The budding yeast protein Chl1p is required for delaying progression through G1/S phase after DNA damage.

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Research

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"The budding yeast protein Chl1p is required for delaying progression through G1/S phase after DNA damage."

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Running title: abrogation of G1-S transition in DNA repair mutants

Key words: Yeast, Chl1p, checkpoint, bud-emergence, DNA damage, G1/S phase, repair.

The budding yeast protein Chl1p is required for delaying progression through G1/S phase after DNA damage.

Abstract

Background: The helicase Chl1p is a nuclear protein required for sister-chromatid cohesion, transcriptional silencing, rDNA recombination, ageing and plays an instrumental role in chromatin remodeling. This budding yeast protein is known to preserve genome integrity and spindle length in S-phase. Here we show additional roles of Chl1p at G1/S phase of the cell cycle following DNA damage. **Results:** G1 arrested cells when exposed to DNA damage are more sensitive and show bud emergence with a faster kinetics in *chl1* mutants compared to wild-type cells. This role of Chl1p in G1 phase is Rad9p dependent and independent of Rad24 and Rad53. *rad9chl1* shows similar bud emergence as the single mutants *chl1* and *rad9* whereas *rad24chl1* and *rad53chl1* shows faster bud emergence compared to the single mutants *rad24*, *rad53* and *chl1*. In case of damage induced by genotoxic agent like hydroxyurea, Chl1p acts as a checkpoint at G1/S. The faster movement of DNA content through G1/S phase and difference in phosphorylation profile of Rad53p in wild type and *chl1* cells confirms the checkpoint defect in *chl1* mutant cells. Further we have observed that the checkpoint defect is synergistic with the replication checkpoint Sgs1p and functions in prallel to the checkpoint pathway of Rad24p. **Conclusion:** Chl1p shows Rad53p independent bud emergence and Rad53p dependent checkpoint, confirms its requirement in two different pathways to maintain the G1/S arrest when cells are exposed to damaging agents. The bud emergence kinetics and DNA segregation were similar to wild type when given the same damage in nocodazole treated *chl1* cells which establishes the absence of any role of Chl1p at the G2/M phase. The novelty of this paper lies in revealing the versatile role of Chl1p in checkpoints as well as repair towards regulating G1/S

transition. Chl1 thus regulates the G1/S phase by affecting the G1 replication checkpoint pathway and shows an additive effect with Rad24p as well as Rad53p activation when damaging agents perturbs the DNA.

Key words: Yeast, Chl1p, checkpoint, bud-emergence, DNA damage, G1/S phase, repair

Background:

The helicase Chl1p is a nuclear protein required for sister-chromatid cohesion, transcriptional silencing, rDNA recombination, ageing and plays an instrumental role in chromatin remodeling (1- 4). It preserves genome integrity upon DNA damage in S-phase (5). The three highly related human homologs of Chl1p are BACH1, hChlR1 and hChlR2. hChlR1 and hChlR2 are expressed only in proliferating human cell lines. Of these, hChlR1 shows in vitro DNA helicase activity and binds to both single- and double-stranded DNA (6,7). BACH1 is a member of the DEAH helicase family and binds to the Rad9 homolog BRCA1, contributing towards DNA repair activity (8).

RAD9 was the first DNA damage checkpoint gene identified in the yeast *Saccharomyces cerevisiae* and was found to play a role in ionizing radiation induced G2/M cell cycle arrest (9,10,11). Throughout the cell cycle, it is required for activation of kinase Rad53 in response to DNA double stranded breaks. Two independent mechanisms exists for the Rad9 activity- the Tudor/BRCT domains of Rad9 plays the role of Rad53 activation at G1/S phase and the CDK consensus sites of Rad9 activates Rad53 at G2/M (12,13). Rad9 homologs 53BP1, MDC1 and BRCA1 also modulates the checkpoint pathways at two phases of the cell cycle. Activation of Rad53 at G1/S depends on the association of Rad9 with the modified chromatin surrounding the double strand breaks, which is mediated by the binding of Tudor/BRCT domain of Rad9 with di-methylated histone H3 and to phosphorylated histone H2A respectively (12). Any mutation in

the pocket fail to execute the G1 checkpoint delay, but the same mutations doesn't affect nocodazole-induced G2/M arrested cells. Furthermore the binding of Rad9 to histone H2A maintains the G1 checkpoint delay instead of the phosphorylation of H2A, when challenged with xenotoxic agents (12,14).

In this paper we have observed the same characteristics in *chl1* mutants. Like *rad9*, *chl1* mutants also fail to execute the G1 arrest when treated with MMS. This study shows that Chl1p is essential for G1/S arrest in response to DNA damage and it acts synergistically with Rad9. In presence of a pulse of damage, the *chl1* cells show faster kinetics of bud emergence when compared to the wild type cells indicative of a compromised checkpoint function. To understand the status of checkpoints at G1/S in presence of damage, G1 arrested cells were exposed to genotoxic agent HU. We observed the bulk DNA accumulation along with compromised Rad53p phosphorylation in *chl1* mutant cells at G1/S phase of the cell cycle, which are the hallmark characteristics of checkpoint proteins. The above mentioned observations confirms the early entry into S-phase for *chl1* mutant cells is due to defect in checkpoints compared to wild-type cells. We also observed that apart from the checkpoint defect of Chl1p which is Rad53p dependent, it follows an additional pathway to regulate the bud emergence at G1/S upon DNA damage as the bud emergence of *rad53chl1* is additive to single mutants *rad53* and *chl1*. All these findings confirm the dual role of this protein in controlling the G1 to S transition in the cell cycle on exposure to DNA damage.

