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# NEDDYLATION AFFECTS CELL CYCLE CONTROL AND GENOME INTEGRITY

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**ABSTRACT**

Neddylation is a process that similar to ubiquitylation results in covalent attachment of a small protein called Rub1 -homologue of human Nedd8- to targeted proteins. Importantly, this process is essential in the majority of eukaryotes with the exception of the budding yeast Saccharomyces cerevisiae. The disregulation of neddylation has been described in several cancers and neurodegenerative disorders. To date, only a limited number of Rub1/NEDD8 targets have been identified. Thus, how neddylation mechanistically affects cellular processes is largely unclear. Here, we found in yeast that components of the neddylation machinery cooperate with DNA damage checkpoint proteins to promote genome stability and protect cells against DNA damage. We further showed that neddylation facilitates G2/M progression in the presence of DNA damage induced by the topoisomerase-1 inhibitor camptothecin. Finally, we found that neddylation regulates the steady state levels of DNA damage response factors such as Mms22 and Nhp10, providing an explanation for how this process controls cell cycle progression.

## INTRODUCTION

Neddylation is a process by which the Rub1 protein (NEDD8 in humans) is conjugated to target proteins in a cascade of reactions that involves E1 activating, E2 conjugating (in S. cerevisiae only Ubc12) and E3 ligating enzymes in a manner analogous to ubiquitylation and SUMOylation [1]. Neddylation is an essential modification for cellular function in all eukaryotes, except in S. cerevisiae. Whereas ubiquitylation and SUMOylation have been shown to regulate a myriad of cellular processes, including DDR [2], those that involve neddylation remain largely unknown due to the limited number of neddylation substrates that have been identified [3]. The best-studied Rub1/Nedd8 targets are cullin-RING ubiquitin ligases (CRLs). The three yeast cullins Cdc53, Rtt101 and Cul3, as well as the eight mammalian cullins CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7 and Parc are neddylated in vivo [4, 5]. Cullin neddylation results in conformational changes that help to anchor the E2 ubiquitin-conjugating enzyme to the E3 ligase complex. This new complex conformation is thought to facilitate ubiquitin transfer to CRL substrates and stimulate CRL ubiquitylation activity [6]. Importantly, multiple CRL targets are key components of processes that have been found to be misregulated in several types of cancer. The DNA replication licensing factor Cdt-1 is an edifying example. Mis-regulated $CRL1^{Skp2}$/$CRL4^{Cdt2}$ results in Cdt-1 accumulation in several human tumors [7]. In addition, disruption of the adaptor protein Skp2 leads to high levels of cyclin E and cyclin-dependent kinase inhibitor p27, which gives rise to polyploid and poly-centromeric cancer cells [8].

Recent work suggests that proteins other than cullins can also be modified through neddylation. Xirodimas and coworkers first reported that the tumor suppressor p53 is ubiquitylated by the E3 ubiquitin ligase Mdm2 and targeted for proteasomal degradation in unperturbed cells [9]. However, upon cellular stress, p53 is stabilized and induces a transcriptional program that results in cell growth inhibition and apoptosis [10]. More recently, Mdm2 was found to be neddylated and required for p53 neddylation [11]. In both cases, neddylation promotes degradation of the targeted protein. Additionally, neddylated-forms of p53 were detected transiently in cells treated with UV, which shows that neddylation is a process that can be triggered by DNA damage [11]. Thus, it becomes apparent that neddylation can also directly affect the level of proteins, including those that are key factors involved in cellular stress responses.

Here, we found that defects in neddylation and in the DNA damage checkpoint have a synergistic effects on cell survival after induction of DNA damage and on genome stability maintenance. Additionally, we show that neddylation promotes G2/M transition in response to the topoisomerase 1-inhibitor camptothecin, which induces DNA damage during replication. Finally, we demonstrate that neddylation affects the levels of two DNA damage response factors, Mms22 and Nhp10. Collectively, our data suggest that neddylation plays a role in cell-cycle control by regulating the levels of particular DNA damage response factors.

