Posttranscriptional Regulation of the \( \text{RAD}^{5} \) DNA Repair Gene by the Dun1 Kinase and the Pan2-Pan3 Poly(A)-Nuclease Complex Contributes to Survival of Replication Blocks*

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The yeast Dun1 kinase has complex checkpoint functions including DNA damage-dependent cell cycle arrest in G2/M, transcriptional induction of repair genes, and regulation of postreplicative DNA repair pathways. Here we report that the Dun1 forkhead-associated domain interacts with the Pan3 subunit of the poly(A)-nuclease complex and that \( \text{dun1pan2} \) and \( \text{dun1pan3} \) double mutants are dramatically hypersensitive to replicational stress. This phenotype was independent of the function of Dun1 in regulating deoxyribonucleotide levels as it was also observed in strains lacking the ribonucleotide reductase inhibitor Sm1l. \( \text{dun1pan2} \) mutants initially arrested normally in response to replication blocks but died in the presence of persistent replication blocks with considerably delayed kinetics compared with mutants lacking the Rad53 kinase, indicating that the double mutation does not compromise the intra-S phase checkpoint. Interestingly, the \( \text{RAD}^{5} \) gene involved in error-free postreplication repair pathways was specifically up-regulated in \( \text{dun1pan2} \) double mutants. Moreover, inducible overexpression of \( \text{RAD}^{5} \) mimicked the double mutant phenotype by hypersensitizing \( \text{dun1} \) mutants to replication blocks. The data indicate that Dun1 and Pan2-Pan3 cooperate to regulate the stoichiometry and thereby the activity of postreplication repair complexes, suggesting that posttranscriptional mechanisms complement the transcriptional response in the regulation of gene expression by checkpoint signaling pathways in \textit{Saccharomyces cerevisiae}.

Eukaryotic cells contain highly conserved checkpoint signaling pathways that prevent genomic instability by regulating the cellular response to DNA damage and replication blocks. Checkpoints involve slowing or arresting the cell cycle until the damage is repaired, the transcriptional induction of repair enzymes, and the direct activation of repair processes (1). The yeast Dun1 protein is a member of a family of protein kinases closely related to the human Chk2/HuCds1 kinase (2, 3) that is mutated in a subset of patients suffering from the Li-Fraumeni multicancer syndrome (4). These kinases are characterized by the presence of at least one FHA domain, a protein-protein interaction module present in more than 200 different proteins (5) that seems to specifically bind to phosphorylated amino acids (preferentially phosphothreonine) in target sequences (6–9). \( \text{dun1} \) mutant strains have a reduced replication block/DNA damage-dependent induction of repair genes (10), a reduced cell cycle arrest function in the \( G_{2}/M \) checkpoint (11, 12), and increased rates of spontaneous chromosome rearrangements (13, 14). The Dun1 kinase is activated through phosphorylation by checkpoint signals in a \( \text{MEC1} \)- and \( \text{RAD}^{53} \)-dependent manner (15), and its positive effect on transcription involves the phosphorylation and inactivation of the Crt1 transcriptional repressor (16). In contrast to the cell cycle arrest function of the S phase checkpoint that is \( \text{RAD}^{53} \)-dependent and largely \( \text{DUN1} \)-independent (12), \( \text{DUN1} \) seems to play a much more crucial role than \( \text{RAD}^{53} \) in preventing gross chromosomal rearrangements (particularly \textit{de novo} telomere additions to chromosome breakpoints) after spontaneous DNA replication errors during normal cell cycles, indicating that it has a specific function in regulating postreplicative DNA repair pathways (13).

