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Impaired tRNA Nuclear Export Links DNA Damage and Cell-Cycle Checkpoint

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SUMMARY

In response to genotoxic stress, cells evoke a plethora of physiological responses collectively aimed at enhancing viability and maintaining the integrity of the genome. Here, we report that unspliced tRNA rapidly accumulates in the nuclei of yeast *Saccharomyces cerevisiae* after DNA damage. This response requires an intact *MEC1*- and *RAD53*-dependent signaling pathway that impedes the nuclear export of intron-containing tRNA via differential relocalization of the karyopherin Los1 to the cytoplasm. The accumulation of unspliced tRNA in the nucleus signals the activation of Gcn4 transcription factor, which, in turn, contributes to cell-cycle arrest in G1 in part by delaying accumulation of the cyclin Cln2. The regulated nucleocytoplasmic tRNA trafficking thus constitutes an integral physiological adaptation to DNA damage. These data further illustrate how signal-mediated crosstalk between distinct functional modules, namely, tRNA nucleocytoplasmic trafficking, protein synthesis, and checkpoint execution, allows for functional coupling of tRNA biogenesis and cell-cycle progression.

INTRODUCTION

In order to maintain the fidelity of genetic information, organisms have evolved surveillance mechanisms that monitor the integrity of the chromosomes. These conserved checkpoints comprise a network of regulatory signaling pathways that initiate downstream events principally aimed at repairing DNA lesions, while inducing a transient arrest in cell division (Weinert and Hartwell, 1988).

Cell-cycle progression normally comprises a series of tightly integrated events initiated via differential activation of the cyclin-dependent kinases (CDKs). In budding yeast *S. cerevisiae*, entry into the cell-division cycle, termed START, is operationally defined as a point at which cells become committed to the mitotic cell cycle. This requires the accumulation of threshold levels of the G1 cyclins Cln1 and Cln2 (Tyers et al., 1992). Activation of the CDK Cdc28 by cyclins signals a host of events that culminate in replication and the subsequent mitotic segregation of the chromosomes.

However, several stages in the cell cycle, including the G1-to-S phase transition, are subject to strict checkpoint arrest in response to both ectopic and endogenous sources of DNA damage. The significance of the G1 checkpoint arrest is highlighted by the observation that genes that function in G1 progression, or surveillance mechanisms that monitor its proper execution, are amongst the most commonly mutated genes found in human cancers (Sherr and McCormick, 2002).

Progression through the G1 phase is normally tightly coupled to the rate of active protein synthesis (Polymenis and Schmidt, 1999). Owing to its ubiquitous requirement in protein translation, the biogenesis of mature transfer RNA (tRNA) plays an intrinsic role in this process (Mann et al., 1992; Volta et al., 2005). After its synthesis by RNA polymerase III, the primary tRNA transcripts undergo extensive processing (reviewed in [Hopper and Phizicky, 2003]), which, in the case of intron-containing tRNAs, includes splicing. The yeast genome encodes 272 tRNA genes, of which 59, encoding 10 different tRNA species, contain introns (O'Connor and Peebles, 1991). Splicing is a prerequisite for the biogenesis of functional tRNA because all introns disrupt the adjacent anticodon loops. Although tRNA processing occurs primarily in the nucleus, splicing in yeast is accomplished in the cytoplasm by a conserved heterotetrameric complex.

Using yeast as a model system, we report that downregulation of tRNA export represents a previously unanticipated physiological response to genotoxic stress. In the

presence of DNA damage, Los1, the principal nuclear export factor for intron-containing tRNA, becomes differentially localized to the cytoplasm in a signal-dependent manner. This relocalization causes rapid accumulation of unspliced precursor tRNA in the nucleus in vivo because Los1 is limiting for the export process. The nuclear retention of tRNA autonomously signals the activation of the Gcn4 transcription factor, which, in turn, mediates the delayed accumulation of the key cell-cycle regulator cyclin Cln2 and the execution of a transient G1 arrest. Disruption of this regulatory network in null mutants of the upstream signaling components or the downstream effectors is associated with aberrant cell-cycle progression and enhanced loss of viability after DNA damage.

RESULTS

Downregulation of tRNA Splicing in Response to DNA Damage

Defects in cellular protein-synthesis machinery (Mann et al., 1992; Polymenis and Schmidt, 1999), including those of the tRNA splicing components (Figure S1, see Supplemental Data available with this article online), are primarily manifested as a slow G1 progression. Since DNA damage also elicits a transient G1 arrest, we examined whether tRNA processing is subject to regulation during checkpoint-induced G1 arrest.

In yeast, the primary tRNA transcript undergoes 5′ and 3′ end removal in the nucleus and, in intron-containing species, splicing after export to the cytosol (Figure 1A). The subsequent ligation of the spliced exons gives rise to functionally mature tRNA. We monitored tRNA end processing and splicing by using oligonucleotide probes that hybridize specifically to the 5′ or 3′ splicing junctions of tRNA $^{Ile}_{UAU}$, as a representative tRNA, or to the intron itself. The results demonstrate that whereas end processing remains intact, there is an accumulation of intron-containing, but end-processed, tRNA after treatment with the DNA alkylating agent methylmethane sulfonate (MMS) (Figure 1B). The accumulation of this processing intermediate is a reflection of an in vivo defect in the splicing step (O'Connor and Peebles, 1991). To confirm the causal basis for this effect, we exposed yeast cultures to UV irradiation, which induces DNA lesions distinct from those generated by MMS. Probing $tRNA^{Ile}$ revealed a similar defect in splicing in UV-irradiated cells, as seen after MMS treatment (Figure 1C, upper row).

Since the splicing junctions in intron-containing tRNAs are not conserved and the introns themselves vary in sequence and length (Abelson et al., 1998), we similarly probed several other intron-containing tRNAs to verify the ubiquity of the DNA-damage response. All newly transcribed tRNA species examined displayed comparable splicing defects after UV exposure (Figure 1C). The ratio of end-processed to primary tRNA transcript serves as a measure of in vivo splicing efficiency (O'Connor and Peebles, 1991). Determining this ratio in $tRNA^{Ile}$ and $tRNA^{Leu}$ before and after DNA damage (Figure 1D) revealed that the in vivo defect in splicing after UV treatment was not intron specific, was comparable in magnitude, and occurred with similar kinetics.

To ensure that the impaired tRNA splicing was not due to spurious effects of MMS or UV other than generating DNA lesions, we introduced genomic DNA double-strand breaks by heterologous expression of the restriction endonuclease EcoRI in vivo. A CEN-based plasmid that expresses EcoRI in the presence of galactose is known to activate DNA damage signaling (Mills et al., 1999). Genomic DNA prepared from yeast cells grown in galactose that harbor this plasmid display lower average molecular weights as compared to glucose controls (Figure 2A, upper panel), indicating the presence of sporadic DNA double-strand breaks. Importantly, these cells also displayed defective tRNA splicing similar in magnitude (Figure 2A, lower panel, quantifications at the bottom) to that detected after exposure to UV. Collectively, these data demonstrate that tRNA splicing is impaired in cells with DNA damage.

The rapid inhibition of tRNA splicing in UV-irradiated cells (Figure 1D), which was complete within 15 min, indicated that it was not secondary to DNA-damage-induced cell-cycle arrest since the irradiated cells do not display a predominant cell-cycle phenotype within this time course (not shown). Consistent with this, wild-type cells arrested in early G1 by α-factor treatment exhibited wild-type splicing activity and, moreover, executed a UV-induced reduction in tRNA splicing similar to that observed in cycling cells (Figure 2B). The reduction in tRNA splicing after DNA damage was therefore not a passive consequence of the induction of cell-cycle arrest.