## RESULTS & DISCUSSION

We performed a large-scale genetic interaction screen, called dE-MAP (for differential epistatic mapping), in the presence of three different DNA damaging compounds: the DNA alkylating agent methyl methanesulfonate (MMS), the topoisomerase-1 inhibitor camptothecin (CPT), and the radiomimetic antibiotic zeocin (ZEO). To identify which of the changes in genetic interactions between conditions were statistically significant, we used a previously published metric to assess the difference in genetic interaction scores (S score) for each gene pair before versus after treatment [12]. We call this network 'differential' genetic network as it is derived from the difference between two static networks (Chapter 1, Figure 1). We then examined the genes which were highly responding to the drugs. The gene with the greatest overall number of interactions was RAD17, a component of the 9-1-1 checkpoint complex which is recruited to double-stranded break (DSB) sites to activate the Mec1-kinase signaling cascade, resulting in cell cycle arrest and repair [13]. Consistent with the role of Rad17 in the DSB

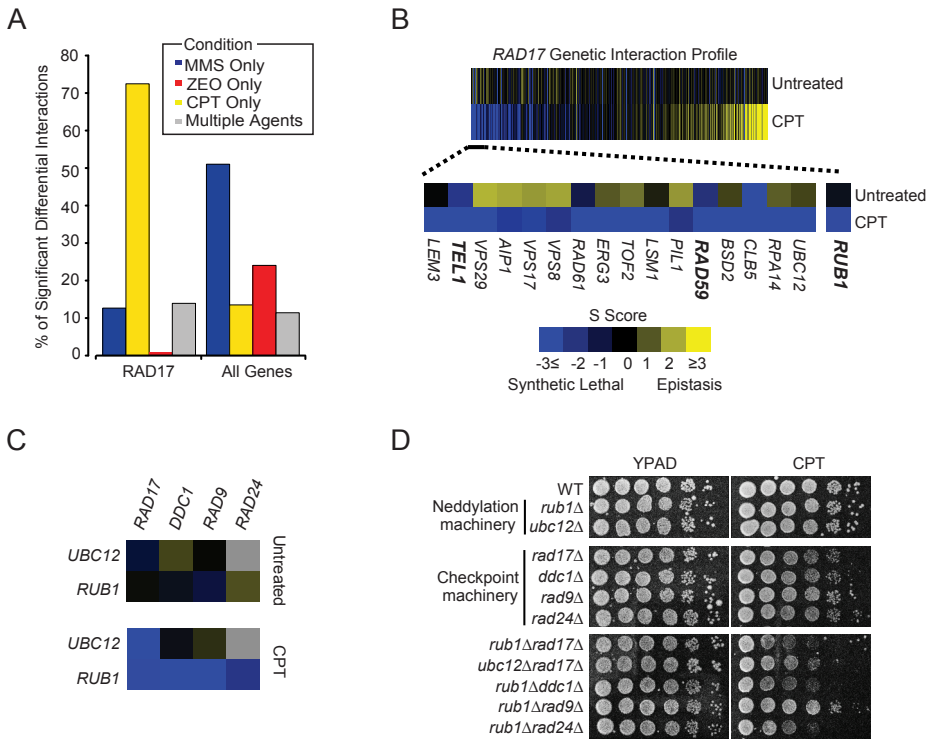


**Figure 1**. **Cells deficient for both neddylation and DNA damage checkpoints show reduced viability in the presence of CPT-induced DNA damage** (A) Percentage of RAD17's significant differential genetic interactions arising in response to MMS, CPT, ZEO, or multiple agents. As a control, the average percentage of significant differential interactions in each of these categories across all genes is shown. (B) Entire CPT-induced genetic interaction profile for RAD17 sorted (left to right) in order of most differential negative to most differential positive. A subset of the top differential negative interactions is also shown. (C) Genetic interactions between components of the neddylation machinery and the DNA damage checkpoint. (D) Viability of cells deficient for both neddylation and DNA damage checkpoints is strongly impaired in the presence of CPT. 10-fold serial dilutions of log-phase cells of the indicated genotypes were spotted onto YPAD and YPAD containing CPT (15 μM) and incubated for 3 days at 30°C.

response [14], we found that the majority of its interactions were induced specifically in response to CPT (73%, Figure 1A). To gain further insight into potential CPT-induced pathways involving the checkpoint, we examined the entire CPT-induced genetic interaction profile of RAD17 (Figure 1B), which revealed strong negative interactions with prominent DSB repair genes (RAD59) and checkpoint regulators, such as TEL1. This is consistent with reports showing that Tel1 functions parallel to Rad17 to regulate checkpoint activation following DSBs [15]. Two additional genes, RUB1 and UBC12, which encode key components of the yeast neddylation machinery, displayed strong negative interactions with RAD17 (Figure 1B). In further support of a potential link between neddylation and checkpoint pathways, the CPT network revealed a number of additional negative interactions between RUB1/UBC12 and other checkpoint genes, including DDC1, RAD9 and RAD24 (Figure 1C). These interactions were also observed via spot dilution assays, confirming that cells defective for neddylation and DNA damage checkpoints are hypersensitive to CPT (Figure 1D).