Results

Chl1p is required for G1/S arrest after DNA damage by MMS

Exponentially growing mutant and wild-type cells were arrested in G1 by alpha factor for 90 minutes, treated with 0.2% MMS at the last 10 minutes of arrest and washed free of cell cycle

block. MMS was quenched by 10% v/v sodium thiosulphate and released in fresh medium. Thereafter, at different time intervals bud emergence was scored as a measure for functioning of the G1/S arrest. The budding kinetics of *chl1* cells is significantly faster then the wild type cells leading us to conclude that Chl1 disrupted mutant cells were deficient in G1/S arrest when their DNA was damaged with MMS, (Fig.1 A). There was no significant difference in the kinetics of bud emergence in control cells (without MMS treatment) (Fig1A). Budding cells are more in *chl1* mutant cells compared to wild-type cells after 1 hour and 2 hours of MMS treatment as shown by randomly taken representative fields (Fig.1B). Thus, Chl1p is required for G1/S arrest in response to DNA damage at the G1 phase. The fast movement *chl1* mutants through G1 phase indicates that the cells are spending less time for repair and have compromised checkpoint arrest at G1. To confirm the defect in G1/S arrest and justifying the progression in cell cycle of the mutant cells with more damage as a result of compromised repair, we performed the sensitivity analysis of *chl1* cells towards genotoxic agents. Mutant and wild-type cells were arrested in G1 using α-factor and then released in S-phase in the presence of 0.2 M HU. Aliquots were removed at various time intervals, cells were counted and plated on YEPD plates to determine viability. Fig. 1C shows nearly 50% loss in the viability of *chl1* mutant cells after 3.5 hours of HU treatment. The loss in cell viability of *chl1* compared to wild-type cells in the presence of 0.2M HU confirmed these results (Fig1c).

Chl1p is not required at G2/M for MMS-induced DNA damage repair

In presence of DNA damage caused by MMS, G2/M-arrested wild-type cells delay nuclear division (15,16). To determine if Chl1p is required in this delay, mutant and wild-type cells were arrested at G2/M by nocodazole, treated with MMS, washed free of cell cycle block including MMS and released into fresh medium. Percentage of cells, which had divided their nuclei, was

scored at different time intervals to measure G2/M arrest. Fig.2 shows that *chl1* mutant cells were proficient for G2/M arrest as they delayed nuclear division when their DNA was damaged with MMS. Also, the control cells did not show any significant differences in the timings of nuclear. Therefore, Chl1p is not required at the G2/M transition for MMS-induced DNA damage division repair.

Chl1p plays a role in regulating the checkpoints at G1/S phase of the cell cycle.

The observation that the *chl1* null mutations shows sensitivity to genotoxic agents like hydroxy-urea (HU), faster kinetics of bud emergence compared to the wild-type suggests that Chl1p could be involved in checkpoint function or in DNA damage repair or in both. As the preliminary observations gives a clue of affected checkpoint function in *chl1* mutant cells we decided to confirm this by more direct experiments, as described below.

When replication forks stall in the presence of HU, S-phase is slowed down (17,18). However, when DNA is damaged in some S-phase checkpoint mutants such as *mec1, rad9, rad17, rad24* and *rad53*, S-phase appears to progress faster because late origins fire inappropriately, causing additional DNA synthesis, which can be detected by flow cytometry (17,18). To test wheather Chl1p has a G1/S phase checkpoint function, the progression of cell cycle by monitoring the DNA content was observed through flow-cytometry in HU treated G1 synchronized cells. After the cells are released from alpha-factor arrest, the cells were re-suspended in fresh media containing 0.2 M HU and the progression of DNA synthesis from G1 to S was monitored by flow cytometry. The *chl1* cells entered S-phase from G1 by 30 minutes of treatment with HU whereas in case of wild type the entry in S-phase from G1 took place around an hour of HU treatment (Fig.3A). Since the G1 to S-phase progression in *chl1* was faster compared to wild-

type cells in the presence of HU, it suggests that the DNA damage checkpoint pathway was perturbed in these cells leading to bulk DNA synthesis.

To confirm the checkpoint role of Chl1p, Rad53p activation was studied directly by assaying for its phosphorylation in HU-treated cells. Cells were synchronized with alpha-factor and released in YEPD in the presence of 0.2 M HU. Aliquots were withdrawn at indicated times. Figure 3B and 3C shows that *chl1* cells had compromised Rad53p phosphorylation compared to the wild type in G1/S-phase. Thus, this confirms that Chl1p is required to activate the DNA damage checkpoint pathway when cells are treated with HU in G1/S-phase.

Chl1p acts independently of the DNA damage checkpoint pathway

The sensitivity of *chl1* cells towards xenotoxic agents proves the accumulation of damaged DNA due to perturbed repair or checkpoint arrest at G1. To do a pathway analysis of Chl1p's checkpoint activity the following experiments were performed. The intra-S-phase checkpoint proteins Sgs1 and Rad24 act in parallel in the DNA replication and damage checkpoint pathways, respectively, to maintain cell viability and activate Rad53p in the presence of hydroxyurea (16, 19). In the viability studies the single mutants *sgs1* and *rad24* were included along with *chl1*. The double mutants *rad24chl1* and *sgs1chl1* were also included to determine if *chl1* showed any synergistic loss in viability with either of these two mutations after releae from G1 arrest in 0.2 M HU. The results (Fig.1C) show that there is a synergistic drop in cell viability in *rad24chl1* double mutants but not in *sgs1 chl1*. The *rad24 sgs1* double mutant exhibited an expected fall in cell viability. This shows that Chl1 acts independenty of the Rad24 pathway. To further confirm the pathway of Chl1p for G1- arrest we performed the bud emergence experiments with mutant genes, which regulates the effect of genetic insults on cell cycle