Downregulation of tRNA Splicing after DNA Damage Requires an Intact Signaling Pathway

The rapid kinetics of the inhibition of tRNA splicing (Figure 1D) was highly reminiscent of a signal-mediated response. Since much of the cellular response to DNA damage is mediated via conserved checkpoint signaling pathways, we explored this possibility by evaluating tRNA splicing after DNA damage in strains bearing null alleles of select genes encoding core components of the checkpoint signaling apparatus in yeast. We first examined in vivo tRNA splicing in strains that harbor deletion of *RAD17*, *RAD24*, or *MEC3*, which encode interacting subunits of a protein complex that functions as a DNA lesion sensor, and in a strain bearing a null allele of *MEC1*, which encodes a distinct sensor that functions in parallel (Majka and Burgers, 2005; Melo and Toczyski, 2002). We also examined splicing in a mutant harboring a deletion of *RAD53*, which encodes the principal downstream signal transducer.

The *rad17*, *rad24*, and *mec3* mutants maintained the ability to downregulate tRNA splicing after MMS treatment (Figure 2C, left panel), indicating that none of these genes is singly required for activating this response. In contrast, the MMS-treated *mec1* or *rad53* mutants did not

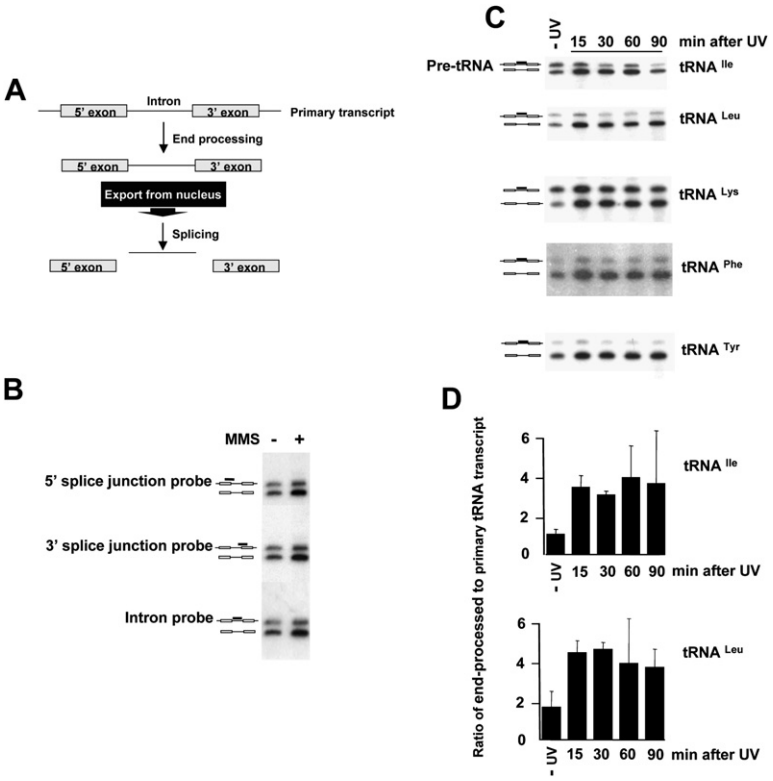

Figure 1. DNA Damage Induces Downregulation of tRNA Splicing

(A) A simplified schematic representation of tRNA primary transcript processing.

(B) Defective tRNA intron excision in log-phase cultures of S288C wild-type yeast in rich media (YPD) after MMS treatment (0.04% v/v) for 80 min. Northern blot of the $tRNA^{Ile}$ transcript with probes, denoted by short, thick lines, complementary to 5′ and 3′ splice junctions or to the intron itself. Primary precursor and end-processed, unspliced transcripts are depicted.

(C) Time course analysis of splicing of the indicated tRNA species after UV irradiation (60 J/m^2).

(D) Quantification of $tRNA^{Ile}$ and $tRNA^{Leu}$ splicing efficiency after UV exposure. Data were normalized to untreated controls (not shown); bars indicate standard deviation across three independent experiments.

accumulate end-processed, intron-containing intermediates (Figure 2C, right panel), indicating that the perturbation in tRNA splicing after DNA damage was Mec1/Rad53-dependent.

The requirement for intact Mec1 and Rad53, the sequential components of a conserved DNA-damage signaling pathway, indicated that the downregulation of tRNA splicing is an active cellular response. Moreover, the unperturbed tRNA splicing activity exhibited by *mec1Δ* and *rad53Δ* mutants, which are highly sensitive to DNA damage, indicated that the impaired tRNA splicing activity exhibited by wild-type cells after exposure to MMS was not due to a simple loss of cellular viability after genotoxic stress.

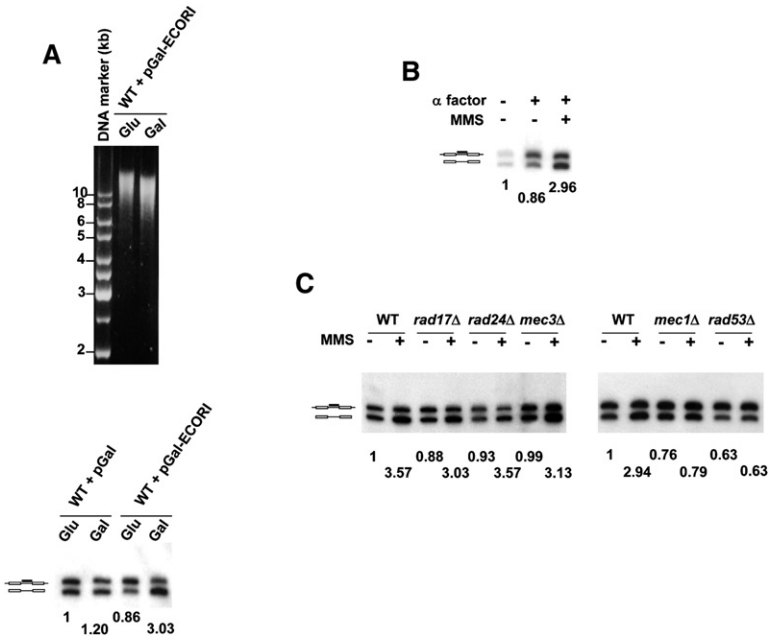

Figure 2. Downregulation of tRNA Splicing Requires an Intact Signaling Pathway

(A) Induction of DNA double-strand breaks perturbs tRNA splicing. (Top) Genomic DNA from S288C wild-type cells harboring a galactose-inducible pGal-EcoRI, expressing the endonuclease EcoRI, grown in glucose or galactose, and stained with ethidium bromide. (Bottom) $tRNA^{Ile}$ splicing, with a probe complementary to its intron, in cells harboring control (pGal) or pGal-EcoRI. Ratio of end-processed to primary unspliced $tRNA^{Ile}$ is denoted; control is assigned an arbitrary value of 1.

(B) MMS (0.04%) reduces $tRNA^{Ile}$ splicing in α-factor-arrested cells. Quantification as in (A).

(C) $tRNA^{Ile}$ splicing in select checkpoint mutants exposed to MMS (0.04%) for 80 min.

