Dissection of DNA damage responses using multiconditional genetic interaction maps

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IRC21 IS A GENERAL RESPONSE FACTOR IN CHECKPOINT CONTROL, REPAIR AND GENOME STABILITY

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ABSTRACT

Due to multiple forms of genotoxic attacks, cells accumulate a large variety of DNA lesions. To protect their genome, cells use molecular pathways that signal and repair the lesions. Collectively, these pathways compose the DNA damage response. How these pathways are orchestrated and connected to enable an appropriate cellular response to the distinct type of DNA lesions that are encountered still remains to be discovered. We used a genome-wide genetic approach called EMAP as a mean to dissect the responses to different types of DNA lesions induced either by the alkylating agent methyl methanesulfonate (MMS) or the topoisomerase-1 inhibitor camptothecin (CPT) and the DNA intercalating agent zeocin (ZEO). Although we observed clear drug-specific responses, the limited overlap between them was significant enough to define a common DNA damage response network. In that conserved space, we not only identified genetic interactions for known DNA damage response factors but also for several poorly characterized genes. Here, we describe that loss of one of these genes, called IRC21, alleviates several DNA damage response defects observed in DNA damage checkpoint deficient cells, including DNA damage sensitivity, impaired checkpoint activation and DNA repair, as well as genome instability. Thus, we identify Irc21 as a novel factor involved in regulating DNA damage responses and genome stability maintenance.

INTRODUCTION

Cells are under constant threat by endogenous and exogenous factors that induce multiple types of DNA damage. To protect against the deleterious effects that DNA damage can have on genome integrity, cells use a combination of pathways that signal and repair these lesions, collectively referred to as DNA damage responses (DDR). These responses to DNA damage typically involve a detection step that initiates signaling cascades that in turn coordinate several processes including cell cycle progression and DNA repair. However, how the pathways and factors involved in the DDR are orchestrated to allow cells to respond properly to different types of DNA lesions remained largely unclear.

In yeast, recent developments of high throughput genetic screens have enabled the dissection of pathways involved in cell organization. However, the genetic understanding of cellular functions has come mainly from static observations where cells were studied under a single standard condition [1, 2]. Recently, Bandyopadhyay et al. have developed a technology called differential epistatic mapping (dE-MAP), which allows to address the dynamic aspect of the cellular response to a perturbation [3]. In order to understand the genetic interaction changes that occur upon induction of different types of DNA damage, we previously generated d-EMAPs after exposure of cells to three different DNA genotoxic drugs: the alkylating agent methyl methanesulfonate (MMS), the topoisomerase-1 inhibitor camptothecin (CPT) and the DNA intercalating agent zeocin (ZEO). The genetic networks induced by each drug were very specific, which suggests that a unique set of DNA damage response pathways is triggered depending on the type of DNA damage that is induced. However, we also found a significant number of genetic changes that were induced by at least two of the genotoxic compounds. We called this overlap between the genetic networks the common DDR network. Indeed, in that common space, the response involves the same genes irrespective of the type of DNA damage induced. Importantly, the common network not only included several known DDR factors, but also revealed unanticipated and poorly characterized genes such as IRC21.

Irc21 was previously identified in a genome-wide study in which mutants were screened for their ability to form spontaneous Rad52 foci. In that screen, deletion of IRC21 led to an increase in spontaneous Rad52 foci, thus the gene was called Increased Recombination Center 21, IRC21 [4]. In another genome-wide screen, *irc21*Δ mutants were found to be resistant to cisplatin and carboplatin [5]. Although this work may implicate a role for Irc21 in the DDR, a precise understanding of its function this response is lacking. Here, we demonstrate that Irc21 is an important novel DDR factor that affects cell cycle regulation, DNA damage repair and genome stability maintenance.

RESULTS & DISCUSSION

We used a recently-developed technology to analyze epistasis between pairs of genes in yeast (EMAP) and investigate the genetic changes induced by three different DNA damaging compounds: methyl methanesulfonate (MMS), camptothecin (CPT) and zeocin (ZEO). To center our study, we employed 55 query genes, all involved in different aspects of the DDR. The queries were crossed against an array of 2022 genes, implicated in the DDR as well as in processes, such as cell cycle regulation, chromatin organization, replication, transcription, and protein transport. The mated strains passed through several selection steps, which resulted in a collection of haploid double mutant strains. All these mutants were left untreated or exposed to MMS, CPT or ZEO and their growth rates were measured 48h later. Eventually, genetic interaction scores were obtained by normalization and statistical analysis of the measured colony sizes (Chapter 1).