To investigate a role for the neddylation machinery in DNA damage checkpoint control, we assessed *rub1*Δ and *ubc12*Δ mutants for their progression through the cell cycle in the presence of CPT. After arrest in G1 and release into medium containing CPT, *rub1*Δ and *ubc12*Δ mutants had significant accumulation of cells in the G2 phase at 90 and 105 minutes, whereas wild-type cells efficiently progressed through G2 and M-phase into the next cell cycle (Figures 2A-B). As this delay was not observed in the absence of CPT (Figure 2C), we demonstrate for the first time that neddylation mutants display perturbations in cell cycle progression upon CPT treatment.

Since defects in cell cycle checkpoints have been shown to contribute to genome instability [16], we decided to measure the rate of gross chromosomal rearrangements (GCR) in the neddylation mutants. The assay utilized determines GCR rates by monitoring the loss of two counter-selectable markers, CAN1 and URA3, which are present on the left arm of the chromosome V (Figure 3A). The rate of GCR events in the *ubc12*Δ mutant was nearly 2.7-fold greater than in wild type, whereas the *rad17*Δ*ubc12*Δ double mutant showed, respectively, a 7- and 2-fold increase in GCR rates when compared to the *ubc12*Δ and *rad17*Δ mutants (Figure

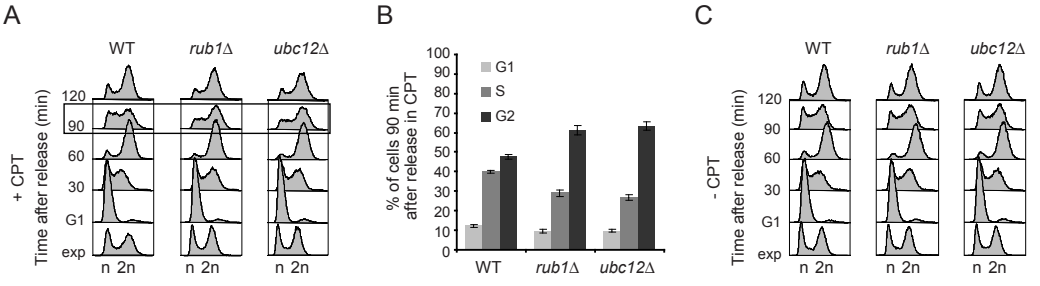


**Figure 2. Neddylation mutants show a delayed G2/M transition in the presence of CPT-induced lesions** (A) WT, *rub1*Δ, *ubc12*Δ cells were arrested in G1 with α-factor and released in S-phase in YPAD plus 50 μM CPT. (B) The percentage of cells in G1, S and G2 phases 90 minutes after release in CPT was determined. Data represent the mean ± standard deviation from 3 independent experiments. (C) as in (A) except that cells were release in YPAD. Aliquots were taken at the indicated time for FACS analysis.

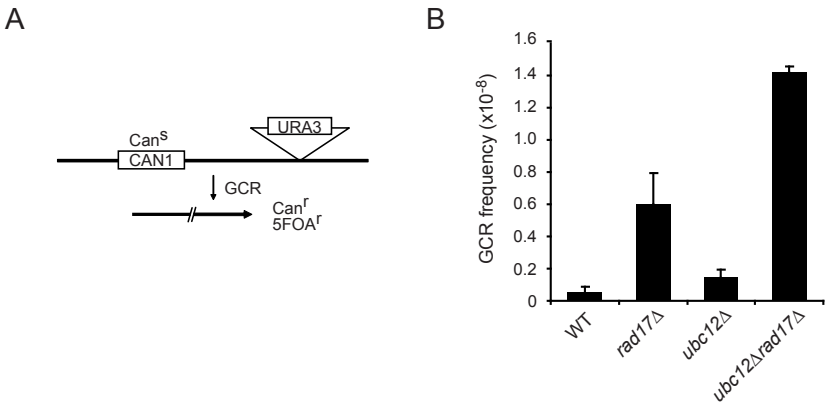


**Figure 3. Cells deficient for both neddylation and DNA checkpoints display increased Gross Chromosomal Rearrangements (GCR)**. (A) Scheme of the working principle of the GCR assay developed by Chen and Kolodner, 1999. (B) Cells deficient for both neddylation and DNA damage checkpoints have increased rates of Gross Chromosomal Rearrangements (GCR). GCR frequencies were determined as previously described in the Experimental Procedures. The mean ± standard deviation of three independent experiments is presented.