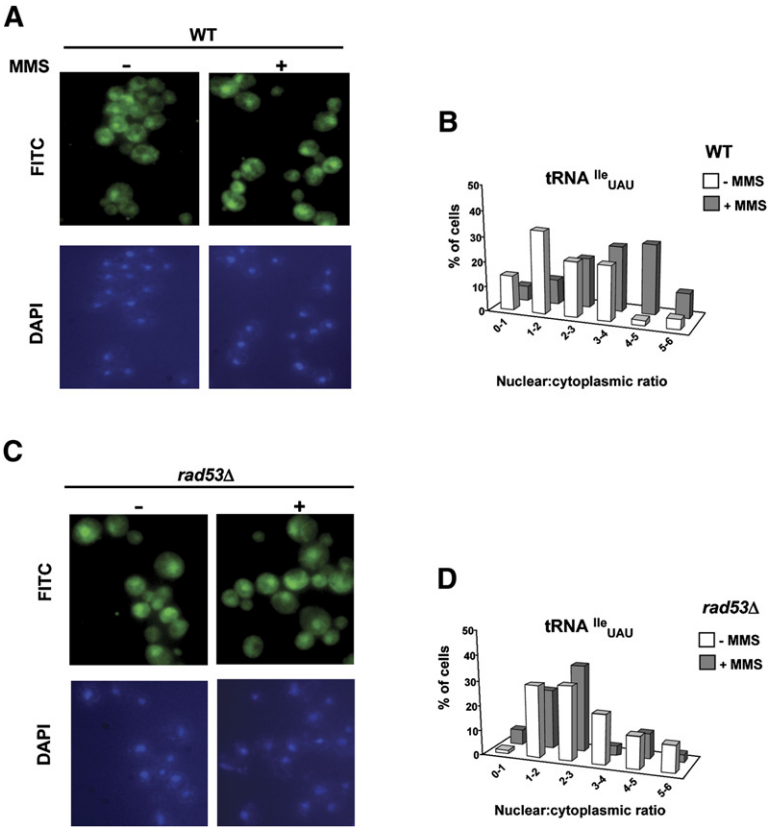

Figure 3. Induction of DNA Damage Specifically Attenuates Nuclear Export of Intron-Containing tRNA

(A) MMS-induced nuclear retention of precursor tRNA. Log-phase wild-type cells were treated with 0.04% MMS for 80 min. The subcellular distribution of intron-containing $tRNA^{Ile}_{UAU}$ transcripts was monitored in situ by fluorescence microscopy (FITC) by using a DIG-labeled oligo complementary to its intron. The nuclei were counterstained with DAPI.

(B) Quantification of the nucleocytoplasmic distribution of $tRNA^{Ile}_{UAU}$. The subcellular abundance of the $tRNA^{Ile}$ transcript in a population of cells was determined by quantifying the FITC signal intensity in the nuclear and cytoplasmic compartments (grouped into bins on the x axis). The number of cells in each bin, as a percentage of the total cell population assayed, is plotted on the y axis.

(C) The subcellular distribution of intron-containing $tRNA^{Ile}_{UAU}$ in a *rad53Δ* mutant.

(D) Quantification of (C).

Checkpoint-Induced Inhibition of tRNA Splicing Is due to Defective tRNA Nuclear Export

To determine the mechanism underlying the impaired tRNA splicing after DNA damage, we initially examined if the defective splicing was due to a reduction in the enzymatic activity of the basal tRNA splicing machinery. Using an in vitro tRNA splicing assay (Reyes and Abelson, 1987) that provided a qualitative reflection of in vivo splicing activity (Figure S2A), lysates prepared from control untreated or MMS-treated wild-type yeast displayed comparable activity over a range of protein concentrations (Figure S2B). Moreover, the relative abundance of the C-terminally TAP-tagged enzymatic subunits Sen2 and Sen34 proteins was unperturbed after DNA damage (Figure S2C), a finding in line with a previous report showing that the level of all four *SEN* gene transcripts remain unchanged after MMS treatment (Gasch et al., 2001). The accumulation of unspliced tRNA after DNA damage was therefore unlikely due to a reduction in the core enzymatic activity of the tRNA splicing machinery.

Although tRNA is transcribed in the nucleus, the yeast splicing machinery is localized to the cytoplasm (Yoshihisa et al., 2003). Consequently, mutations in the nuclear export machinery lead to the accumulation of intron-containing tRNA in the nucleus (Hellmuth et al., 1998; Simos and Hurt, 1999). Therefore, we monitored the integrity of tRNA nuclear export after DNA damage. In a fluorescence in situ hybridization assay (FISH), the newly transcribed intron-containing $tRNA^{Ile}$ was predominantly localized to the nucleus during unperturbed growth (Figure 3A), a localization pattern in line with what was seen in a previous report (Sarkar and Hopper, 1998). After MMS treatment, the wild-type cells displayed a more pronounced tRNA nuclear accumulation (Figure 3A). We determined the subcellular distribution of intron-containing $tRNA^{Ile}$ transcripts by rigorously quantifying the ratio of nuclear to cytoplasmic fluorescence (FITC) signals in a cell population (see Experimental Procedures for details). This quantitative approach confirmed that the precursor tRNA is nuclear enriched in MMS-treated cells (Figure 3B), thereby illustrating that the subcellular distribution of tRNA was subject to fine regulation in cells with DNA damage. The MMS-induced nuclear retention of newly transcribed tRNA in situ was correlated with the accumulation of unspliced tRNA in vivo (Figure 2C, WT). Unlike the wild-type cells, the *rad53* mutants did not exhibit differential tRNA nuclear accumulation after MMS treatment (Figure 3C, quantified in Figure 3D), in agreement with the observation that these mutants maintained the ongoing rate of tRNA splicing after DNA damage (Figure 2C, *rad53Δ*). The Rad53-mediated sequestering of precursor transcripts in the nucleus provided a plausible mechanistic basis for the accumulation of unspliced tRNA in vivo after DNA damage in wild-type cells.

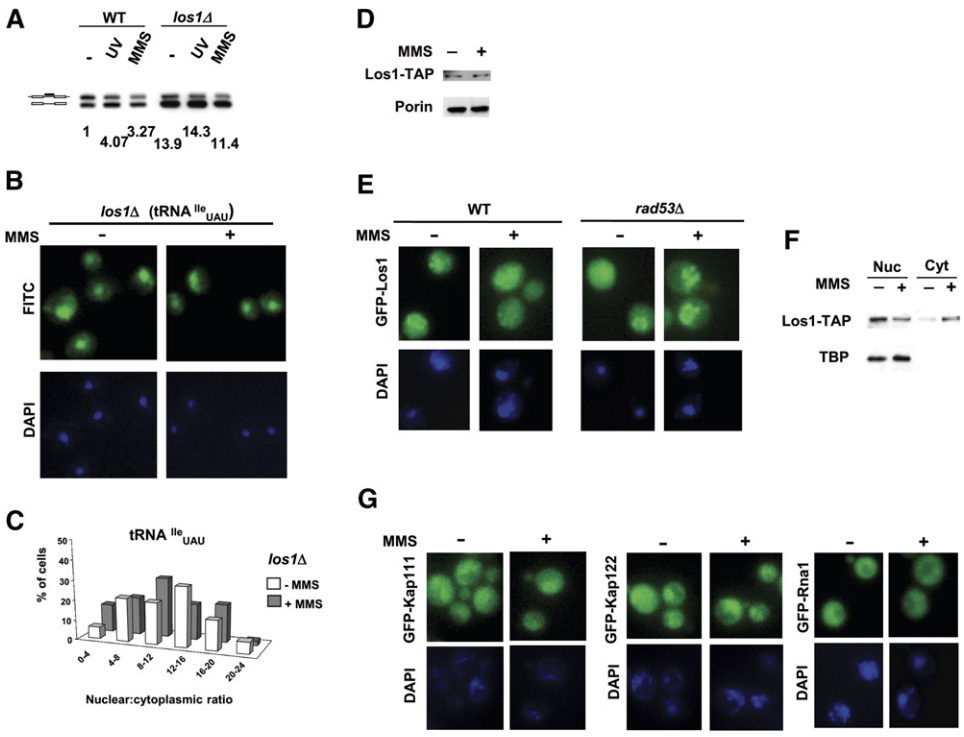

Figure 4. MMS Treatment Results in Subcellular Redistribution of tRNA Export Factor Los1

(A) In vivo $tRNA^{Ile}_{UAU}$ splicing in wild-type and *los1Δ* cells harvested 30 min after UV irradiation (60 J/m^2) or 80 min after MMS (0.04%) treatment. Total RNA was assayed with an oligo complementary to the $tRNA^{Ile}$ intron.