To precisely highlight the interactions that changed in response to DNA damage, we made use of a metric previously developed to measure the difference between the genetic score in untreated versus treated conditions [3]. The resulting score is called a differential interaction score. The analysis of our differential genetic networks showed that while the interactions induced by the three agents were largely divergent, it did implicate a "common" network of 584 interactions that were altered in response to at least two agents (Figure 1A). Many known DNA repair factors were highly connected within this network including DSB repair factors (RAD52, SAE2, MRE11, RAD59), post replication repair (PRR) genes (RAD18), and chromatin remodelers (SWR1) which have well-documented roles in the DDR [6, 7]. In particular, our analysis highlighted the damage checkpoint gene RAD17 as a hub not only of the CPT network (see above), but also of conserved interactions across agents (Figure 1A, top inset). These included a differential positive interaction with IRC21, an as yet uncharacterized gene, in response to both CPT (differential $P = 4.7x10^{-7}$) and MMS ($P = 8.3x10^{-7}$), but not ZEO ($P = 0.53$). We confirmed that Irc21 is expressed in vivo in yeast (Figure 1B), and that deletion of IRC21 in a *rad17*Δ mutant suppresses its sensitivity to CPT and MMS (Figure 1C). Importantly, this suppressive effect was also observed in other checkpoint mutants, including *ddc1*Δ (another mutant of the Rad17-Ddc1-Mec3 (9-1-1) complex) and *rad9*Δ (Figure 1D). Analysis of the Irc21 protein sequence revealed the presence of a cytochrome b5-like domain (Figure 1E), which is usually found in proteins that are involved in cytochrome P450-dependent metabolic processes [8]. To rule out that the suppression was due to Irc21 affecting drug metabolism via its cytochrome b5 domain, we exposed cells to ultraviolet light (UV) and ionizing radiation (IR) and were able to re-produce the suppressive phenotype in both cases (Figure 1F). Ectopic expression of Irc21 in the *rad17*Δ*irc21*Δ mutant restored the sensitivity to DNA damaging agents to that observed for the *rad17*Δ mutant (Figure 1B and F). These results suggest that Irc21 affects cell survival in response to genotoxic insult by modulating the DNA damage checkpoint rather than by affecting drug metabolism.

To further explore this possibility, we profiled *rad17*Δ, *irc21*Δ and *rad17*Δ*irc21*Δ mutants for their cell cycle progression in the presence of MMS. While the wild-type and *irc21*Δ strains displayed slow S-phase progression and accumulated in G2 two hours after release from G1, the checkpoint-deficient *rad17*Δ strain rapidly progressed through S-phase and accumulated in G2 within an hour (Figures 2A-B). Remarkably, deletion of IRC21 in the *rad17*Δ strain partially suppressed the checkpoint deficiency as we noted, from 60 to 120