3B), suggesting that neddylation and checkpoint pathways are likely to cooperate in promoting genome stability.

We next asked whether the perturbations in cell cycle progression observed in the neddylation mutants were due to abnormal activation DNA damage checkpoints. It has been shown previously that CPT-induced DNA damage does not trigger activation of Rad53 [17]. It is not known whether Chk1 is also not activated under the same damaging condition. We found that wild-type cells neither showed Rad53 nor Chk1 activation upon release from G1 into CPT ([17] and Figure 4A-B). We then monitored the presence of phosphorylated forms of Rad53 and Chk1 in *rub1*Δ. Surprisingly, also in *rub1*Δ mutants Rad53 and Chk1 were not activated upon DNA damage induced by CPT (Figure 4A-B), which suggests that the G2 delay seen in these mutants is not the consequence of DNA damage checkpoint activation.

The best-studied NEDD8/Rub1 targets are cullin proteins, which are

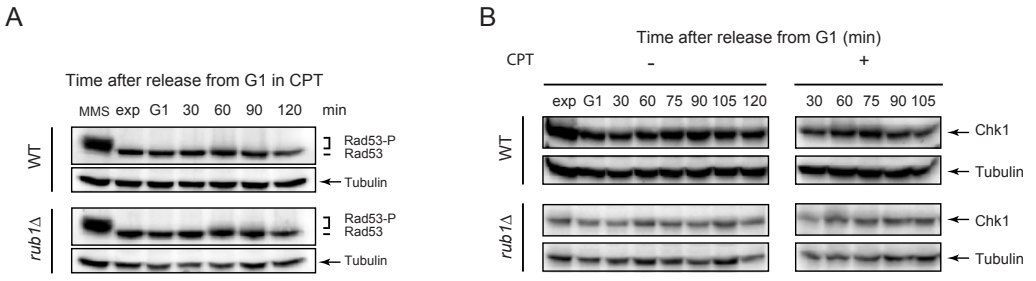


**Figure 4. Neddylation defects do not lead to DNA damage checkpoint activation.** Exponentially (exp) growing WT and *rub1*Δ cells were arrested in G1 with α-factor and released in fresh medium containing CPT (50 μM). MMS-treated exponentially growing cells served as a positive control. The phosphorylation status of (A) Rad53 and (B) Chk1 was monitored using Western blot analysis at the indicated time-points.