(B) Subcellular distribution of intron-containing $tRNA^{Ile}_{UAU}$ in a *los1* mutant in situ by fluorescence microscopy as in Figure 3A.

(C) Quantification of the nucleocytoplasmic distribution of $tRNA^{Ile}_{UAU}$ as in Figure 3B.

(D) Los1-TAP abundance monitored by western blot of whole-cell lysates from untreated and MMS-treated cells. The mitochondrial porin served as the loading control.

(E) In situ localization of Los1-GFP in wild-type and *rad53Δ* cells before or after MMS exposure (0.04% for 80 min). Successive images for GFP and DAPI are shown.

(F) Differential recovery of Los1-TAP in cytoplasmic fractions after MMS treatment determined by western blot of the cytosolic fractions and lysates from Ficol-gradient-purified nuclei. The canonical TATA-box binding protein (TBP) served as a nuclear marker.

(G) In situ localization of C-terminally GFP-tagged Kap111, Kap122, and Rna1 before and after MMS treatment.

The defect in nucleocytoplasmic trafficking was not ubiquitous because two intronless tRNAs, in addition to poly(A)-containing mRNA, maintained a persistent subcellular localization after MMS treatment (Figure S3). The intact nucleocytoplasmic trafficking of these RNA species suggested that the nuclear accumulation of intron-containing tRNA after MMS was a targeted response, an observation that is in line with its requirement for a signaling pathway (Figure 2C).

The nuclear accumulation of tRNA after MMS treatment is not due to elevated synthesis of primary transcripts after DNA damage, because the polymerase III-directed tRNA transcription is globally downregulated after DNA damage in yeast (Ghavidel and Schultz, 2001). This raised the possibility that the nuclear retention of tRNA was due to its attenuated export from the nucleus.

As a member of the importin β family of karyopherins, yeast Los1 (Hopper et al., 1980) and its mammalian counterpart, exportin-t (Arts et al., 1998), are the principal tRNA export receptors. In keeping with its gross defect in tRNA splicing in vivo (Figure 4A), *los1* deletion mutants displayed a constitutive nuclear accumulation of intron-containing $tRNA^{Ile}_{UAU}$ in unperturbed growth (Figure 4B, quantifications in Figure 4C). However, unlike the wild-type cells, induction of DNA damage in *los1Δ* cells did not enhance the basal defect in tRNA export (Figure 4C) or splicing (Figure 4A, quantifications shown at the bottom). The failure to elicit a response in these mutants suggested that the nuclear accumulation of tRNA primary transcripts in wild-type cells after DNA damage was due to impaired function of the Los1 export factor.

Western blot of the whole-cell lysates from a strain with C-terminally TAP-tagged Los1 demonstrated that the apparent involvement of Los1 in defective tRNA export after DNA damage was not due to a reduction in its relative abundance (Figure 4D). Given the likelihood of Los1 nucleocytoplasmic shuttling (Hellmuth et al., 1998), we examined its in situ localization both before and after DNA damage in cells harboring a C-terminally GFP-tagged allele of *LOS1*. Similar to its wild-type counterpart (Simos et al.,

1996), and in keeping with its principal function as an export factor, Los1-GFP was predominantly localized to the nuclear periphery during normal growth in wild-type cells (Figure 4E, left panel). By contrast, a fraction of Los1-GFP became preferentially redistributed to the cytoplasm after exposure to MMS, giving rise to its homogenous cellular appearance (Figure 4E, left panel). In an independent experimental approach, parallel western blot examination of nuclear and cytosolic protein fractions prepared from TAP-tagged Los1 cells confirmed the differential relocalization of Los1 to the cytoplasm after MMS exposure (Figure 4F). In addition to Los1, we also examined the in situ localization of two other members of the yeast karyopherin β family, Kap111 and Kap122, along with that of Rna1, a Ran-specific GTPase-activating protein (RanGAP1), involved in RNA export. The apparent persistent in situ localization of the C-terminally GFP-tagged karyopherins to the nucleus and Rna1 to the cytoplasm (Figure 4G) suggested that the DNA-damage-induced relocalization of Los1 was a targeted response. Consistent with this, the nuclear localization of Los1-GFP remained unperturbed in MMS-treated *rad53Δ* mutants (Figure 4E, right panel), demonstrating a requirement for Rad53-dependent signaling in mediating this response. The significance of the DNA-damage-induced Los1 relocalization was demonstrated by the observation that *rad53Δ* cells, which maintained a constitutive nuclear pool of Los1, displayed no obvious defect in tRNA export (Figure 3C) or splicing (Figure 2C) after MMS treatment. Collectively, these data indicate that the signal-mediated relocalization of Los1 from the nucleus was the likely determinant of the impaired tRNA export after DNA damage.

Constitutive Reduction in tRNA Export through *LOS1* Deletion Restores the Impaired G1 Arrest and Enhances the Viability of *rad53* Checkpoint Mutants

Delay in progression through the G1 phase of the cell cycle is a hallmark of cells with unrepaired genomic lesions (Siede et al., 1994). Although G1 arrest appears to be a transient delay en route to mitotic arrest, from a theoretical viewpoint, failure to delay cell-cycle progression before the initiation of DNA replication can lead to gross chromosomal anomalies (reviewed in Paulovich et al., 1997b). Accordingly, premature progression through G1 partly accounts for the increased genomic instability in checkpoint mutants. We therefore examined whether the failure of *rad53* mutants to downregulate tRNA export after DNA damage (Figure 3D) contributes to the defective G1 checkpoint execution in these mutants.

As a downstream target of Rad53 signaling (Figure 4E), *LOS1* is functionally epistatic to *RAD53* in downregulation of splicing, because mutation in either gene alone abolishes the MMS-induced reduction in tRNA splicing (Figures 2C and 4A). We reasoned that deletion of *LOS1* in a *rad53* null mutant should functionally mimic its negative regulation by intact Rad53 signaling in MMS-treated wild-type cells. This experimental approach is of utility in deciphering the downstream effectors of signaling pathways. For instance, reducing histone gene dosage, a target of Rad53-mediated signaling that is normally degraded in wild-type cells after DNA damage, partially restores viability in a *rad53* mutant (Gunjan and Verreault, 2003).

A constitutive reduction in tRNA splicing via targeted deletion of *LOS1* resulted in marked accumulation of unspliced tRNA in both *rad53Δ* and isogenic wild-type control strains (Figure 5A, with relative quantifications indicated). In order to examine the integrity of the G1 checkpoint in these mutants, logarithmically growing (log) cells were blocked in G1 with α-factor, treated with MMS, and then released into fresh media. Cell-cycle progression was then monitored by FACS.