In addition to the transcriptional control of gene expression, posttranscriptional mRNA modifications and mRNA decay pathways play crucial roles in the eukaryotic regulation of protein levels (17). A major posttranscriptional modification is the addition of the poly(A) tail at the 3′-end of pre-mRNAs that contributes to the regulation of protein translation (18, 19) and mRNA stability (17). In \textit{Saccharomyces cerevisiae}, mRNA degradation is usually initiated by the 3′ → 5′ exonucleolytic digest of the poly(A) tail by a cytoplasmic mRNA deadenylase containing the Ccr4 and Caf1 proteins (20). In addition to Ccr4-Caf1, poly(A) tail length distribution is also regulated by the poly(A)-nuclease (PAN) complex, which consists of the catalytic 135-kDa Pan2 subunit with sequence motifs characteristic of RNase D-like 3′ → 5′ exonucleases (21) and the 72-kDa Pan3 subunit of unknown function. The primary function of PAN seems to be to “preset” poly(A) tails to message-specific lengths before or during the nucleocyttoplasmic export of mRNAs (22), but it also contributes to cytoplasmic mRNA turnover as an alternative or complementing pathway to Ccr4-Caf1 (20).

Here we report that Dun1 cooperates with PAN in the regulation of \( \text{RAD}^{5} \) mRNA levels and cell survival in response to replicational stress. The data suggest that posttranscriptional mechanisms contribute to the regulation of gene expression by checkpoint signaling pathways.

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1 The abbreviations used are: FHA, forkhead-associated; PAN, poly(A)-nucl ease; HA, hemagglutinin; HU, hydroxyurea; MMS, methyl methanesulfonate; RNR, ribonucleotide reductase; YPD, yeast extract/peptone/glucose.
EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast two-hybrid assays were performed in PJ46–4A (MATa, trp1–901, leu2–3,112, ura3–52, his3–200, gal4Δ, gal80Δ, lys2Δ::GAL1-HIS3, GAL2-AD2E, met2::GAL7-lacZ) (23). Functional experiments were performed in the BY4741 genetic background (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) (24) except for the rad53Δasml1A strain (25), which was in the W303-1A background (26). BY4741 strains containing pan2Δ or pan3Δ deletions (pan2Δ::kanMX4 or pan3Δ::kanMX4) were obtained from Research Genetics. Dun1 was disrupted in these strains using standard PCR-based methods and LEU2 as a selectable marker. Double and triple mutants with smi1Δ were obtained by mating dun1Δpan2Δ or dun1Δpan3Δ strains with a smi1Δ strain in the BY4742 background (MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, smi1Δ::kanMX4) followed by sporulation and tetrad dissection. For generation of the PAN2-p416-Gal1 transactivation (pan2Δ::GAL1-PAN2), a hemagglutinin (HA) tag was introduced at the 3′-end of the coding sequence by PCR and pop-in/pop-out selection and was confirmed by PCR and immunoprecipitation/immunoblot analysis. For overexpression experiments, RAD5 residues corresponding to the full-length cDNA were generated by PCR and cloned into p146GAL1 (27).

Yeast Two-hybrid Assays—Randomly primed yeast cDNA was synthesized using standard conditions, cloned into pGAD GH (CLONTECH) using EcoRI adapters (Promega), and transformed into the Escherichia coli strain XL10-Gold (Stratagene). The library contained ∼110,000 independent clones. The Dun1 FHA domain (residues 19–159) cloned into pAS2 was used as bait to screen the library for FHA-interacting proteins. Interacting clones were selected on plates lacking Trp, Leu, and His and containing 2 mM 3-aminotriazole. Positive clones were isolated after 5 days, patched onto identical plates also lacking adenine as an additional reporter, and grouped by restriction enzyme mapping and cDNA sequence analysis. Clones were also tested for β-galactosidase activity of liquid cultures as a third reporter. Color development was monitored by absorbance measurements at 420 nm and corrected for protein levels by Bradford assay. To confirm the interaction, the original interacting Pan3 clone as well as various fragments and a full-length cDNA generated by PCR were cotransformed into yeast strains with a full-length cDNA generated by PCR were cotransformed with pAS2-Dun1FH or empty pAS2. HU and MMS Sensitivity Assays—For plate assays, 2 µl of 10-fold serial dilutions of yeast cultures (starting A600 was 0.5) were spotted onto yeast extract/peptone/dextrose (YPD) plates containing 100 µM HU and incubated for 4 days at 30 °C. Similar experiments were performed with yeast strains transformed with p146GAL1 or p146GAL1-RAD5 plasmids that were plated on synthetic medium lacking uracil and containing either 2% sucrose or 2% sucrose plus 4% galactose. For solution treatments and a full-length cDNA generated by PCR were cotransformed with pAS2-Dun1FH or empty pAS2. HU and MMS Sensitivity Assays—For plate assays, 2 µl of 10-fold serial dilutions of yeast cultures (starting A600 was 0.5) were spotted onto yeast extract/peptone/dextrose (YPD) plates containing 100 µM HU and incubated for 4 days at 30 °C. Similar experiments were performed with yeast strains transformed with p146GAL1 or p146GAL1-RAD5 plasmids that were plated on synthetic medium lacking uracil and containing either 2% sucrose or 2% sucrose plus 4% galactose. For solution treatments and a full-length cDNA generated by PCR were cotransformed with pAS2-Dun1FH or empty pAS2.