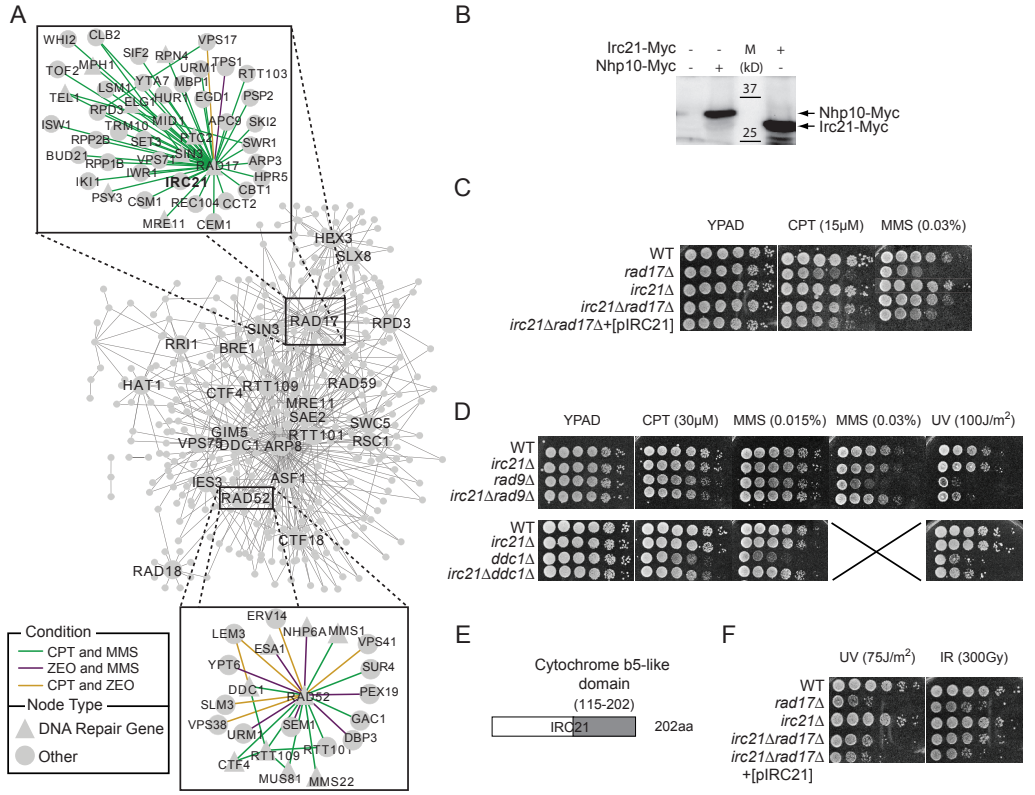

Figure 1. **Irc21 loss alleviates the sensitivity of rad17Δ cells to DNA damage** (A) Network of all 584 differential genetic interactions induced by at least two agents. The top 25 hubs in this network have been labeled. The sub-networks of interactions involving RAD17 and RAD52 are also shown. (B) Western blot analysis of cells expressing Myc-tagged Irc21. Cells from non-tagged and Nhp10-Myc expressing strains were used as negative and positive controls, respectively. (C) Effect of IRC21 deletion on the viability of *rad17*Δ cells. 10-fold serial dilutions of log-phase cells of the indicated genotypes were either spotted onto YPAD plates containing MMS or CPT. (D) as in C, except that *rad9*Δ and *ddc1*Δ cells were used and that cells were also spotted on YPAD and exposed to UV. (E) Schematic of the Irc21 protein showing a putative cytochrome b5-like domain in its C-terminus. (F) as in (C) except cells were exposed to UV and IR.

minutes after release in MMS, an increased fraction of cells remaining in S-phase (20.7% versus 10.7% at two hours after release; Figure 2B). Moreover, we observed that the *rad17*Δ mutant failed to activate the central checkpoint kinase Rad53, denoted by the absence of phosphorylated forms of Rad53 (Figure 2C). However the *rad17*Δ*irc21*Δ double mutant displayed a moderate restoration of this phenotype with Rad53 becoming slightly phosphorylated already 60 minutes after release from G1 arrest in MMS-containing media (Figure 2C).

Checkpoint proteins detect DNA lesions, arrest the cell cycle and trigger DNA repair [6, 9]. Given that Irc21 modulates the DNA damage checkpoint, we examined whether it also functions in DNA damage repair. Rad52 is a key repair protein in yeast that is not only involved in the response to stalled or collapsed replication forks in S-phase, but also facilitates the repair of DSBs and single-stranded gaps [4]. It has been shown to accumulate into DNA damage-induced subnuclear foci that are thought to represent active repair centers