In the absence of DNA damage, all four strains displayed comparable rates of cell-cycle progression (Figure 5B, cf. lanes 1, 3, 5, and 7) since arrest in response to, or recovery from, α-factor arrest is not checkpoint dependent. The MMS-treated wild-type cells displayed a prolonged G1 progression, indicated by the delay in accumulation of cells with a 2N DNA content (Figure 5B, lanes 1 and 2). The intact Rad53-dependent signaling in wild-type cells normally impinges on Los1 and alters its nucleocytoplasmic distribution (Figure 4E). Accordingly, a *los1* null mutant displayed a G1 delay (Figure 5B, lanes 2 and 4) and viability (Figure 5C) comparable to that of the wild-type cells after MMS treatment.

Given the requirement for Rad53 signaling in the G1 checkpoint (Sidorova and Breeden, 1997), the MMS-treated *rad53* null mutant exited G1 unimpeded, indicated by rapid accumulation of cells with a 2N DNA content (Figure 5B, cf. lanes 2 and 6). Remarkably, *rad53Δ los1Δ* double mutants displayed a G1 profile similar to that of the wild-type cells (Figure 5B, cf. lanes 2, 6, and 8). In principle, deletion of *LOS1*, a negatively regulated downstream target of Rad53 in wild-type cells, acts as a suppressor of the defect in G1 checkpoint execution in a *rad53* mutant. Concomitant with the restoration of the G1 checkpoint, there was a partial recovery in viability in the *rad53Δ los1Δ* double mutants after MMS treatment relative to the *rad53*Δ parental strain (Figure 5C).

In addition to prolonging G1 progression, treatment of yeast with MMS also delays DNA replication as a result of impaired firing of replication origins (Tercero et al., 2003). Since determining the cellular DNA content by FACS does not distinguish between slow passage through START and delayed initiation of replication, we performed an α-factor trap experiment (Gerald et al., 2002). Deletion of *LOS1* extended the window of α-factor sensitivity in *rad53Δ los1Δ* double mutants after MMS treatment, demonstrating that Rad53-mediated downregulation of tRNA export is a determinant of progression through START (Figure S5).

Downregulation of tRNA Export Contributes to G1 Arrest by Delaying Translation of Cln2

We sought to examine how impaired tRNA export contributes to the execution of the G1 checkpoint. In unperturbed

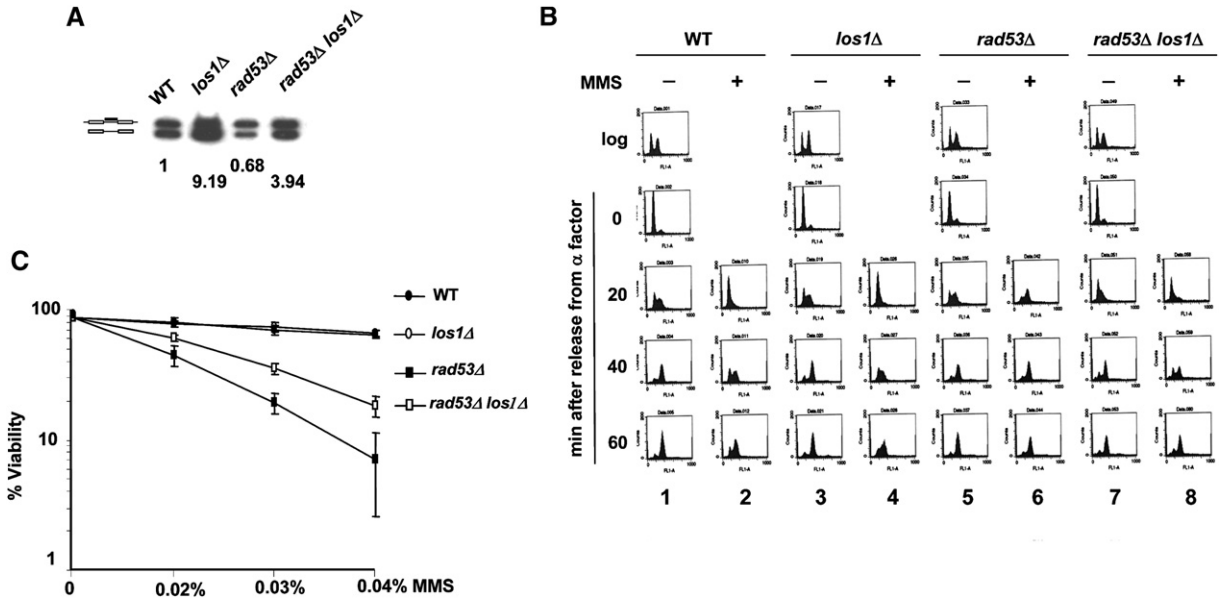

Figure 5. Deletion of *LOS1* Restores G1 Arrest and Enhances Viability in a *rad53* Mutant in Response to DNA Damage

(A) $tRNA^{Ile}_{UAU}$ splicing in wild-type, *los1Δ*, *rad53Δ*, and *rad53Δ los1Δ* mutants assayed by northern blot with an oligo complementary to its intron. The ratio of end-processed to primary unspliced $tRNA^{Ile}$ is denoted; untreated wild-type cells are assigned an arbitrary value of 1.

(B) Delayed G1 progression in *rad53Δ* mutants by deleting *LOS1*. Logarithmically growing (log) cells were arrested in G1 by α-factor and were treated with MMS or left untreated. These cells were subsequently released into fresh media and analyzed by FACS.

(C) Reduction in MMS hypersensitivity of *rad53Δ* mutants by deleting *LOS1*. After MMS treatment, viability was determined by counting colonies after a 3 day incubation at 30°C.

growth, the G1 cyclins Cln1, Cln2, and Cln3 largely govern progression through START (Tyers et al., 1991). In particular, the accumulation of Cln1 and Cln2 proteins to threshold levels precedes G1 progression. Given the clear delay in G1 progression in MMS-treated wild-type cells (Figure 5B, lanes 1 and 2), we explored the possibility that a defect in the accumulation of the G1 cyclins plays a role in execution of the G1 checkpoint after DNA damage. To this end, we monitored the accumulation of Cln2 by quantitative western blot by using a Cln2-TAP strain that harbors a C-terminally TAP-tagged allele of *CLN2* (Ghaemmaghami et al., 2003). Cln2-TAP cells displayed wild-type growth rates (data not shown), and the canonical cell-cycle-regulated expression of Cln2 mRNA and protein (Figures 6A and 6B, upper panels).

In order to monitor the accumulation of Cln2-TAP after DNA damage, cells were synchronized in G1 by α-factor and then left unperturbed or treated with MMS. After release into fresh media to allow for resumption of the cell cycle, cells were harvested at the indicated time points, and whole-cell protein lysates and mRNA were prepared for analysis (Figures 6A and 6B). Since it was previously reported that the Cln2 mRNA level decreases after MMS treatment (Sidorova and Breeden, 1997), we normalized Cln2-TAP protein abundance to that of its cognate mRNA in order to monitor the rate of Cln2-TAP protein translation independent of its transcript levels. Figure 6C depicts the quantification of Cln2-TAP protein accumulation after release from α-factor arrest. In untreated cells, Cln2-TAP protein steadily accumulated and was maximally expressed within 40 min after release, whereafter it declined sharply as cells exited G1 (Figure 5B, lane 1). By contrast, the accumulation of Cln2-TAP protein was markedly slower in MMS-treated cells (Figure 6C), coincident with the delayed G1 progression in these cells (Figure 5B, lane 2). While we cannot rule out a role for transcriptional regulation of G1 cyclins as part of a broader cellular response, our data are consistent with the involvement of a posttranscriptional mechanism in regulating Cln2 expression. Notably, this delay was not due to aberrant proteolysis of Cln2 since the half-life of the G1 cyclins remains unchanged after genotoxic stress (Germain et al., 1997).