RESULTS

Interaction of the Dun1 FHA Domain with Pan3—The Dun1 FHA domain contains ∼137 amino acid residues (28) and can bind to a phosphorylated model peptide in vitro (25). To understand the physiological function of this domain, we performed a yeast two-hybrid screen to identify interacting yeast proteins. In this screen, we isolated two identical clones that contained residues 223–237 of Pan3. This Pan3 construct supported growth on reporter plates lacking histidine and adenine only when cotransformed with the Dun1 FHA domain construct (Fig. 1) but not with the corresponding empty vector or the Chk2 FHA domain (data not shown). Similar results were also obtained with a full-length Pan3 construct (which had a 6-fold higher activity than the original clone in β-galactosidase induction as a third reporter gene; data not shown) but not several truncated constructs (Fig. 1), indicating that this interaction depends on the proper three-dimensional fold of the Pan3 N terminus. To confirm this interaction, we introduced a HA tag into the chromosomal PAN3 gene for coimmunoprecipitation experiments. However, Pan3-HA could only be detected in immunoprecipitations from large culture volumes (0.25–1 liter in log phase), and we failed to detect Pan3 in Pan3-HA immunoprecipitates from untreated cultures or after treatment with the replication-blocking agent HU or the DNA-damaging agent MMS (data not shown). This indicates that the interaction between Dun1 and Pan3 involves only a small fraction of these proteins or that it is either weak or very transient, reminiscent of the recently reported two-hybrid interaction between the Schizosaccharomyces pombe Cds1 FHA domain and the replication checkpoint protein Mrc1 (30).

Synthetic Lethality of dun1 and pan2/pan3 in Response to Replication Stress—dun1 mutants grow poorly on plates containing HU, an inhibitor of ribonucleotide reductase (RNR) that causes replication blocks due to dNTP depletion (10, 26). To independently evaluate the interaction of Dun1 with Pan3, we used a genetic approach to investigate whether the PAN complex modifies this HU-dependent growth defect. Deletion of the PAN3 gene or the gene for the catalytic Pan2 subunit had no gross effect on cell growth in an otherwise wild type background—i.e. in the dun1Δ background on control plates (Fig. 2A, top panel). Deletion of either PAN2 or PAN3 alone also did not affect cell growth in the presence of 100 mM HU (Fig. 2A, bottom panel). However, when the single dun1Δ mutation the growth defect on HU plates was increased by >100-fold in dun1Δpan2A and dun1Δpan3Δ double mutant strains (Fig. 2A, bottom panel). The inability of the double mutants to form colonies on HU plates could reflect slower cell growth or...
increased lethality. We therefore analyzed cell viability in the presence of 200 mM HU over a 24-h time course. In this experiment (Fig. 2B), viable wild type cells as well as pan2Δ mutants increased in number despite the presence of HU, dun1Δ mutants failed to proliferate, but >90% of cells remained viable even after 24 h in 200 mM HU (Fig. 2B). In contrast, viability of dun1Δpan2Δ double mutants decreased after 8 h, and after 24 h only ~7% of cells were able to form colonies when plated on normal medium (Fig. 2B). As the majority of synthetic lethal interactions occur between genes that act in a common biochemical pathway (31), the more than additive defect of dun1Δ and pan2Δ or pan3Δ in response to replication blocks supports the interaction of the Dun1 FHA domain with Pan3 observed in the two-hybrid screen.