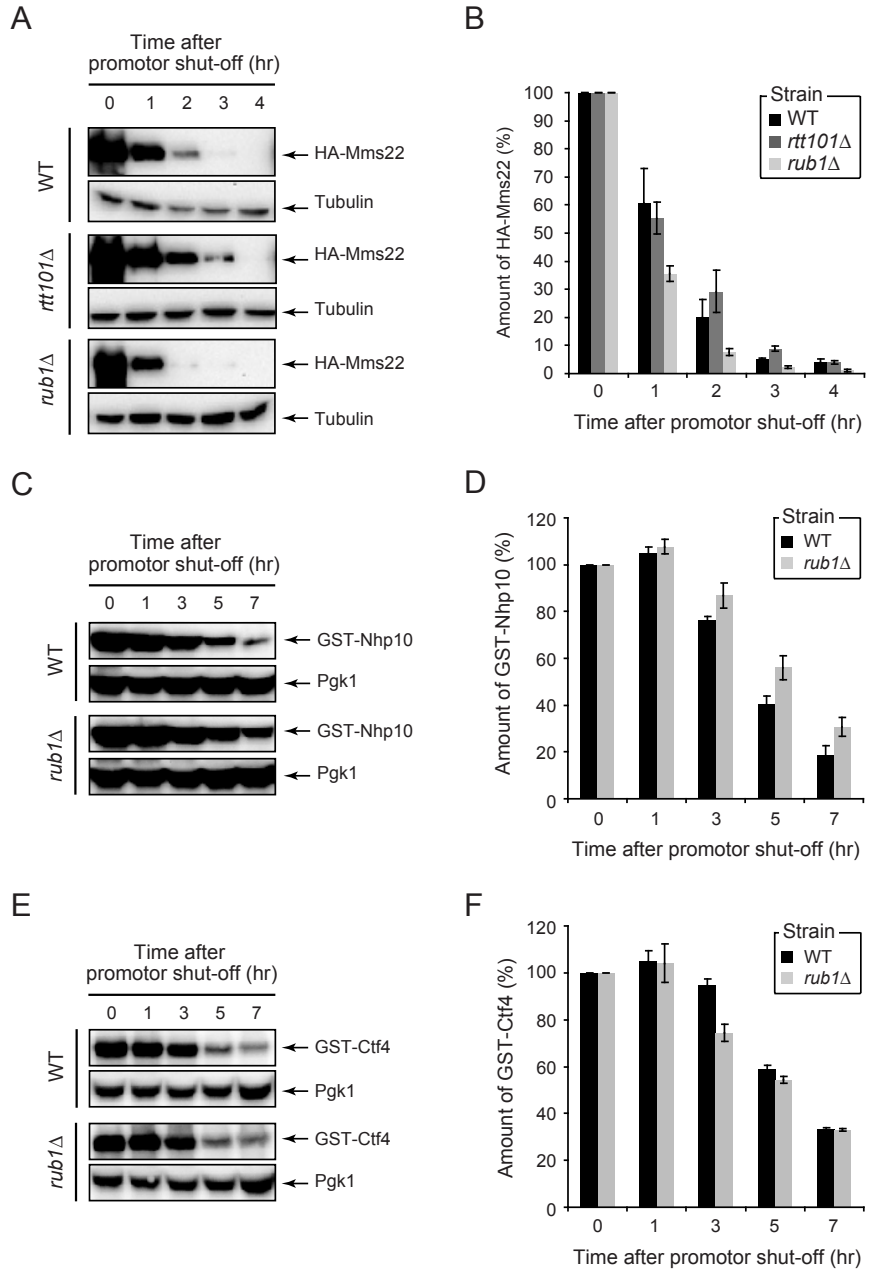


**Figure 5. Neddylation affects the turnover of the DNA damage response proteins Mms22 and Nhp10** (A) Expression of GAL1-HA-Mms22 was induced in WT, *rtt101*Δ and *rub1*Δ cells by growing the cells in 2% galactose for 3 hours. Cells were released in 2% glucose to shut-off expression of HA-Mms22, after which levels of HA-Mms22 were monitored by Western blot analysis. (B) Bar-plot showing the rate of HA-Mms22 protein degradation in WT, *rtt101*Δ and *rub1*Δ cells. The levels of HA-Mms22 protein were quantified and normalized to tubulin. The ratio at the start of shut-off was set to 100%. (C) as in (A) except that the expression of GAL1-GST-Nhp10 was monitored. (D) as in (B) except that the rate of GST-Nhp10 protein degradation is shown. The levels of GST-Nhp10 protein were quantified and normalized to Pgk1. In (E) as in (C) except that the expression of GAL1-GST-Ctf4 was monitored. (F) as in (D) except that the rate of GST-Ctf4 protein degradation is shown. The mean ± standard deviation of three to four independent experiments is presented.

scaffolds for the assembly of multi-subunit cullin-RING ubiquitin ligases (CRLs) [1, 4]. Interestingly, the yeast cullin Rtt101 has been shown to play a critical role in regulating the G2/M checkpoint by promoting proteasomal degradation of Mms22 [18]. Given the role of neddylation in CRL modification, we examined whether this process would affect the steady state levels of Mms22. We observed a faster degradation of Mms22 in a *rub1*Δ strain when compared to wild-type, suggesting that neddylation, in contrast to Rtt101-dependent ubiquitylation [18], promotes Mms22 stability (Figure 5A-B).