The paralogous *CLN1* and *CLN2* genes are coordinately expressed throughout the cell cycle and function redundantly in G1 progression (Tyers et al., 1991). Given their extensive functional and regulatory overlap, we anticipate that Cln1 expression is subject to a similar mode of translational regulation after DNA damage. Despite their apparent functional redundancy, Cln3 is distinct from Cln2 (and Cln1) both in its primary sequence and largely constitutive expression pattern throughout the cell cycle (Stuart and Wittenberg, 1994). Therefore, we monitored the abundance of TAP-tagged Cln3 in parallel. In accordance with its constitutive cell-cycle expression, Cln3-TAP levels remained unchanged in α-factor-arrested cells (Figure S6). Moreover, unlike Cln2-TAP, Cln3-TAP protein expression was undisturbed in MMS-treated cells

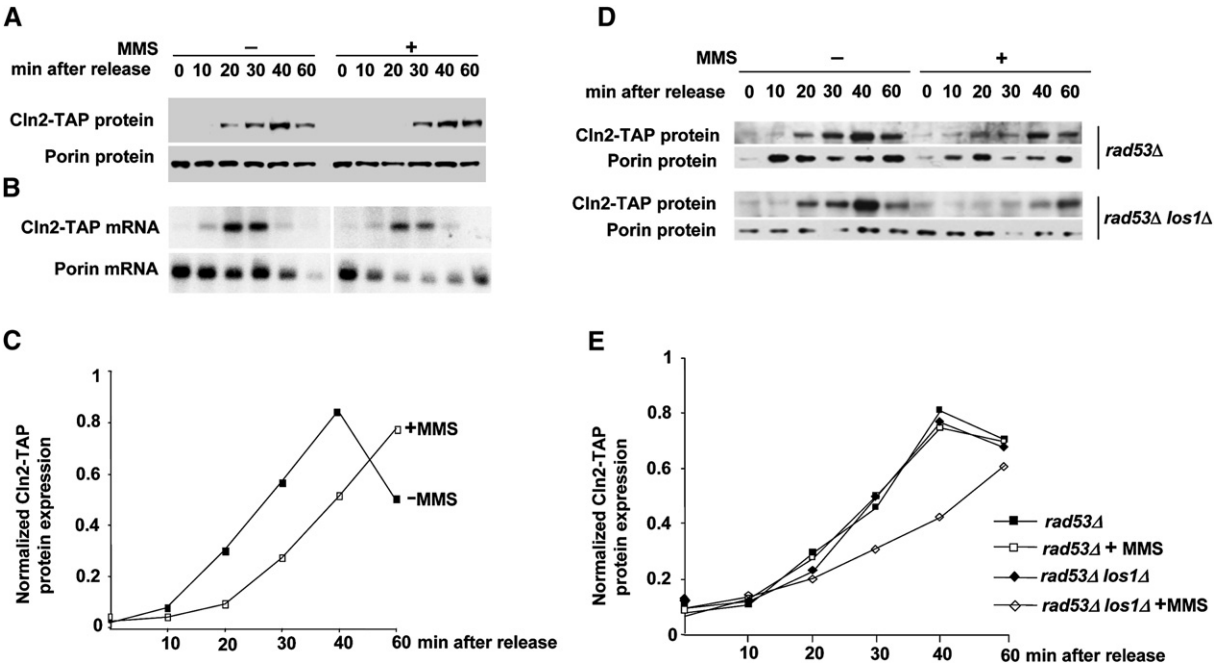

Figure 6. Impaired tRNA Export Contributes to G1 Checkpoint by Delaying Accumulation of Cln2

(A) Time course analysis of Cln2-TAP accumulation during cell-cycle recovery of wild-type cells after synchronization with α-factor, with or without exposure to MMS. Porin served as a loading control.

(B) Cln2-TAP and Porin mRNA levels prepared from cells in (A).

(C) Quantification of Cln2-TAP protein, normalized to its cognate mRNA, in unperturbed or MMS-treated cells as depicted in (A) and (B) (see text for detail).

(D) Cln2-TAP protein levels in control and MMS-treated *rad53Δ* or *rad53Δ los1Δ* mutants.

(E) Quantification of Cln2-TAP protein levels depicted in (D).

(Figure S6, quantifications in the lower panel). These results argue for a measure of specificity in the targeted perturbation in Cln2 protein translation after DNA damage.

The delayed accumulation of Cln2 in MMS-treated wild-type cells was highly concordant with the transient G1 arrest in these cells (Figure 5B, lanes 1and 2). Since *rad53Δ* cells do not arrest in G1 after MMS treatment (Figure 5B, cf. lanes 2 and 6), we reasoned that aberrant Cln2 accumulation may underlie the failure to execute a G1 checkpoint in these mutants. We therefore examined Cln2-TAP protein accumulation in untreated and MMS-treated *rad53Δ* cells after recovery from α-factor arrest.

In contrast to the wild-type cells, Cln2-TAP accumulated in MMS-treated *rad53Δ* cells with a kinetic profile indistinguishable from that observed in untreated cells (Figure 6D). Since Cln2 accumulation was comparable in the wild-type and the *rad53* mutant during unperturbed growth, intact Rad53 signaling was specifically required for the delayed accumulation of Cln2-TAP after DNA damage (Figure 6D, quantification in Figure 6E). Remarkably, deletion of *LOS1* in a *rad53* null mutant delayed Cln2-TAP accumulation after MMS (Figure 6D, quantification in Figure 6E), while restoring the defective G1 checkpoint in these cells (Figure 5B, cf. lanes 6 and 8). Collectively, these data provide evidence that Rad53-dependent regulation of tRNA export plays a key role in executing the G1 checkpoint by delaying the accumulation of the key cell-cycle regulator Cln2.

Impaired tRNA Export Contributes to G1 Checkpoint via Activation of GCN4

We sought to examine how the attenuated nuclear export of intron-containing tRNA contributes to the delayed accumulation of Cln2 and the subsequent G1 arrest. Since the delay in Cln2 accumulation is primarily at the translation level, we reasoned that the defect in tRNA export leads to impaired translation by lowering the abundance of the mature tRNA pool available to the protein-synthesis machinery. Quantitative northern blot, however, revealed that the defect in flow of the intron-containing transcripts form the nucleus after UV or MMS treatment had no measurable impact on the level of cognate mature tRNAs (Figure S7B), a likely reflection of an inordinately long tRNA half-life (Colby et al., 1981).

Physiological perturbations, such as nutritional or genotoxic stress, induce translational derepression of the ubiquitous transcription factor Gcn4, a response conserved from yeast to mammals (Dever et al., 1993; Engelberg et al., 1994). Gcn4 activation reduces the rate of general protein synthesis while simultaneously increasing the expression of proteins whose functions are required under conditions of starvation or stress (reviewed in Hinnebusch