The dun1Δpan2Δ3 Phenotype Is Independent of RNR Regulation and the Intra-S Phase Checkpoint—dun1Δ mutants are severely compromised but not entirely deficient in up-regulating RNR genes in response to HU treatment (10). We therefore tested whether further reduced RNR levels could cause the increased HU sensitivity of the double mutants. However, Northern blot analysis demonstrated that HU-induced RNR3 mRNA levels in the dun1Δpan2Δ strain were similar to those in the dun1Δ strain (Fig. 3A). We also tested the effect of the sml1Δ mutation on the double mutants. Sml1 inhibits RNR activity, and sml1Δ results in 2.5-fold higher dNTP levels and extragenic suppression of several dun1 phenotypes (26). However, although sml1Δ improved cell growth on HU plates, it did not alleviate the HU hypersensitivity of the dun1Δpan2Δ and dun1Δpan3Δ mutants relative to dun1Δ (Fig. 2C).

Another explanation for the increased lethality could be an insufficient cell cycle arrest function of the S phase checkpoint. We therefore analyzed cells from HU time course experiments by flow cytometry. Fig. 3B shows that all strains initially synchronized in S phase after HU addition and that this arrest was maintained in the dun1Δpan2Δ strain for about 6 h. However, while wild type and pan2Δ cells reassumed a normal cell cycle profile after 24 h (presumably due to RNR up-regulation to generate sufficient dNTPs), analysis of the dun1Δpan2Δ strain revealed a dramatic accumulation of cells with <1n DNA content after 12 and 24 h, consistent with increased genome instability as a cause of the HU-dependent lethality. Rad53 plays a central role in arresting the cell cycle in response to replication blocks, and Fig. 2B shows that the lethality of dun1Δpan2Δ double mutants occurred with a considerable delay compared with rad53Δ cells (in a non-isogenic wild type background). Likewise, Rad53 activation by hyperphosphorylation (which can be detected as slower migrating bands in immunoblots; Ref. 32) in response to HU treatment was uncompromised in the dun1Δpan2Δ strain (Fig. 3C).

Taken together these results demonstrate that the increased replication block sensitivity of the double mutant is independent of dNTP levels and the S phase cell cycle arrest function. Finally, in contrast to the HU hypersensitivity, dun1Δpan2Δ and dun1Δpan3Δ double mutants had no increased DNA damage sensitivity after treatment with MMS (Fig. 2D), indicating that the genetic interaction of Dun1 and Pan2/3 is specifically required for the survival of replicative DNA damage.

**Regulation of RAD5 mRNA Levels by Dun1 and PAN—** To test whether poly(A) tails may be a checkpoint target in vivo, we analyzed poly(A) tail length distribution profiles in response to replication blocks and DNA damage in wild type, dun1Δ,
pan2Δ and dun1Δ/pan2Δ strains (Fig. 4A). In these experiments, deletion of PAN2 increased the maximal length of poly(A) tails by ~20 bases, similar to previous reports (22). Interestingly, HU treatment caused a slight decrease in the maximal poly(A) tail length in all strains, whereas DNA damage by MMS treatment caused a noticeable increase (particularly in dun1Δ/pan2Δ) of the maximal length of poly(A) tails (Fig. 4A). While these data are consistent with the modulation of poly(A) tail length distribution profiles by checkpoint signals, we cannot exclude the possibility that these are secondary effects of synchronizing cells in S phase (HU) with a corresponding shift in gene expression profiles or possible slowing of nucleocytoplasmic export of mRNAs that could result in longer poly(A) tails (33). Moreover, the overall effects were still maintained in the pan2Δ and double mutant strains. Therefore, these changes are either independent of the Pan2-Pan3 complex or compensated by an alternative mRNA deadenylase, for example the Ccr4-Caf1 complex (20). Importantly, the overall poly(A) tail length profile after HU treatment was essentially identical in the pan2Δ strain (which remains viable after replication blocks) and the dun1Δ/pan2Δ strain (for which replication blocks are lethal), indicating that the increased lethality of the dun1Δ/pan2Δ double mutant is not simply the result of globally deregulated poly(A) tail length profiles.