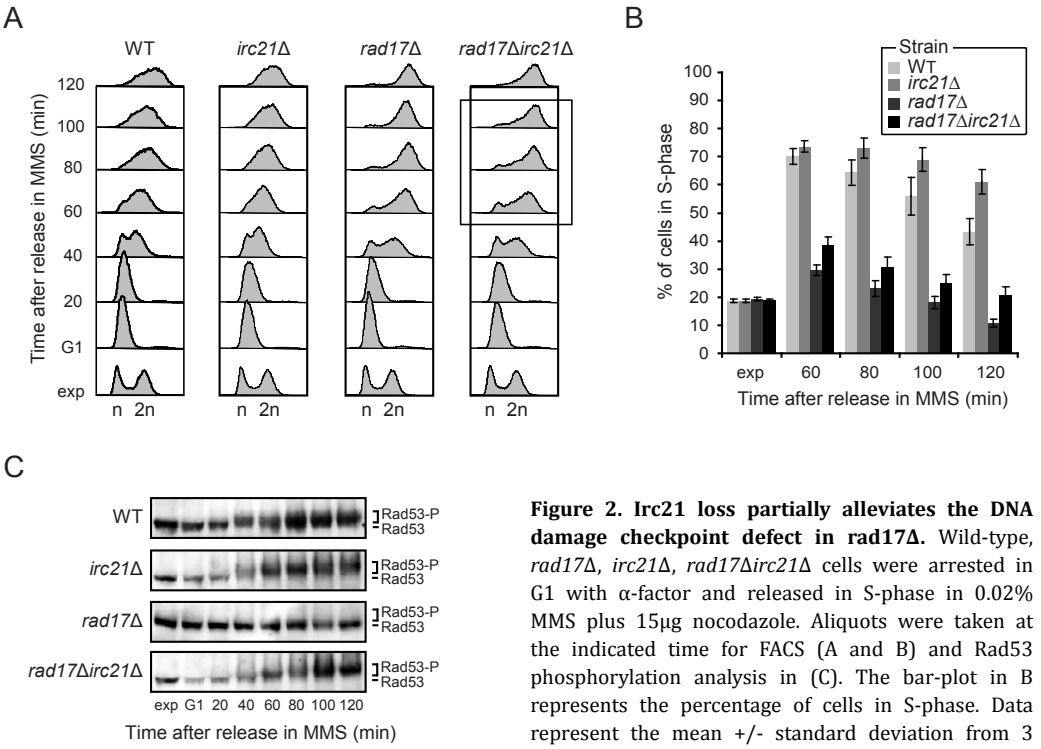

Figure 2. Irc21 loss partially alleviates the DNA damage checkpoint defect in rad17Δ. Wild-type, *rad17*Δ, *irc21*Δ, *rad17*Δ*irc21*Δ cells were arrested in G1 with α-factor and released in S-phase in 0.02% MMS plus 15μg nocodazole. Aliquots were taken at the indicated time for FACS (A and B) and Rad53 phosphorylation analysis in (C). The bar-plot in B represents the percentage of cells in S-phase. Data represent the mean +/- standard deviation from 3 independent experiments.

[10]. We used this phenotype to investigate the capacity of wild-type, *rad17*Δ, *irc21*Δ and *rad17*Δ*irc21*Δ strains to repair DNA damage. To this end, we monitored the accumulation of Rad52 foci following exposure to MMS and found that the rate of assembly of Rad52 foci was similar in all strains as they all reached a maximum number of Rad52 foci one hour after exposure to MMS (Figure 3A-E). Interestingly, *irc21*Δ mutant have an early repair delay attested by a larger number of cells that contain Rad52 foci (60%) compared to wild-type cells (40%) two hours after release from MMS (Figure 3E). However, while Rad52 foci gradually disappeared by 2–4 hours in wild-type and *irc21*Δ cells, persistent foci were observed in the *rad17*Δ mutant, indicating abrogation of repair (Figure 3A-C and E). Surprisingly, deletion of IRC21 alleviated the repair defect seen in the *rad17*Δ strain, as indicated by the enhanced dissolution of Rad52 foci in the *rad17*Δ*irc21*Δ strain compared to that in the *rad17*Δ strain (4 hour time point, Figure 3A-E).

Finally we found that, whereas *irc21*Δ cells showed no alterations in genomic stability, *rad17*Δ cells displayed a 8.2-fold increase in GCR events compared to wild-type (Figure 4A-B). However, *rad17*Δ*irc21*Δ cells only showed a 4.5-fold increase, suggesting that deletion of IRC21 partially rescues the deleterious impact of Rad17 loss on GCR (Figure 4B). Together, these results suggest that Irc21 not only modulates DNA damage checkpoint, but also promotes efficient repair of DNA damage and contributes to genome stability.

In contrast to previous high-throughput localization studies, which reported Irc21 localization in the cytoplasm [11], we found that Irc21-GFP localizes in