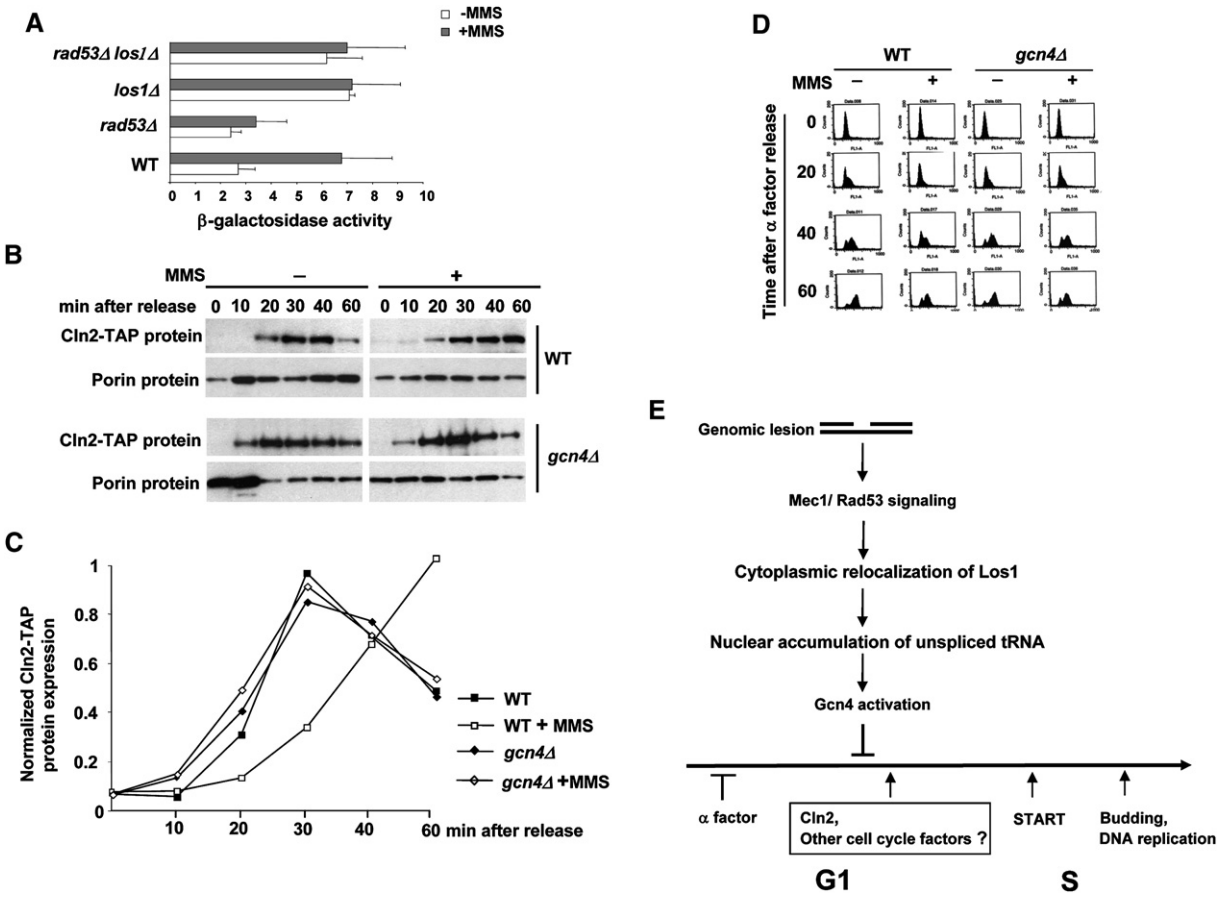

Figure 7. Impaired tRNA Export Signals MMS-Induced G1 Arrest by Activating Gcn4

(A) β-galactosidase activity in strains that harbor a *GCN4-lacZ* reporter plasmid. Logarithmically growing cells, grown in Ura– media, were treated with 0.04% MMS for 80 min prior to being processed for β-galactosidase activity, denoted on the x axis (Miller units*10^{-1}).

(B and C) (B) Time course analysis of Cln2-TAP accumulation during recovery of a wild-type and an isogenic *gcn4Δ* strain after synchronization with α-factor, with or without exposure to MMS. Porin served as a loading control for quantification in (C).

(D) Cell-cycle progression in MMS-treated *gcn4Δ* mutants. Logarithmically growing cells were synchronized in G1 by α-factor and were treated with MMS or left untreated. These cells were subsequently released into fresh media, and their progression through the cell cycle was monitored at the indicated time points by FACS.

(E) A working model for regulated nucleocytoplasmic trafficking of tRNA after DNA damage. In cells with DNA damage, a Mec1/Rad53-dependent signaling pathway, via yet to be identified downstream effector(s), impinges on the export process via differential relocalization of Los1 to the cytoplasm. The ensuing nuclear accumulation of tRNA signals activation of Gcn4, which, in turn, contributes to the execution of the G1 checkpoint by delaying the accumulation of cyclin Cln2, and likely other key regulators of G1 progression.

and Natarajan, 2002). Remarkably, aberrant tRNA accumulation in the nucleus in mutants with defects in tRNA processing or export also leads to translational derepression of Gcn4 in the cytoplasm (Qiu et al., 2000). While the intermediary components of this response remain unknown, it is postulated that it serves as a nuclear surveillance system that detects defects in tRNA biogenesis and elicits a Gcn4-dependent reduction in the initiation step of protein translation. In particular, deletion of *LOS1* leads to constitutive activation of Gcn4 (Qiu et al., 2000). Hence, we investigated whether the less pronounced accumulation of intron-containing tRNA in nuclei of cells with DNA damage similarly induces activation of Gcn4.

We utilized a widely employed CEN-based reporter plasmid, carrying a *GCN4* promoter driving the expression of a *lacZ* gene (*GCN4-lacZ*), that provided a quantitative measure of Gcn4 activity in a β-galactosidase assay (Dever et al., 1992). Wild-type cells that harbor this heterologous plasmid displayed a basal β-galactosidase activity in vivo (Figure 7A). MMS treatment led to the expected Gcn4 activation, reflected in the increase in β-galactosidase activity (Figure 7A). The nearly 3-fold MMS-induced increase in plasmid-borne *lacZ* expression was in line with that in cells that harbor a genomic copy of *GCN4-lacZ* (Natarajan et al., 2001).

While *rad53Δ* mutants exhibited a level of β-galactosidase activity comparable to that of the wild-type in

unperturbed growth, they did not display the MMS-induced increase in β-galactosidase activity normally seen in wild-type cells (Figure 7A). Parenthetically, the requirement for Rad53 is a salient feature that distinguishes the MMS-induced response, with respect to upstream signaling, from a general stress response (Figure S8). Moreover, deletion of *LOS1* led to the constitutive activation of Gcn4 and concomitantly abrogated an additional MMS-induced increase in β-galactosidase activity (Figure 7A). These results illustrate that Gcn4 activation after MMS treatment occurred via a regulatory network that minimally comprised Rad53 signaling and Los1 as a downstream effector. It follows that, as with the gross defect in tRNA export in a *los1* null mutant, the more subtle perturbations in tRNA trafficking also autonomously signaled Gcn4 activation after DNA damage.

We next examined how the Rad53-mediated activation of Gcn4 contributes to maintaining homeostasis in cells with DNA damage. As a downstream effector of Rad53, deletion of *GCN4*, similar to that in a *rad53* null mutant (Figure 6D), is expected to result in premature accumulation of Cln2 after MMS treatment, To examine this idea, wild-type and an isogenic *gcn4Δ* mutant that harbor a TAP-tagged allele of Cln2 were synchronized in G1 by α-factor treatment and subsequently treated with MMS prior to release from G1 arrest. While untreated wild-type and *gcn4* strains accumulated Cln2-TAP at comparable rates, accumulation of Cln2-TAP in MMS-treated *gcn4Δ* mutants followed a considerably faster kinetic relative to that of the wild-type cells (Figure 7B, quantification in Figure 7C). In line with their premature accumulation of Cln2-TAP, *gcn4Δ* cells also failed to execute a G1 checkpoint (Figure 7D), thereby providing an explanation for their enhanced MMS sensitivity (Begley et al., 2004). These data illustrate that deletion of *GCN4* phenocopies the G1 checkpoint deficiency normally observed in a *rad53* mutant, consistent with its role as a downstream component of DNA-damage signaling.