kinetics, like *rad9*, *rad24* and the corresponding double mutants. Rad9 and Rad24 epistasis group are required for efficient cell-cycle arrest after DNA damage in G1/S (20,21) and G2/M (16, 22). To determine if Chl1p is in Rad9p or Rad24p pathway at this phase of the cell cycle, experiments were carried out to monitor the kinetics of bud emergence. WT, *chl1*, *rad9*, *rad24*, *rad24 chl1* and *rad9chl1* cells were arrested in G1 by alpha factor, treated with 0.2% MMS, washed free of cell cycle block and MMS, and released into fresh medium to score for bud emergence. Fig.4A shows that the double mutant *rad24 chl1* emerged from the arrest faster than either of the single mutants *chl1* and *rad24* and the effect appeared to be additive with *chl1* mutation. This confirms that Chl1p acts independently of Rad24p to arrest damaged cells at G1/S phase. On contrary Fig.4B shows that the double mutant *rad9 chl1* doesn't emerge from the arrest any faster than either of the single mutants, *rad9* and *chl1*. Thus, Chl1p acts through the Rad9 pathway. Representative fields of budding cellsBud emergence of the single mutants *rad24*, *rad9* and the double mutants *rad24chl1*, *rad9chl1* also proves that *chl1* mutant cells have more buds compared to wild-type cells after 2 hours of MMS treatment and the number of buds in case of *rad24chl1* is significantly more compared to *rad24* and *chl1* alone (Fig. 4C)

Chl1p plays role in dual mode of arrest upon DNA damage in G1/S phase of the cell cycle

The pathway analysis (shown in Figure 4) and the sensitivity studies towards genotoxic agents (as shown in Figure1) of Chl1p give a clue that Chl1 may act independently of the DNA damage checkpoint pathway. To further confirm that Chl1p acts independently and in addition to the damage checkpoint pathway and is synergistic to replication checkpoint pathway, we monitored Rad53p phosphorylation both in wild type, single and double mutant cells. The checkpoint mutants *rad24* and *sgs1* were included in the Rad53 phosphorylation studies as they have roles in

damage checkpoint and replication checkpoint pathways (23,24). *rad24* cells, as expected, showed lower levels of Rad53p phosphorylation (Fig.5A,5B). Interestingly *rad24chl1* mutant was even more compromised in phosphorylating Rad53p than *rad24* and *chl1* alone (Fig. 5A, 5B). In case of replication checkpoint, *sgs1* cells showed much lower levels of Rad53p phosphorylation while *sgs1chl1* mutant was no different than *sgs1* (Supplementary Fig. 1 A, B). We also performed the cell cycle analysis of G1 synchronized HU treated checkpoint mutant cells. We observed that after 2 hours of HU treatment the *chl1* cells were progressing faster like the *rad24* checkpoint mutant cells (Fig.5C). The double mutant *rad24chl1* was even faster (Fig.5C). So in this section we confirmed the role of Chl1p, independent of Rad24p, in checkpoint pathway through Rad53p activation in G1/S.

In S-phase, Chl1p plays a role in repair pathway upon DNA damage (25). As Chl1p acts as a repair protein in S-phase, we opted to determine if Chl1p has some additional role in G1 phase in addition to Rad53p checkpoint pathway. The bud emmergence experiments with mutant genes *rad53*, *chl1* and the corresponding double mutants were performed. WT, *chl1*, *rad53* and *rad53chl1* cells were arrested in G1 by alpha factor, treated with 0.2% MMS, washed free of cell cycle block and MMS, and released into fresh medium to score for bud emergence. Bud emergence was scored after exposure to damage following the same protocol. Fig.5D shows that the double mutant *rad53 chl1* emerges significantly faster from G1 arrest than the single mutants *chl1* and *rad53*. The randomly captured representative fields of budding cells of *chl1*, *rad53* and *rad53chl1* also confirm the same (Fig. 5E). The faster bud emergence of the double mutant suggests that Chl1p acts independently of Rad53p checkpoint response and may be following a parallel pathway of arresting at G1 along with Rad53p checkpoint arrest to maintain the genomic integrity on exposure to different types of genomic insults.

Discussion

The functioning of Rad9 as G1/S checkpoint is dependent on its TUDOR and BRCT domains and is independent of its auto-phosphorylation through CDK (26). Rad53 activation in G1 and S phase depends on the association of Rad9 with the modified chromatin adjacent to DSBs. Rad9-chromatin association is mediated by binding of TUDOR domains to histone di-methylated H3 and BRCT domains binding to phosphorylated histone H2A (12). If the interaction is broken the activation of phosphorylated Rad53 is compromised in presence of genotoxic agent like MMS and HU. The Rad9 BRCT mutant fails to perform the G1 checkpoint delay post DNA insult but they were proficient in checkpoint response upon DNA damage in Nocodazole treated cells. So, the recruitment and retention of Rad9 at the damage sites through BRCT domain play a vital role for the G1/S arrest. The interactor proteins of Rad9 at BRCT domain are also instrumental in maintaining the arrest for proper repair of the damage.