As another means of identifying potential DDR factors whose stability might be modulated by the neddylation machinery we examined the set of positive genetic interactions containing RUB1 in our CPT network, as previous work suggested that linear signal transduction pathways are often enriched for positive genetic interaction [19, 20]. The highest positive interaction exhibited by RUB1 in response to CPT was with NHP10 ($P < 7.8x10-8$), a component of the INO80 chromatin remodeling complex with known roles in DNA repair and cell cycle control [21, 22]. In contrast to the faster turnover of Mms22, we found that Nhp10 degradation was slower in the rub1Δ strain compared to wild-type (Figure 5C-D). As a negative control, we selected the sister-chromatids cohesion factor, Ctf4, which displayed a very weak differential positive interaction with RUB1 in the CPT network, and found that the steady-state levels of this protein were not altered in a *rub1*Δ strain (Figure 5E-F). Taken together, these data implicate the neddylation machinery as a novel factor that regulates cell cycle progression in response to DNA damage and contributes to genome stability, most likely by regulating the steady state levels of DDR factors such as Mms22 and Nhp10.

While CRLs are the most well studied Rub1 substrates to-date, emerging evidence suggests that many other proteins may be modified by neddylation [3]. We infer from this that the stability of DDR factors such as Mms22 or Nhp10 may be regulated either by direct neddylation, or indirectly by the neddylation of E3 ubiquitin ligases or CRLs (Figure 6).

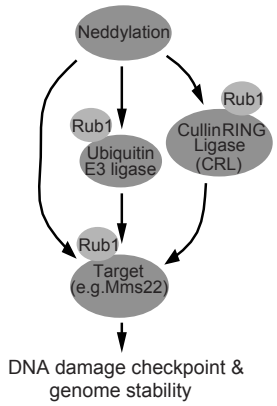


**Figure 6. Schematic illustrating mechanisms by which the neddylation machinery may regulate cell cycle progression and genome stability.** See text for details.

## CONCLUSION

Here we provide an intriguing connection between neddylation, control of the steady state levels of DDR factors, and cell cycle regulation. However, how regulation of Mms22 or Nhp10 by the neddylation pathway affects cell cycle progression is not clear. Nhp10 is a subunit of the chromatin remodeling INO80 complex, which has been found to associate with origins of replication [23, 24]. Vincent et al. showed that nhp10Δ mutants were less efficient in

replicating late regions of the genome after MMS, which means that under replication stress the presence of Nhp10 on DNA might be prolonged till late S beginning of G2 phase. As we observed that Rub1 promotes Nhp10 degradation, we envisage that neddylation is critical for the timely removal of Nhp10 from the DNA when replication stress has been overcome, allowing cells to progress through mitosis. Support for such a scenario also comes from work of Ben-Aroya et al., who showed that Mms22 is recruited to damaged chromatin but needs to be removed after repair in order to allow cells to enter mitosis [18]. However, to our surprise we found that instead of promoting degradation of Mms22, neddylation is required for its stabilization. Since neddylation has been mainly associated with protein degradation, it is tempting to propose that the regulation of Mms22 by Rub1 occurs in an indirect manner. The CRL Rtt101 has been proposed to target Mms22 for proteasomal degradation [18]. However, data suggest that Rub1 does not affect Rtt101 activity [25]. Thus, Rub1 may promote the degradation of another factor than Rtt101 that directly mediates Mms22 turnover. In line with this idea, the E3 ligase Mdm2 is targeted for degradation upon neddylation, which leads to the stabilization of its main target p53 [11].



Preliminary data suggest that Mms22 and Nhp10 may not be the only factors that are regulated by neddylation. Pds1 secures the attachment of sister-chromatids after DNA replication and becomes degraded by the anaphase-promoting complex (APC) to ensure separation of sister-chromatids before entry into mitosis. We hypothesized that Pds1 is another factor whose levels could be affected by neddylation. Indeed, we observed a slight delay in degradation of the anaphase inhibitor Pds1 in *rub1*Δ cells synchronized in G1 and released in CPT (data not shown). This suggests that Rub1-mediated degradation of Pds1 may prevent prolonged cell cycle arrest in G2/M. In addition, we found that the Cdk1 inhibitor SIC1 displays a high positive interaction with RUB1 after CPT treatment. Sic1 inhibition of Cdk1 is necessary for cells to exit mitosis [26]. We could envisage that neddylated-Sic1 is degraded after CPT to prevent premature entry to mitosis. Thus, defects in neddylation would induce mitosis. However, we observed the opposite effect in *rub1*Δ cells, suggesting that the G2/M delay observed in CPT-treated *rub1*Δ cells is most likely the combined effect of changes in the steady state levels of several factors, which reveals a novel complex regulatory mechanism for cell cycle control in response to DNA damage.