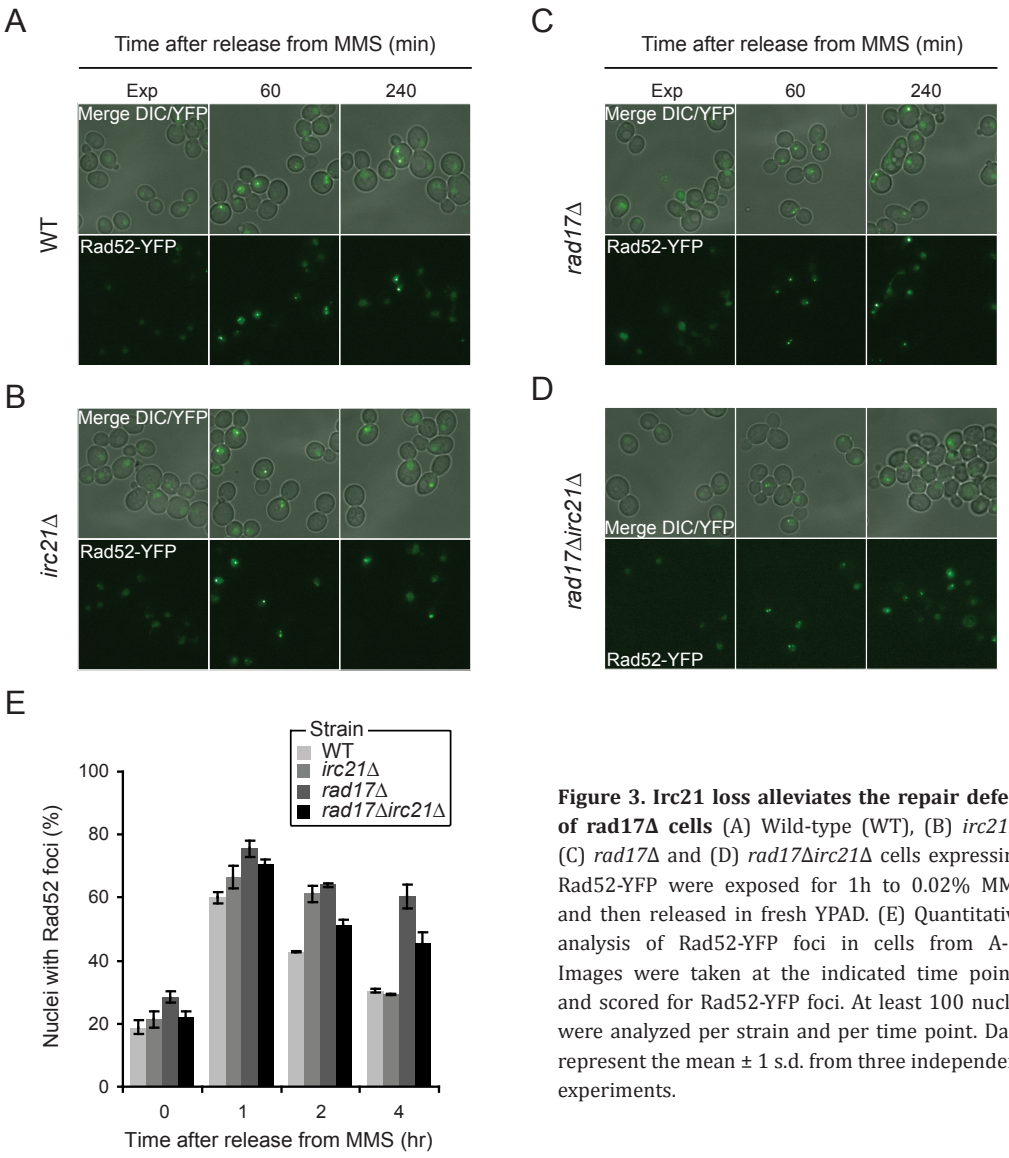

Figure 3. Irc21 loss alleviates the repair defect of rad17Δ cells (A) Wild-type (WT), (B) *irc21*Δ, (C) *rad17*Δ and (D) *rad17*Δ*irc21*Δ cells expressing Rad52-YFP were exposed for 1h to 0.02% MMS and then released in fresh YPAD. (E) Quantitative analysis of Rad52-YFP foci in cells from A-D. Images were taken at the indicated time points and scored for Rad52-YFP foci. At least 100 nuclei were analyzed per strain and per time point. Data represent the mean ± 1 s.d. from three independent experiments.

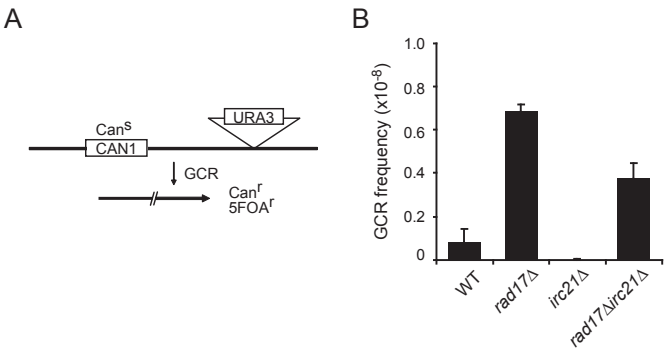

Figure 4: Irc21 loss alleviates genome instability in rad17Δ cells. (A) Scheme of the working principle of the GCR assay developed by Chen and Kolodner, 1999. (B) Effect of IRC21 deletion on GCR frequencies in rad17Δ cells. The mean GCR frequency ± standard deviation of three independent experiments is presented.