In this paper we show evidence that, like Rad9, *chl1* mutants also fail to execute the G1 checkpoints and the delay in bud emergence is perturbed in G1-arrested cells when treated with MMS. In the presence of damage, Chl1 acts as a checkpoint and executes the G1/S phase arrest. In *chl1* mutants, the immediate repair process of the damaged DNA is compromised and as a result the cells doesn't stay arrested at G1 phase for the repair activities as evident from faster bud emergence, compared to *rad53* leading to accumulation of more damage. In this paper we have also shown that *Chl1* are more sensitive in response to DNA damaging agents that may be because of the presence of accumulated damage. The observations like faster movement through G1/S and compromised Rad53 activation in *chl1* mutant cells confirms the Rad53p dependent checkpoint function of Chl1p at G1 phase. It plays the checkpoint role parallel to the damage checkpoint pathway in G1/S phase of the cell cycle as the Rad53p phosphorylation of *chl1*

mutants is even more compromised in absence of *rad24*. Apart from the checkpoint role, the repair function of Chl1p also regulates the G1 phase arrest when DNA is perturbed. Our data confirmed that Chl1p acts independently of Rad53p in arresting the cells at G1/S by observing the faster bud emergence in *rad53chl1* compared to the single mutants. Our data also indicates that prior to activation of the checkpoint proteins in late G1/S phase (FACS profile, Fig. 4A and Western blot, Fig. 4B), Chl1p regulates the arrest of cells at early G1 phase in presence of damage. So Chl1p plays a role as a checkpoint at G1/S phase, which leads to Rad53 activation and prevents bulk DNA synthesis. Apart from its checkpoint function at G1/S phase, it plays some additional function, may be the repair function independent of Rad53p and in synchrony with Rad9p to regulate the budding kinetics following insult to the genetic material (Fig. 6)

G2/M phase arrest is executed by the auto-phosphorylation of Rad9 and is independent of the BRCT domain (27). Establishment of sister chromatid cohesion occurs for the repair of double strand breaks at G2/M (28,29). Since Chl1p is required for the establishment of sister chromatid cohesion (2), resistance of *chl1* mutant towards killing by MMS treatment at G2/M suggests that the repair of this damage is not critically dependent on the cohesion function of Chl1p.

Conclusion:

In summary, this paper brings to light additional cell cycle regulation roles of Chl1p in the budding yeast. In absence of Chl1p, double strand break induced cells fail to perform the G1/S checkpoint delay. Chl1p also leads to Rad53 activation, the major effector checkpoint kinase in presence of any damage due to blocks at G1. The Rad53p checkpoint activation by Chl1p at G1/S is independent of the Rad24p mediated damage checkpoint pathway. We also show that the role of Chl1p in G1 phase is Rad9p dependent and independent of Rad24p and Rad53p. The, double mutant *rad9ch1* shows similar bud emergence as the single mutants *chl1* and *rad9*

whereas the double mutant *rad24chl1* and *rad53chl1* shows faster bud emergence than the single mutants. This budding kinetics explains an additional role of Chl1p independent of Rad53p checkpoint activation. This paper supports a model in which Chl1p plays a critical role in regulating the G1/S transition along with Rad9 when cells are compromised with DNA damaging agents. Consistent with our data and the supporting experimental findings from other groups, we predict that the helicase Chl1p plays a role in modulating the chromatin structure of the damaged DNA, aids Rad9 BRCT domain to access phosphorylated H2A S129 residue at the double strand break region followed by engagement of repair machinery. The repair process is further supported by the checkpoint function of Chl1p. The checkpoint property further activates downstream regulators and key checkpoint proteins and keeps the cells arrested at early G1 as well as G1/S transition to provide some time for proper repair of the perturbed DNA at DSBs or blocks.

As the mammalian homologs of Rad9p (BRCA1) and Chl1p (BACH1) interacts at the BRCT domain (30), helicase Chl1p is suspected to be the Rad9p interactor and presumed to play the role of repair and remodeling of the damaged DNA along with Rad9 at the damaged sites. The findings of this paper gives a clue that the association of Rad9 to the modified chromatin at the DSB's helps to bring Chl1p repair protein through interaction with BRCT domain and repair damage by delaying G1 to S transition. During damage, the interaction between BRCT domain of Rad9 and phospho-H2A brings in the repair protein Chl1p helicase to the proximity of the damaged sites. As Chl1p also acts as a chromatin-remodeling factor (4), this in turn helps to remodel the chromatin bound Rad9 and initiate repair activity by arresting the cells at G1. The G1/S phase arrest is further supported by its Rad53p dependent checkpoint activity.

Materials and methods

Media and chemicals

All media, chemicals and enzymes have been described before (5,20,31). DAPI, alpha-factor, HU and goat anti-rat AP-conjugated antibody were from Sigma. Goat anti-mouse TRITC-conjugated antibody and NBT/BCIP was from Bangalore Genei Pvt. Ltd. Rad53 goat polyclonal antibody, raised against a carboxy terminus peptide of yeast Rad53p, and secondary AP-conjugated anti-goat antibody were from Santa Cruz Biotechnology, USA. MMS was from SRL (India).

Construction of single and double mutant strains

Gene disruptions and deletions of Chl1 are described in (32). Construction of double mutants and PCR based deletion of *CHL1* and *BAR1* were carried out as described in (5, 33). 699 and all the strains listed in Table 1 are in W303 background while the parent strains of the remaining were from G. Fink.

Cell synchronization, bud emergence and nuclear segregation

Cells were synchronized in G1 using alpha-factor as described in (34). Briefly, log phase cells were arrested with 0.025 μg/ml α-factor for 90 minutes and treated with 0.2% MMS in the last 10 minutes of arrest at 30°C. MMS was quenched by 10% v/v sodium thiosulphate. Cells were washed free of cell cycle block (α-factor) and released into fresh medium. Thereafter, at different time intervals bud emergence post DNA damage was scored as a measure of G1/S arrest (35).

For G2/M arrest exponentially growing cells were treated with 15 μg/ml nocodazole for 3 hours at 30°C. The arrested cells were treated with 0.15% MMS during last half-hour of nocodazole arrest. After treatment, MMS was quenched with 10% sodium thiosulfate (v/v) and released from

block. Nuclear stain was done with DAPI (36). Around 150–200 cells were counted for nuclear morphologies, using a fluorescence microscope (Leica fitted with DC 300F camera).