To test whether the increased replication block sensitivity of the dun1Δ/pan2Δ strain may instead be caused by a specific effect on few mRNAs, we analyzed gene expression profiles by subsequent hybridizations of a yeast DNA array (containing 400 open reading frames subsequently hybridized with probes from poly(A) RNA of pan2Δ, dun1Δ/pan2Δ, and dun1Δ strains treated for 3 h with 150 μM HU. Normalized to an intermediate intensity spot (YOR249c, open arrowhead, 1 arbitrary unit), the RAD5 signal (circled) was increased by 65-130-fold in the double mutant strain (10.39 arbitrary units) relative to the dun1Δ (0.16 arbitrary units) and pan2Δ (0.08 arbitrary units) controls. Other spots differed only marginally between these strains (YOL040c, near the lower corner of the open arrowhead: dun1Δ/pan2Δ = 3.42, pan2Δ = 4.43, dun1Δ = 4.62 arbitrary units; YML039w, filled arrowhead, upper left corner: dun1Δ/pan2Δ = 8.74, pan2Δ = 7.74, dun1Δ = 7.37 arbitrary units). C, DNA array analysis of other members (boxed) of the RAD6 epistasis group. D, Northern blot analysis of total RNA isolated from control (−), HU-treated (+), or MMS-treated (+) strains indicated above probed for RAD5 and 18S RNA as a loading control. The graph shows the mean of normalized RAD5/18S ratios based on quantitative phosphorimaging analysis. Error bars indicate the range of two independent experiments.

Up-regulation of RAD5 mRNA Impairs dun1Δ Survival of Replication Stress—Regardless of the quantitative discrepancy between the Northern and array analyses, the experiments described above suggested that up-regulation of RAD5 gene expression could be the molecular mechanism responsible for the increased replication block sensitivity of dun1Δ/pan2Δ double mutants. To test this hypothesis, we tested whether ectopic overexpression of RAD5 could mimic the HU-hypersensitizing effect of pan2Δ in the dun1Δ background. For this purpose, the various yeast strains were transformed with a galactose-inducible RAD5 plasmid (under control of the GAL1 promoter) as well as the corresponding empty vector control and tested for viability on galactose-containing HU plates. Relative to the vector control, RAD5 overexpression markedly reduced the viability of dun1Δ and dun1Δ/pan2Δ strains but had only a modest effect on wild type and pan2Δ strains on HU plates (Fig. 5) and had no effect on control plates lacking HU or galactose (Fig. 5). Interestingly RAD5 overexpression under these conditions had a more dramatic effect than the pan2 deletion on dun1Δ viability. This is most likely the result of much higher RAD5 mRNA levels achieved from the GAL1 promoter compared with the endogenous mRNA that was con-
firmed by Northern blot analysis (data not shown). Therefore, this experiment demonstrates that elevated RAD5 mRNA levels are sufficient to hypersensitize dun1Δ strains to replicational stress and supports the conclusion that elevated RAD5 mRNA levels are the reason for the increased HU-dependent lethality of the dun1Δpan2Δ double mutant.

**DISCUSSION**

**DUN1** plays a crucial role in preventing gross chromosomal rearrangements resulting from inappropriate repair pathways of spontaneous replicative DNA damage even in the absence of exogenous DNA-damaging or replication-blocking agents (13). The data presented here indicate that the regulation of RAD5 mRNA levels by Dun1 in concert with PAN contributes to the function of DUN1 in maintaining genome stability. dun1Δpan2Δ cells remain in S phase for considerable time (Fig. 3B), activate Rad53 as a key player in the intra-S checkpoint normally (Fig. 3C), and die after replication blocks with considerably delayed kinetics compared with rad53Δ cells (Fig. 2B). At the same time, expression of the RAD5 gene, which plays a role in postreplicative DNA repair (35), is specifically deregulated (Fig. 4) and sufficient to cause HU-dependent lethality in the dun1Δ background (Fig. 5), while the accumulation of cells with aberrant DNA contents in flow cytometry profiles (Fig. 3B) coincides temporally with the reduced viability in survival curves (Fig. 2B). The most likely explanation for the dun1Δpan2Δ phenotype is, therefore, that these cells arrest normally in S phase while replicative DNA damage persists but that this damage is inappropriately repaired, which removes the checkpoint signal, allowing for subsequent cell division with loss of genetic material and concomitantly increased lethality.