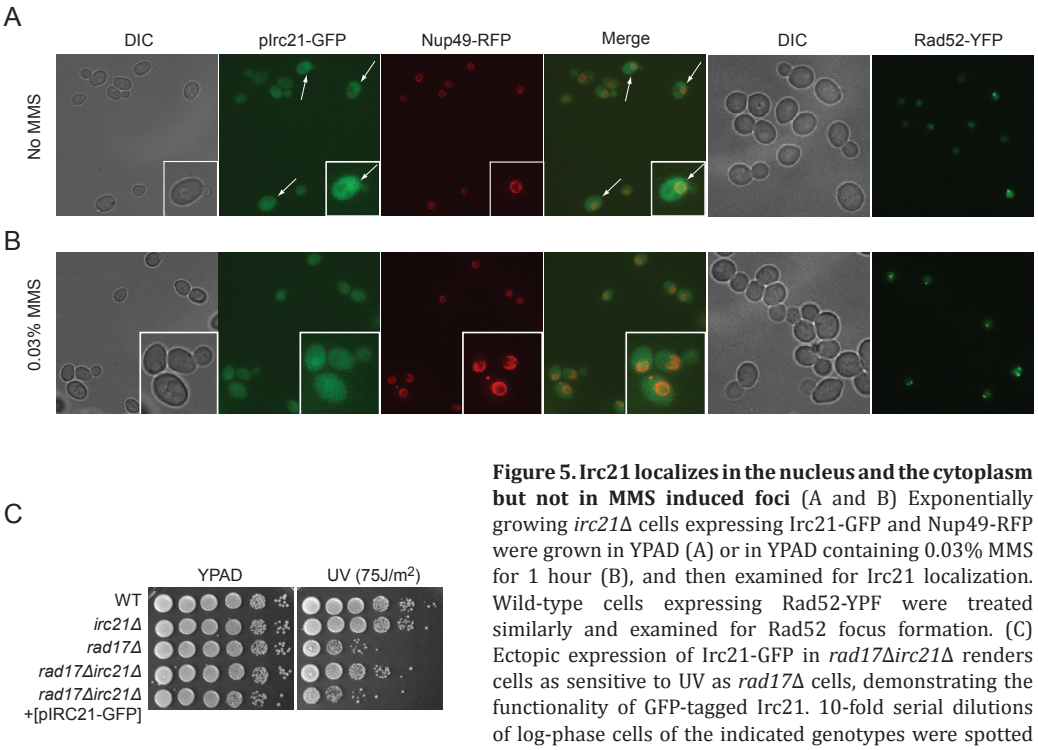

Figure 5. Irc21 localizes in the nucleus and the cytoplasm but not in MMS induced foci (A and B) Exponentially growing *irc21*Δ cells expressing Irc21-GFP and Nup49-RFP were grown in YPAD (A) or in YPAD containing 0.03% MMS for 1 hour (B), and then examined for Irc21 localization. Wild-type cells expressing Rad52-YPF were treated similarly and examined for Rad52 focus formation. (C) Ectopic expression of Irc21-GFP in *rad17*Δ*irc21*Δ renders cells as sensitive to UV as *rad17*Δ cells, demonstrating the functionality of GFP-tagged Irc21. 10-fold serial dilutions of log-phase cells of the indicated genotypes were spotted onto YPAD plates, exposed to UV and incubated for 3 days at 30°C.

both the cytoplasm and nucleus (Figure 5A). We examined the functionality of the Irc21-GFP construct by drop-test analysis (Figure 5C). Irc21-GFP expressing *rad17*Δ*irc21*Δ cells were as UV sensitive as *rad17*Δ cells, suggesting that the Irc21-GFP construct is functional (Figure 5C). We noticed that Irc21-GFP did not accumulate into MMS-induced sub-nuclear foci as observed for Rad52-YFP (Figure 5B), suggesting that it may not operate directly at DNA lesions.

Interestingly, we observed that *irc21*Δ strains are hypersensitive to MMS when combined with the TOR inhibitor rapamycin (Figure 6A), a compound that can lead to increased and decreased abundance of proteins (via the autophagy pathway) including factors involved in the DDR [12, 13]. This may suggest that Irc21 affects the DDR by regulating the steady state levels of distinct DDR proteins. Work from Robert and colleagues demonstrated that the stability of the HR protein Sae2 following double-stranded breaks is dependent on the autophagy machinery [14]. To check whether the autophagy-dependent degradation of Sae2 is mediated by Irc21, we monitored the stability of Sae2 [14]. In agreement with this published work, we found that Sae2 was degraded much quicker after induction of a single DSB when cells are exposed to rapamycin (Figure 6B). However, this rapid degradation was neither dependent on Irc21 nor or Rad17 (Figure 6B). Earlier in the current work, we examined the role of Irc21 in response to MMS, which alkylates DNA bases and can lead to replication fork stalling (and not per se DSBs). We therefore examined the effect of MMS on the autophagy-dependent degradation of Sae2. We found that following exposure to MMS Sae2 was also degraded much more rapidly in rapamycin-treated