Flow cytometry

The phases of the cell cycle were determined by flow cytometry according to the protocol described in 20. Briefly, exponentially growing 1-2 X 10^7 cells were arrested at G1 using alpha-factor. The arrested cells were released in YEPD media containing 0.2M HU. Cells were collected at different time intervals in chilled 70% ethanol to do the cell cycle analysis. The cells fixed from each time point including the exponentials were spun down and fixed overnight in 70% ethanol at 4˚C. Cells were washed and suspended in Tris-EDTA (pH 7.5) buffer for RNaseA treatment at 37°C for 4 hours. Propidium Iodide (50 μg/ml) staining was done overnight at 4°C. Flow cytometry was done in FACS caliber (Becton Dickinson) with the sonicated samples (10 amps for 15 sec).

Protein extractions and western blot analysis:

For western blot analysis, protein extracts were prepared according to (8, 25) from cells synchronized in G1 and released in YEPD medium containing 0.2M HU. Proteins were separated on 8% SDS–PAGE containing an acrylamide to bis-acrylamide ratio of 80:1 and transferred to poly-vinylidene difluoride (PVDF) membrane (Schleicher and Schuell). Rad53 was detected using anti-Rad53 goat polyclonal antibody at 1:1000 dilution in TBS (50 mM Tris buffer pH 7.5, 150 mM NaCl) containing 0.5% BSA for 12–16 h. Secondary alkaline phosphatase-conjugated anti-goat antibody was incubated with the membrane for 2 h at 1:2500 dilution.

Declaration:

Ethics approval and consent to participate

Not applicable

Consent for publication:

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that they have no financial, personal or professional competing interests that could be construed to have influenced this paper

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Author's contribution:

SL contributed towards design and drafting the work, analysis and interpretation of the data and gave the major contribution in writing the manuscript. KMN performed the experiments of budding index counting of different strains, Imaging the strains and analyzed the data. KMN gave the major contribution in making the figures. AZ cultured the different strains and performed the experiments for counting the budding index of yeast.

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Figures

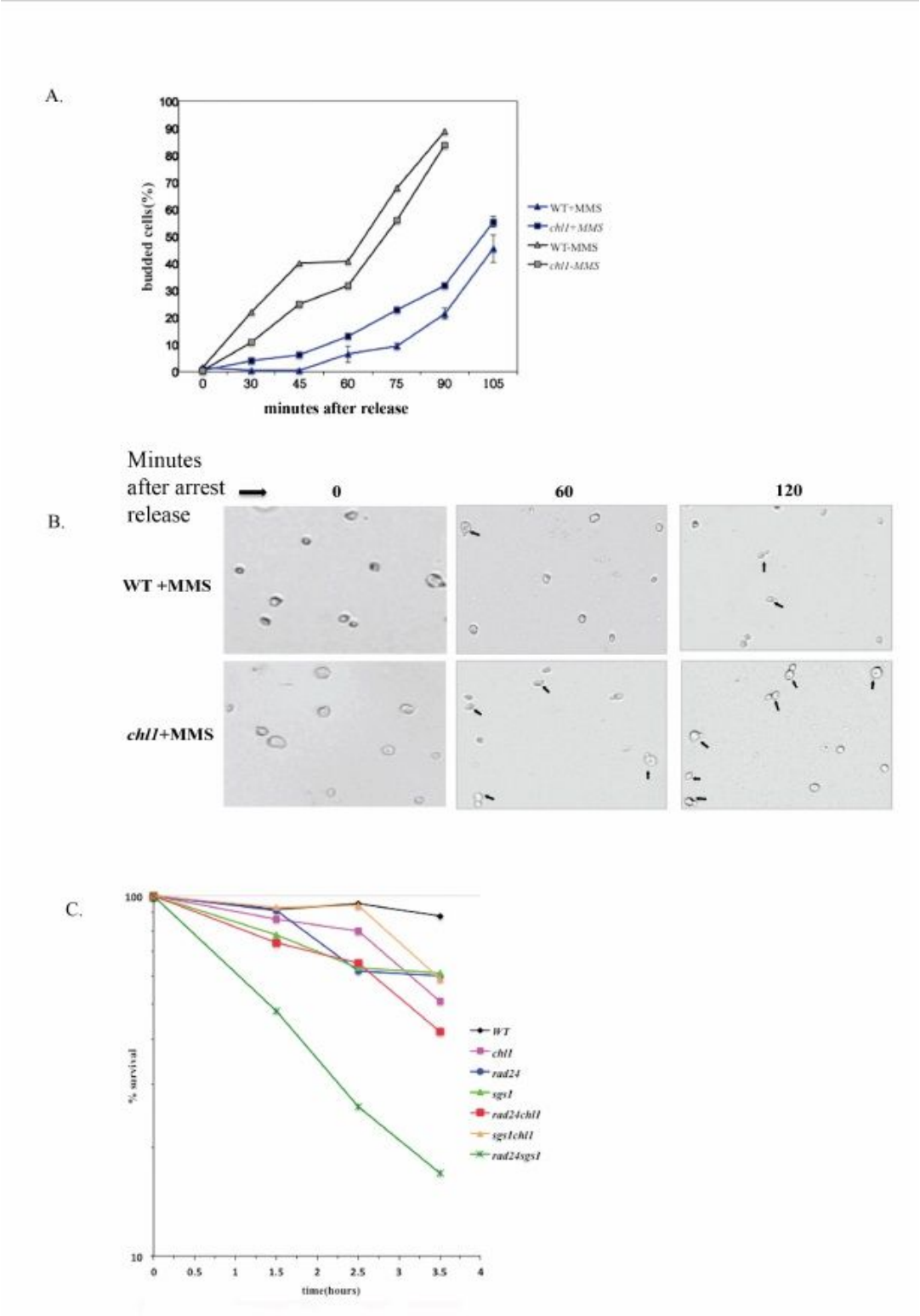

Figure 1

Chl1p is required for G1/S after DNA damage by MMS. A. G1-phase bud emergence kinetics of mutant and wild-type cells after MMS treatment. Wild-type (699) and mutant cell 699Dchl1 (chl1) were grown to exponential phase and arrested with 5 µg/ml α-factor for 90 minutes (G1 arrest) as described in materials

