B) Hug1p-GFP subcellular localization pattern as quantified by fluorescence microscopy (as described in Section 2 and [22]) of wild-type (WT, YMB888), sml1Δ (U952-3B), mec1Δ sml1Δ (U953-61A), dun1Δ (U971) and rad53Δ (U950-5C) expressing GAL1-HUG1-GFP (pMB830) grown in galactose medium with or without 0.1 M HU for 3.5 h. The graph shows means of three replicates with at least 100 cells counted per experiment. Asterisks (*) indicate significant difference when compared to respective wild-type cells (Tukey’s HSD; p < 0.05). (C) Delayed induction and cytoplasmic localization of Hug1p in response to HU treatment may serve to downregulate Rnr complex activity. Rnr complex with solid outline indicates catalytically active form, while the one with dashed outline designates catalytically inactive form. Dif1p mediates the localization of Rnr2p–Rnr4p to the nucleus where Wtm1p anchors it, while Sml1p inhibits the activity of Rnr1p in the cytoplasm. Dif1p and Sml1p are phosphorylated and degraded in response to HU treatment and Rnr2p–Rnr4p localizes to the cytoplasm for catalytic activity. The delayed induction of Hug1p and its cytoplasmic localization and co-compartmentalization with the Rnr complex may serve to downregulate Rnr activity and facilitate recovery from checkpoint response.

We propose that Hug1p may serve to negatively regulate the MEC1 pathway, which unlike DIF1 and SML1, is induced in response to DNA damage and replication arrest. This is based on our results which show that: (a) strains expressing GAL1-HUG1 are sensitized to growth in the presence of HU and DNA damaging agents, (b) the temporal pattern of Hug1p expression in the presence of HU exhibits a lag when compared with Rnr3p, a positive regulator of the MEC1 pathway, and resembles that of Crt1p, a negative regulator of the MEC1 pathway, and (c) suppression of lethality of mec1Δ and HU sensitivity of dun1Δ strains by deletion of HUG1.

We propose that Hug1p may serve to negatively regulate the MEC1 pathway by co-compartmentalization with Rnr2p–Rnr4p to the cytoplasm in response to HU treatment. The cytoplasmic localization is not simply due to overexpression of Hug1p as corroborated by localization analysis of GAL1-HUG1-GFP. Consistent with a requirement of MEC1 pathway genes for the induction of HUG1, cytoplasmic localization of Hug1p was dependent on MEC1, RAD53 and DUN1. Interestingly, Hug1p and Rnr2p–Rnr4p subcellular compartmentalization data share similar dependencies on the MEC1 pathway genes [14]. The localization to the same cellular compartment may allow Hug1p to interact with Rnr2p–Rnr4p through an undetermined, potentially inhibitory mechanism (Fig. 4C) and downregulate Rnr activity. As seen in the model, in cycling cells, Dif1p mediates the localization of Rnr2p–Rnr4p to the nucleus where Wtm1p anchors it, while Sml1p inhibits the activity of Rnr1p in the cytoplasm. After 1.5–2 h of HU induction, Dif1p and Sml1p are phosphorylated and degraded; Rnr2p–Rnr4p is exported from the nucleus to the cytoplasm where it forms the active Rnr complex with the Rnr1p homodimer. After 3.5 h of HU treatment, the high level of Hug1p expression and its localization to the cytoplasm and co-compartmentalization with the Rnr complex serve to downregulate Rnr activity and, potentially, dNTP pools. The delayed expression of Hug1p to replication arrest and co-compartmentalization with Rnr2p–Rnr4p may act to negatively regulate Rnr activity in the absence of negative MEC1 effectors, DIF1 and SML1, and permit cellular recovery in post-stress conditions. Taken together, our data define a novel role for HUG1 in the DNA damage and replication arrest pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.089.
References