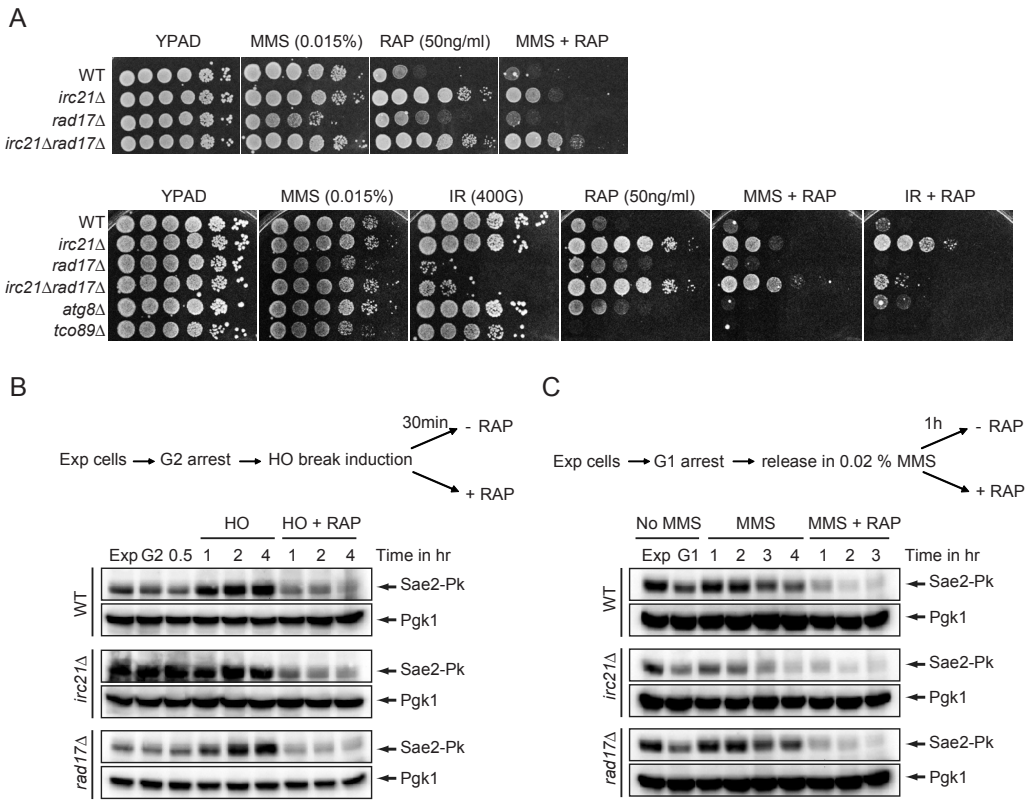

Figure 6. Irc21 does not mediate the autophagy-dependent degradation of Sae2 following DNA damage (A) *irc21*Δ cells are hypersensitive to MMS when combined with the TOR inhibitor rapamycin (RAP). 10-fold serial dilutions of log-phase cells of the indicated genotypes were either spotted onto YPAD plates containing MMS, RAP or both and incubated for 3 days at 30°C. (B) Exponentially (exp) growing WT, *irc21*Δ and *rad17*Δ cells were arrested in G2 with nocodazole after which a single double-stranded break was induced at MAT by the HO endonuclease as described previously [14]. Cells were then exposed to rapamycin (+RAP) or not (-RAP), after which the levels of Sae2-Pk and Pgk1 were monitored by Western blot analysis at the indicated time points. (C) As in B, except that exponentially growing cells were arrested in G1 using α-factor, released into fresh medium containing 0.02% MMS, and then left untreated or exposed to rapamycin.

wild-type cells (Figure 6C). A similar rate of degradation was seen in rapamycin-treated *irc21*Δ cells, suggesting that this autophagy-dependent degradation of Sae2 in response to MMS-induced DNA damage, like that after DSB induction, was not dependent on Irc21 (Figure 6C). Interestingly, Sae2 levels were decreased in *irc21*Δ cells that were only exposed to MMS. A similar decrease in Sae2 levels was observed in wild-type cells exposed to MMS and rapamycin, which suggests that IRC21 deletion mimics the effect of rapamycin on Sae2 levels (Figure 6C). Whether the effect on Irc21 on the steady state levels of Sae2 or other DNA repair factors affects the DDR needs further investigation.

CONCLUSION

Here, we describe the discovery of Irc21 as a new factor that regulates the DRR. However the precise role of Irc21 in this response remain to be determined. Irc21 contains a cytochrome b5 domain. Cytochrome b5

proteins have been shown to positively regulate reactions catalyzed by cytochrome P450 proteins. P450 proteins inactivate multiple hormones and xenobiotic compounds and play key roles in lipid metabolism (reviewed in [15]). Thus, one possible role for Irc21 may be to metabolize drugs or regulate their uptake. Here, we found that deletion of IRC21 alleviates *rad17*Δ cells sensitivity to various source of DNA damage (Figure 1C-D and F). We noticed that the survival of *rad17*Δ*irc21*Δ cells was more efficient after MMS and CPT than after IR and UV treatment, which suggest that the possible effect of Irc21 in drug metabolism may partially affect cell viability. In line with this idea, it was also shown that *irc21*Δ cells are resistant to carboplatin and cisplatin [5]. However, the improved survival of *rad17*Δ*irc21*Δ cells after IR and UV confirmed a role for Irc21 in the cellular response to DNA damage that is independent of its putative drug metabolism activity. Moreover, upon MMS treatment, *irc21*Δ cells accumulate as many repair foci as wild-type cells, suggesting that the load of damage induced by MMS and so the MMS uptake is the same in the two strains (Figure 3A-B and E).