and methods. After 80 minutes of α-factor treatment at 30°C, each culture was divided into two. To one half 0.2% MMS was added and the other was maintained as a control. Cells were kept shaking for a further 10 minutes. After treatment, MMS was inactivated by the addition of one volume of 10% sodium thiosulfate solution, cells were spun down and the pellet was washed quickly with YEPD medium at RT. The cells were released in fresh YEPD medium at 30°C and aliquots were removed at regular times for scoring the percentage of budded cells. The graph represents the percentage of bud emergence in WT and chl1 cells at different time intervals after release from G1 arrest and 0.1% MMS treatment simultaneously. The black filled symbols are given for cells treated with MMS, the grey filled symbols indicates the absence of MMS. Data shown are averages of values obtained from three independent experiments and the deviations from the mean are shown as error bars. B. Budding of mutant and wild-type cells after MMS treatment. The bright fields of WT and chl1 from (A) at 40X resolution shows the budded cells in wild-type (699) and chl1 (699DChl1) mutant cultures after 1hour and 2 hours of release from MMS treatment. The budded cells are indicated with arrows. C. chl1 cells are sensitive towards killing by genotoxic agent in G1/S-phase. 699 (wild-type), 699Dchl1 (chl1), SL3 (rad24), SL3Dchl1 (rad24 chl1), 699Δsgs1 (sgs1), 699Δsgs1Dchl1 (sgs1 chl1) and SL21 (sgs1 rad24) cells were arrested by alpha-factor in G1 and released in fresh YEPD containing 0.2 M HU. Aliquots were removed for cell viabilities at the indicated time points.

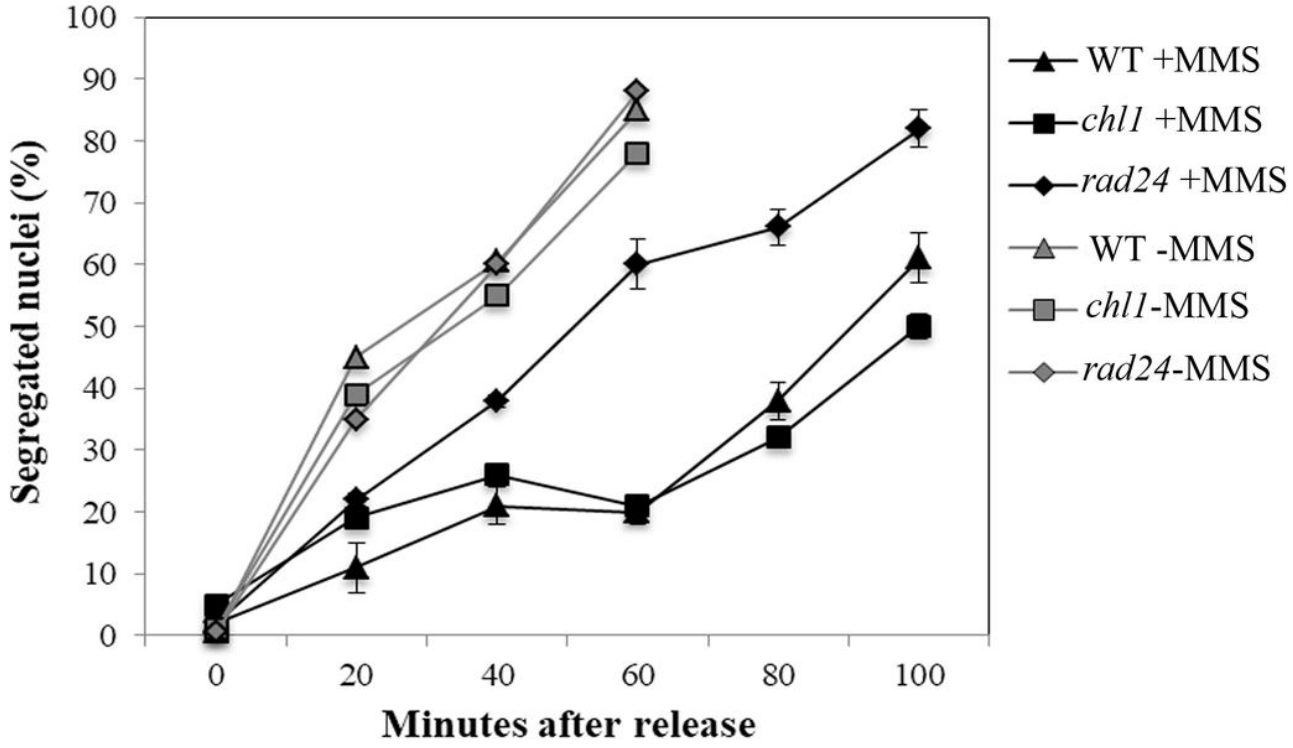

Figure 2

Chl1p is not required for G2/M arrest after DNA 24 damage by MMS. Wild-type (699) and mutant cells 699Dchl1 (chl1) and SL3 (rad24) were grown to exponential phase and arrested with 15 µg/ml nocodazole for 3 hours at 30°C for G2/M arrest. The arrested cells were treated with 0.15% MMS during last half hour of nocodazole arrest and kept shaking. After treatment, MMS was inactivated by the addition of one volume of 10% sodium thiosulfate solution. Cells washed with YEPD medium and released in fresh medium at 30°C and aliquots were removed at regular intervals and stained with DAPI to score for the percentage of cells with divided nuclei. Data shown are average of values obtained from three independent experiments and error bars are standard deviations from the mean value. The filled symbols are given for cells treated with MMS, the empty symbols indicates the absence of MMS. Error bars are not shown for data points pertaining to minus MMS experiments to avoid cluttering.