It is important to note that such a mechanism could go unnoticed in unperturbed cells, yet becomes critical upon cellular stresses. In agreement with this, the MLN4924 inhibitor of the NEDD8 activating enzyme (NAE1) was found to sensitize cancer cells to ionizing radiation (IR) treatment [27]. Mechanistically, it was shown that CRL targets such as the cell cycle regulators p21, p27, Wee1 or Cdt1 are stabilized upon NAE1 inhibition and that IR treatment further enhances this stabilization effect. Knockdown of CDT1 or WEE1 in MLN4924 treated cells rescues the enhanced sensitivity to IR, suggesting that it is the accumulation of cell cycle regulators upon inhibition of neddylation that causes the hypersensitivity of cancer cells to IR.

Neddylation belongs to a group of processes that regulate protein stability such as sumoylation or ubiquitylation, which appear to interact with each other. The Mdm2 E3 ligase is able to both ubiquitylate and neddylate p53 [9, 11]. Moreover, the cullin Rtt101 was found to be ubiquitylated and neddylated on the same lysine K491 [25]. Finally, Xirodimas et al. showed that p53 is differently modified when cells

are exposed to UV. Both neddylated and ubiquitylated forms of p53 appear in unperturbed cells whereas only neddylated forms were present transiently 4hr after UV treatment. Collectively, the work strongly suggests that a tight regulation of these distinct protein modifications is important for the regulation of cellular processes, including those involved in stress responses. Future work will however be required to understand how these modifications are regulated and if they compete or cooperate with other posttranslational modifications such as those induced by sumoylation or phosphorylation.

**MATERIAL & METHODS**

*DNA damage sensitivity assays*

Overnight cultures were diluted 1:20, grown for 3 h at 30 °C and diluted to $1x10^7$ cells/ml. Fivefold dilution series were spotted on plates containing 15 μM CPT and grown at 30 °C for 3 days.

*Cell Cycle Profiling*

Exponentially growing cells were synchronized in G1 with α-factor (7.5μM) and released in the presence or not of 50 μM CPT. Samples were taken every 30 min for 2h. Cells were stained with propidium iodide. Flow cytometry analysis was performed on a BD™ LSRII instrument. BD FACSDiva™ software was used for data analysis.

*GCR assay*

The gross chromosomal rearrangement assay was done according to a previously published protocol [28]. Briefly, cells were grown overnight in YPAD to a density of $2\text{-}5x10^9$ cells/ml. Cells were then spread on SC-Arg plates containing canavanine (60μg/ml) and 5-FOA (0.1%). A fraction of the cells was spread on YPAD to determine the plating efficiency. GCR rates were determined by scoring $Can^r$-$FOA^r$ colonies after loss of URA3 and CAN1 genes on chromosome 5 relatively to the total number of colonies scored on YPAD. Values reported are from three different experiments, which were each started using five independent colonies per strain.

*Rad53 and Chk1 western blot analysis*

Exponentially growing cells were synchronized in G1 with α-factor (7.5μM) and in the presence or not of 50 μM CPT. Whole cell extracts were prepared for western blot analysis to examine Rad53 and Chk1 phosphorylation. Anti-Rad53 (Santa-Cruz, sc-6749), anti-HA (Santa Cruz, sc-7392) and anti-Tubulin (Sigma T6199; Clone DM1A) antibodies were used.

*Mms22, Nhp10 and Ctf4 turnover*

Mms22, Nhp10 and Ctf4 turnover were examined as previously described [18] using cells expressing GAL1-HA-Mms22 [18], pGAL1-GST-NHP10 (Open Biosystems) or pGAL1-GST-NHP10 (Open Biosystems). Briefly, cells were grown to a density of $5x10^6$ cells/ml after which galactose was added to a final concentration of 2%. Cells were then grown for an additional 3h. Next, cells were washed and incubated in YPLGg + 2% glucose or SC-URA for the rest of the experiment to shut down the expression of HA-Mms22, GST-Nhp10 or GST-Ctf4. Samples were taken every hour for 7h after glucose addition after which whole cell extracts were prepared for western blot analysis to examine the HA-Mms22, GST-Nhp10 and GST-Ctf4 levels. Anti-HA (Santa Cruz Biotechnology, SC-7392), anti-Tubulin (Sigma T6199; Clone DM1A), anti-GST (Amersham) and anti-Pgk1 (Invitrogen) antibodies were used.

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