We found that *irc21*Δ cells are resistant to rapamycin (Figure 6A). Rapamycin treatment inhibits the target of rapamycin (TOR) signaling pathway, which causes severe changes in the transcription profile of many genes and stimulates autophagy, thereby affecting the steady state levels of certain proteins. We showed that both deletion of IRC21 and rapamycin treatment lead to a decrease in Sae2 levels after MMS exposure (Figure 6B-C). Interestingly, we found that Rad52-foci disappearance was delayed in *irc21*Δ cells (Figure 3E), indicating that DNA repair occurs at a slower rate that may be potentially caused by a partial loss of Sae2. Whether Irc21 affects Sae2 levels by affecting its transcription or degradation is not clear. Consistent with previous work, we found that rapamycin, which stimulates autophagy by inhibiting TOR provokes Sae2 degradation [14]. However, we showed that this autophagy-mediated degradation of Sae2 was not dependent on Irc21, suggesting that Irc21 does not regulate protein levels via the autophagy machinery (Figure 6C). Beside, the fact that IRC21 deletion mimics rapamycin treatment may imply that Irc21, like rapamycin, is a negative regulator of the TOR pathway. Since TOR inhibition induces transcriptional changes, we could envisage that Irc21 affects the transcription pattern of SAE2 and probably multiple other genes by inhibiting TOR signaling.

Interestingly, we found that IRC21 has a similar differential genetic interaction profile to that of RRD1 on MMS. RRD1 deletion also confers resistance to rapamycin. Rrd1 is a peptidyl propyl isomerase that has been implicated in transcriptional changes in response to rapamycin and other transcriptional stress-inducing compounds [16]. It was proposed that Rrd1 promotes RNA polymerase II isomerisation in response to rapamycin, resulting in RNA pol II dissociation from chromatin [17]. Thus it may be that Irc21 like Rrd1 is involved in transcription regulation upon cellular stress. A number of recent studies in yeast and mammals have suggested that rapamycin treatment of cells and the subsequent inactivation of mTOR plays a role in up-regulating the level of numerous factors involved in DNA repair [12, 18]. Thus, the role of Irc21 in the DDR may be that it controls the mTOR-dependent up or down- transcriptional regulation of DNA repair factors.

MATHERIAL & METHODS

Cell survival assays

Overnight cultures were diluted 1:20, grown for 3 h at 30 °C and diluted to $1x10^7$ cells/ml. 10-fold dilution series were spotted on

plates containing CPT or MMS or spotted on YPAD and exposed to IR or UV after which they were 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 0.02% MMS. Samples were taken every 20 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.

Checkpoint assay

Exponentially growing cells were synchronized in G1 with α-factor (7.5μM) and released in 0.02% MMS. Whole cell extracts were prepared for western blot analysis to examine Rad53 phosphorylation using anti-Rad53 (Santa-Cruz, sc-6749) antibody.

Analysis of Rad52 foci

Cells containing a Rad52-YFP expression vector were grown to mid-log phase, exposed to MMS for 1 hour, washed and concentrated in 1% low melting agar (Cambrex). Images were captured using a Leica AF6000 LX microscope at 100-fold magnification using a HCX PL FLUOTAR 100x 1.3 oil objective lens.

GCR assay

The Gross Chromosomal Rearrangement (GCR) assay was done according to a previously published protocol [19]. Briefly, cells were grown overnight in YPAD to a density of 2-5x10^9 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.

Western blot analysis of Sae2

Exponentially growing cells were arrested in G2 with nocodazole after which a single double-stranded break was induced at MAT by the HO endonuclease as described previously [14]. In the MMS experiment, exponentially growing cells were arrested in G1 α-factor (7.5μM) and released into fresh medium containing 0.02% MMS. Cells were then exposed to rapamycin (+200ng/ml RAP) or not (-RAP), after which the levels of Sae2-Pk and Pgk1 were monitored by Western blot analysis using anti V5-TAG (Invitrogen) and anti-Pgk1 (Abcam 22C5) antibodies.

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