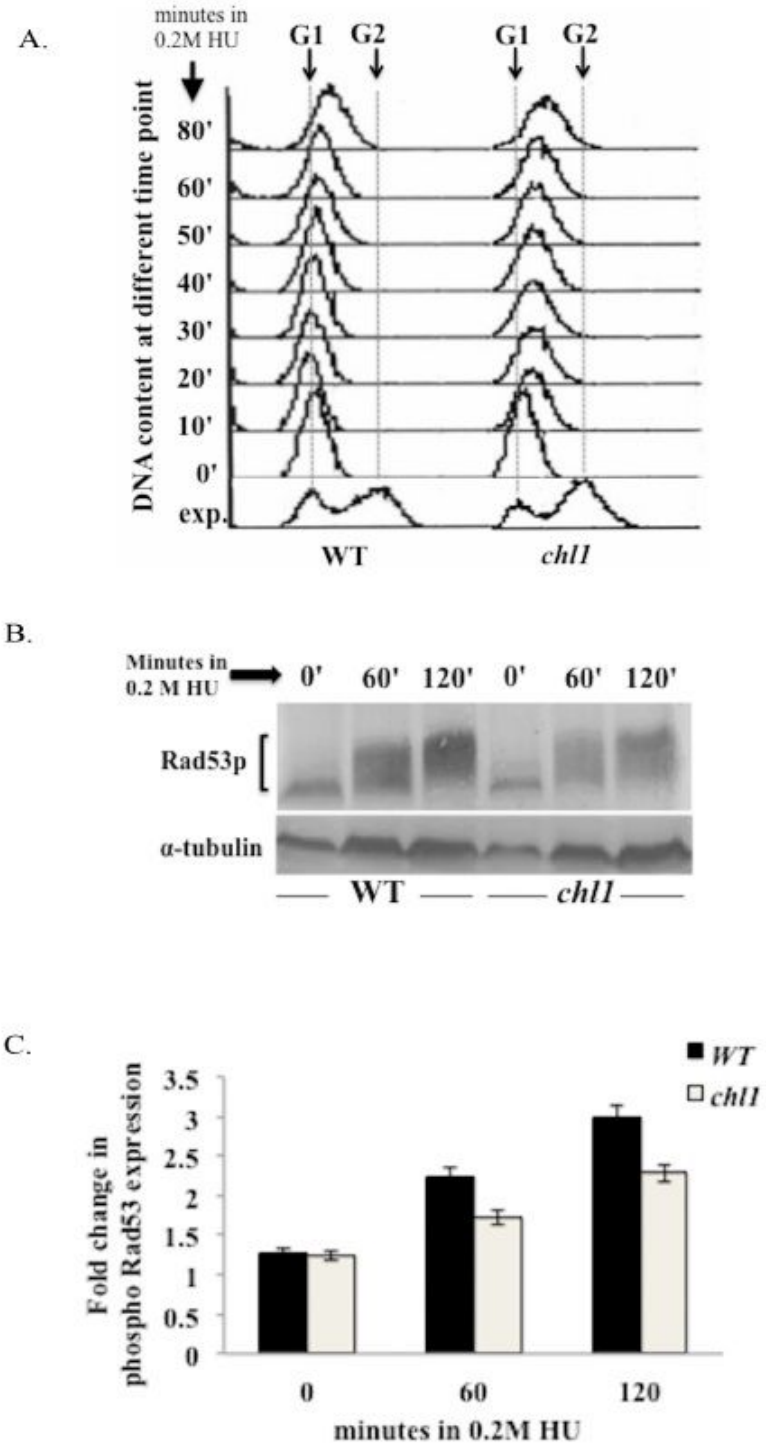

Figure 3

Chl1p plays a role in regulating the checkpoints at G1/S phase of the cell cycle. A. G1/S-phase progression of mutant and wild-type cells in the presence of HU. Wild type (699) and 699Dchl1 (chl1) cells were all synchronized with alpha-factor, washed free of alpha-factor, re-suspended in pre-warmed YEPD medium at 30°C and HU was added to a final concentration of 0.2M. Both the cultures were kept shaking at 30°C. Aliquots were removed at various times for FACS analysis. Arrows indicate G1 and G2

DNA contents. B. chl1 cells are compromised in Rad53p phosphorylation in response to HU treatment in G1/S-phase. Wild type, CHL1(699) and 699Dchl1 (chl1) cells were arrested in G1 phase and released in fresh YEPD medium containing 0.2M HU at 30°C. Rad53p phosphorylation was detected by western blot analysis of proteins extracted from aliquots of cells removed at indicated times, using antibodies directed against the Rad53 protein. C. Quantification of Rad53p expression in chl1 cells. The intensity of the phosphorylated bands of Rad53 in Wild type (699) and 699Dchl1 (chl1) cells in western blot were quantified using Image J software. The values of the Rad53p intensities were normalized with corresponding intensities of alpha-Tubulin to normalize the protein loading in different time points.