DISCUSSION

As essential components of the protein translation machinery, tRNAs function as fundamental regulators of cell growth and proliferation. The primary transcripts of the noncoding tRNAs are subject to extensive processing, which belies their apparent functional simplicity. While the core components of the processing machineries are well defined, the potential for selective regulation of tRNA processing in response to biological cues remains largely unexplored.

Here, we have shown that in budding yeast downregulation of tRNA export constitutes a previously unanticipated regulatory mode that contributes to the proper execution of the G1 checkpoint. Mechanistically, this response is mediated via Rad53-dependent differential localization of the Los1 export factor to the cytoplasm in cells with DNA damage (Figure 4E). This is a compelling example of a regulatory mechanism that elicits a robust cellular response by impinging on Los1, a limiting component of the tRNA export (Figure S4). The resulting nuclear accumulation of unspliced tRNA signals Gcn4 activation and the subsequent execution of the G1 checkpoint. Failure to accumulate tRNA after genotoxic stress in *rad53* mutants (Figure 3C) contributes to their deficiency in the G1 checkpoint. Conversely, deletion of *LOS1* restores the G1 checkpoint in a *rad53* mutant (Figure 5B, lanes 6 and 8) because it autonomously signals the activation of Gcn4 (Figure 7A). These data collectively illustrate that the tRNA export process serves as an entry point for Rad53 signaling, which serves to transduce information about the integrity of the genome to the protein-synthesis machinery in the cytoplasm (Figure 7E). Consistent with its physiological significance, failure to execute this response results in elevated loss of viability after DNA damage (Figure 5C).

Large-scale studies with synthetic genetic arrays (Tong et al., 2004) and proteomics approaches (Gavin et al., 2006) have proven highly informative in in vivo mapping of the components of distinct functional modules in yeast. However, the potential for concerted regulation of these modules via their mutual functional interplay remains largely unknown because these qualitative data primarily portray static pictures of the cellular protein network topology. The data presented here illustrate a remarkable signal-mediated crosstalk between distinct functional modules, namely, tRNA nucleocytoplasmic trafficking, protein synthesis, and checkpoint execution. It highlights an interconnecting pathway that contributes to maintaining homeostasis via subtly quantifying the output of these functional modules. This regulatory network allows for functional coupling of tRNA biogenesis and cell-cycle progression, an observation in agreement with the tight correlation between proliferation rate and cellular protein synthesis capacity (Niwa and Walter, 2000).

The aberrant tRNA accumulation in the nucleus in cells with DNA damage temporally precedes and autonomously signals G1 arrest via activation of the ubiquitous transcription factor Gcn4 (Figure 7A). Expression profiling with cDNA microarray has revealed that Gcn4 activation is accompanied by wide-spread reorganization of the cellular transcriptome (Hinnebusch and Natarajan, 2002). The remodeling of the cellular transcriptional outlook by Gcn4 likely underlies its function in the G1 checkpoint. Genes encoding ribosomal proteins and general translation factors constitute the largest functional cluster among the genes repressed by Gcn4 (Natarajan et al., 2001). This partly accounts for the robust reduction in global protein translation in cells with DNA damage. Recovery from a DNA-damage-induced G1 arrest, unlike resumption of the cell cycle after α-factor arrest, requires de novo protein translation (Siede et al., 1994). The Gcn4-mediated decrease in the output of the protein-synthesis machinery provides a rationale for the differential requirement for Gcn4 in the G1 checkpoint (Figure 7D).

The temporal requirement for protein synthesis in G1 progression is largely due to a requirement for an efficient

rate of protein synthesis in translation of G1 cyclins (Polymenis and Schmidt, 1999). Delayed translation of Cln2 is correlated with a G1 arrest after MMS exposure in wild-type cells (Figure 6A). Conversely, aberrant accumulation of Cln2 is concomitant with premature G1 progression in MMS-treated null mutants of *RAD53* (Figure 5B) and its downstream effector *GCN4* (Figure 7D). Consistent with a role for translational regulation of Cln2 in executing the G1 checkpoint, reducing the rate of Cln2 protein accumulation, by deleting *LOS1*, restores MMS-induced G1 arrest (Figure 5B, lanes 6 and 8) and enhances viability in a checkpoint-defective *rad53* mutant (Figure 5C). These data collectively reiterate the fundamental role of delayed Cln2 translation in the execution of the G1 checkpoint and further provide a plausible mechanistic basis for its execution (Figure 7E). Arguing for the conservation of the Cln2 function in the G1 checkpoint in higher eukaryotes, premature G1 progression via deregulated expression of G1 cyclin E in human epithelial cells is accompanied by increased loss of genomic stability, an observation in line with the elevated expression of cyclin E in a host of human tumors (Spruck et al., 1999).

EXPERIMENTAL PROCEDURES

Yeast Strains

All *rad* mutant strains were haploid isogenic derivatives of the wild-typeYMP10860: ***MATa*** *ura3 leu2 trp1 his3 sml1* (Paulovich et al., 1997a). C-terminally TAP- and GFP-tagged fusion strains, all derivatives of S288C, were as described (Ghaemmaghami et al., 2003; Huh et al., 2003). Haploid yeast strains that harbor conditionally repressible *tet* alleles of the splicing endonucleases (Mnaimneh et al., 2004) were grown in YPD and were transferred to fresh YPD + 10 μg/ml doxycycline at 30°C for 12 hr in order to shut off expression from the *tet* promoters. *LOS1* was disrupted with a *CloneNAT*-selectable cassette by using a standard one-step integration procedure (Wach et al., 1994).

Induction of DNA Damage

Unless indicated otherwise, replicate cultures of log-phase cells in YPD media were treated with 0.04% MMS (Sigma Aldrich) for 80 min. For UV treatment, 30 ml cultures were grown to an A_{600} of 0.2 in minimal SD media and were irradiated for 60 s with a 254 nm germicidal UV lamp at the dose rate of 1 J/m^2/s while being stirred in a 15 cm Petri dish (Ghavidel and Schultz, 2001). Viability was determined by the number of colonies normalized to that observed with untreated controls after 3 days of incubation at 30°C. The CEN-based expression plasmid that harbors the restriction endonuclease EcoRI under a galactose-inducible *GAL* promoter was as described (Mills et al., 1999).

RNA Preparation and Analysis

Total yeast RNA were isolated and analyzed as described (Schmitt et al., 1990). Northern blot procedure, including the sequence for individual oligonucleotide probes used for detecting select tRNA species, are as reported (O'Connor and Peebles, 1991).

Protein Preparation and Analysis

Pelleted cells were disrupted with glass beads in lysis buffer (20 mM HEPES-KOH [pH 7.6], 120 mM ammonium sulfate, 1 mM EDTA, protease inhibitor cocktail [Roche], 2 mM DTT) with a single 30 s burst with a mini-bead beater (Biospec). After adding Triton X-100 to 0.25% (v/v), the lysate was incubated on ice for 30 min. Cellular debris was pelleted by microcentrifugation for 10 min. The protein supernatant was resolved in denaturing gels and examined by western blot essentially as described (Ghavidel and Schultz, 2001).

In Vitro tRNA Splicing Assay

The in vitro splicing assay, performed with a $tRNA^{Phe}$ substrate, was done essentially as described (Reyes and Abelson, 1987), with slight modifications as described in Supplemental Data.

Fluorescence Microscopy

The RNA in situ localization (FISH) was done as described (Sarkar and Hopper, 1998). The quantification scheme for tRNA nucleocytoplasmic abundance is described in detail in Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.cell.org/cgi/content/full/131/5/915/DC1/.

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