Interestingly, **DUN1** has recently been indirectly linked to regulation of the **RAD6** epistasis group of which **RAD5** is a member (38). Rad5 is a RING finger domain protein that mediates the interaction of the Lys-63-specific ubiquitin ligases Ubc13 and Mms2 with Rad18 and the Lys-48-specific ubiquitin ligase Rad6 (35). This heteromeric complex has been proposed to generate a signal that activates pathways for the repair of DNA double strand breaks by homologous recombination (i.e. “error-free”) instead of non-homologous end joining (i.e. “error-prone”) (35–36). The same surfaces involved in the heteromeric interaction of Rad5 and Rad18 can also mediate homodimerization of the respective subunits and thereby dissociate the pentamer into Rad5-Ubc13-Mms2 and Rad6-Rad18 subcomplexes, which may generate a Rad6-dependent signal to activate an error-prone subpathway (35). According to this model, increased Rad5 protein levels in the dun1Δpan2Δ strain could indeed shift the equilibrium away from the heteromeric complex toward an error-prone postreplicative DNA repair pathway that causes the increased lethality (Fig. 6). This pathway seems to be particularly critical in the absence of **DUN1** as **RAD5** overexpression alone had only a subtle effect on replicational stress survival of wild type or **pan2Δ** strains (Fig. 5). This is conceivable given that **DUN1** has several additional DNA damage repair functions, e.g. transcriptional induction of repair enzymes (16) and direct regulation of repair proteins such as Rad55 (39), which may render dun1Δ mutants more sensitive to deleterious effects of either pan2Δ deletion or **RAD5** overexpression.

As synthetic lethality is usually a property of genes whose products interact in a common biochemical pathway (31), the simplest interpretation of our data is that Dun1 and PAN interact at the posttranscriptional level to regulate **RAD5** gene expression. While the two-hybrid data indicate that Dun1 interacts directly with Pan3, a key question remaining to be answered is whether PAN activity is regulated by Dun1 in response to replication blocks. Given the synthetic effect of the two genes, it is quite possible that Dun1 does not directly regulate PAN but another enzyme with a similar function, e.g. Ccr4-Caf1. In this scenario (Fig. 6), PAN would constitutively regulate **RAD5** mRNA levels, whereas Ccr4-Caf1 would be strictly Dun1-dependent and therefore compensate for the loss of PAN as long as **DUN1** is present. Ccr4-Caf1 has multiple functions as an mRNA deadenylase (20) and as part of the CCR4-NOT complex in control of transcriptional initiation and elongation (40). Tucker et al. (20) have suggested that one possible explanation for linking the cytoplasmic Ccr4-Caf1 deadenylase to the transcriptional machinery is that it needs to be cotranscriptionally loaded onto messenger ribonucleoprotein complexes. If this is so, it would transiently be in a complex with PAN during the initial trimming of the poly(A) tail. In this context, Pan3 binding by the Dun1 FHA domain could act as a transient scaffold to bring the kinase domain in contact with Ccr4-Caf1 to phosphorylate it and regulate its substrate specificity before export to the cytoplasm. Interestingly, Caf1 (also known as Pop2) has recently been shown to be regulated by phosphorylation in response to diauxic shifts by the Yak1 kinase, but it is unclear whether this affects its deadenylase function (41). An important goal of future studies will be to elucidate the precise molecular mechanism by which Dun1 contributes to the regulation of **RAD5** mRNA stability.

Transcriptional control of gene expression (1) and cotranscriptional regulation of mRNA 3′-end processing and polyadenylation reactions (42) are established components of cell
cycle checkpoints. Our results indicate that posttranscriptional mechanisms involving poly(A) tail length control are an additional checkpoint target in the regulation of gene expression. The Pan2-Pan3 and Ccr4-Caf1 poly(A)-exonucleases, the Dun1 kinase, and members of the RAD6 epistasis group have reason-
ably conserved orthologs in mammals (2, 20, 35). Therefore, the checkpoint-dependent regulation of posttranscriptional control pathways may be not be restricted to yeast but may also contribute to the prevention of chromosome aberrations in mammalian cells.

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