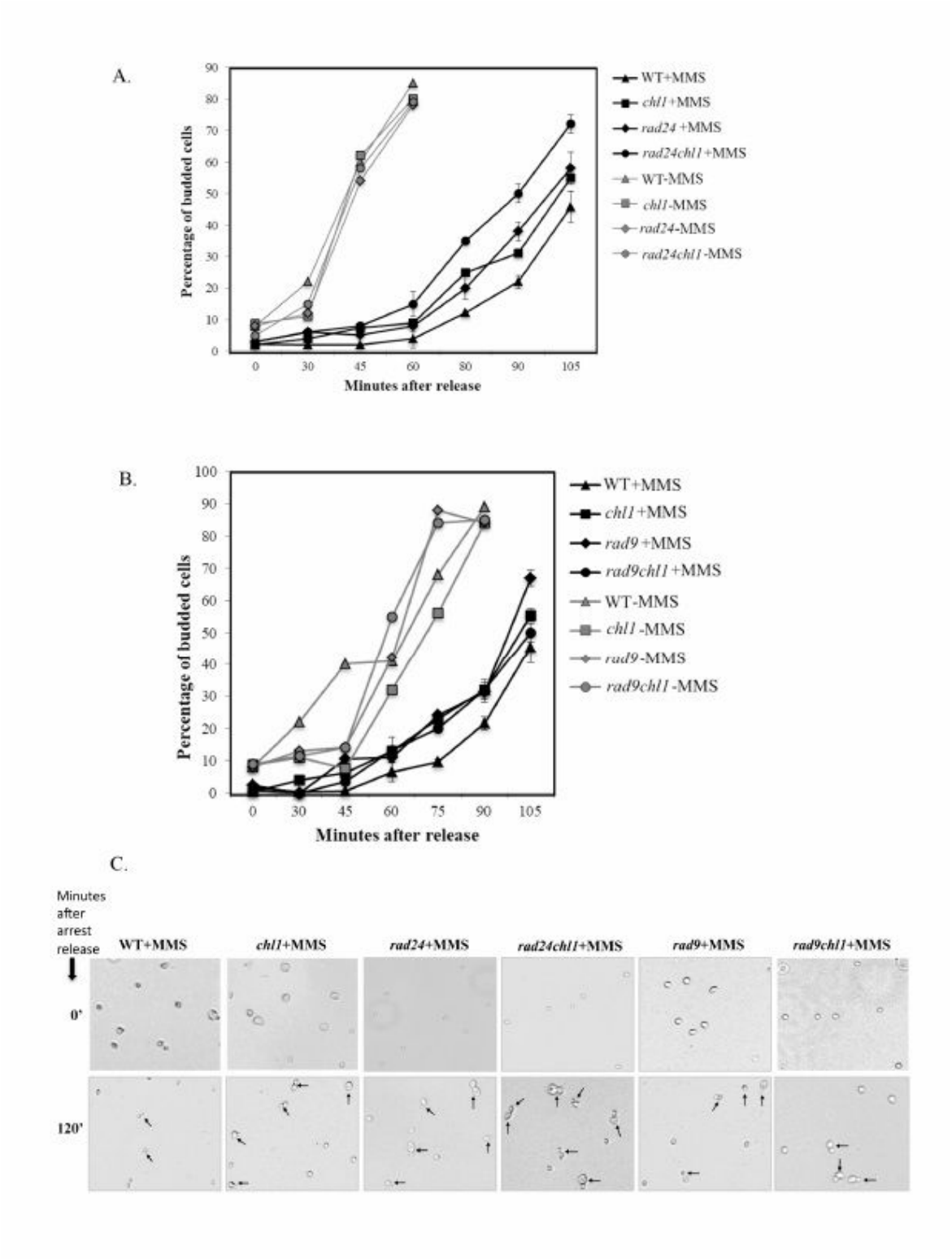

Figure 4

Chl1p acts independently of the DNA damage checkpoint pathway. A. G1-phase bud emergence kinetics of chl1 mutant cells are additive to rad24 after MMS treatment. Wild-type (699) and mutant cells 699Dchl1 (chl1), SL3 (rad24), SL3Dchl1 (rad24 chl1) were grown to exponential phase and arrested with 5 µg/ml α-factor for 90 minutes (G1 arrest) as described in materials and methods. After 80 minutes of α-factor treatment at 30°C, 0.2% MMS was added.Cells were kept shaking for a further 10 minutes. After

treatment, MMS was inactivated by the addition of one volume of 10% sodium thiosulfate solution, cells were spun down and the pellet was washed quickly with YEPD medium at RT. The cells were released in fresh YEPD medium at 30°C and aliquots were removed at regular times for scoring the percentage of budded cells. The graph represents the percentage of bud emergence in WT, chl1, rad24 and rad24chl1 cells at different time intervals after release from G1 arrest and 0.1% MMS treatment simultaneously. The black filled symbols indicate cells treated with MMS, the grey filled symbols indicates the cells without MMS treatment. Data shown are average of values obtained from three independent experiments. B. G1-phase bud emergence kinetics of chl1 mutant cells are synergistic to rad9 after MMS treatment. Wild-type (699) and mutant cells 699Dchl1 (chl1), SL19 (rad9), SL19Dchl1 (rad9 chl1) were grown to exponential phase and follow through same experimental procedures as done in 4A. The graph represents the percentage of bud emergence in WT, chl1, rad9 and rad9chl1 cells at different time intervals after release from G1 arrest and 0.1% MMS treatment simultaneously. The black filled symbols are given for cells 70 treated with MMS, the grey filled symbols indicates absence of MMS. Data shown are average of values obtained from three independent experiments. C. Additive and synergistic budding of mutant and wild-type cells after MMS treatment. The bright fields of WT and mutant cells from (A) and (B) at 40X resolution shows the budded cells in wild-type (699) and the mutant cells 699Dchl1 (chl1), SL3 (rad24), SL3Dchl1 (rad24 chl1), SL19 (rad9), SL19Dchl1 (rad9 chl1) mutant cultures after 2 hours release from MMS treatment. The budded cells are indicated with arrows.

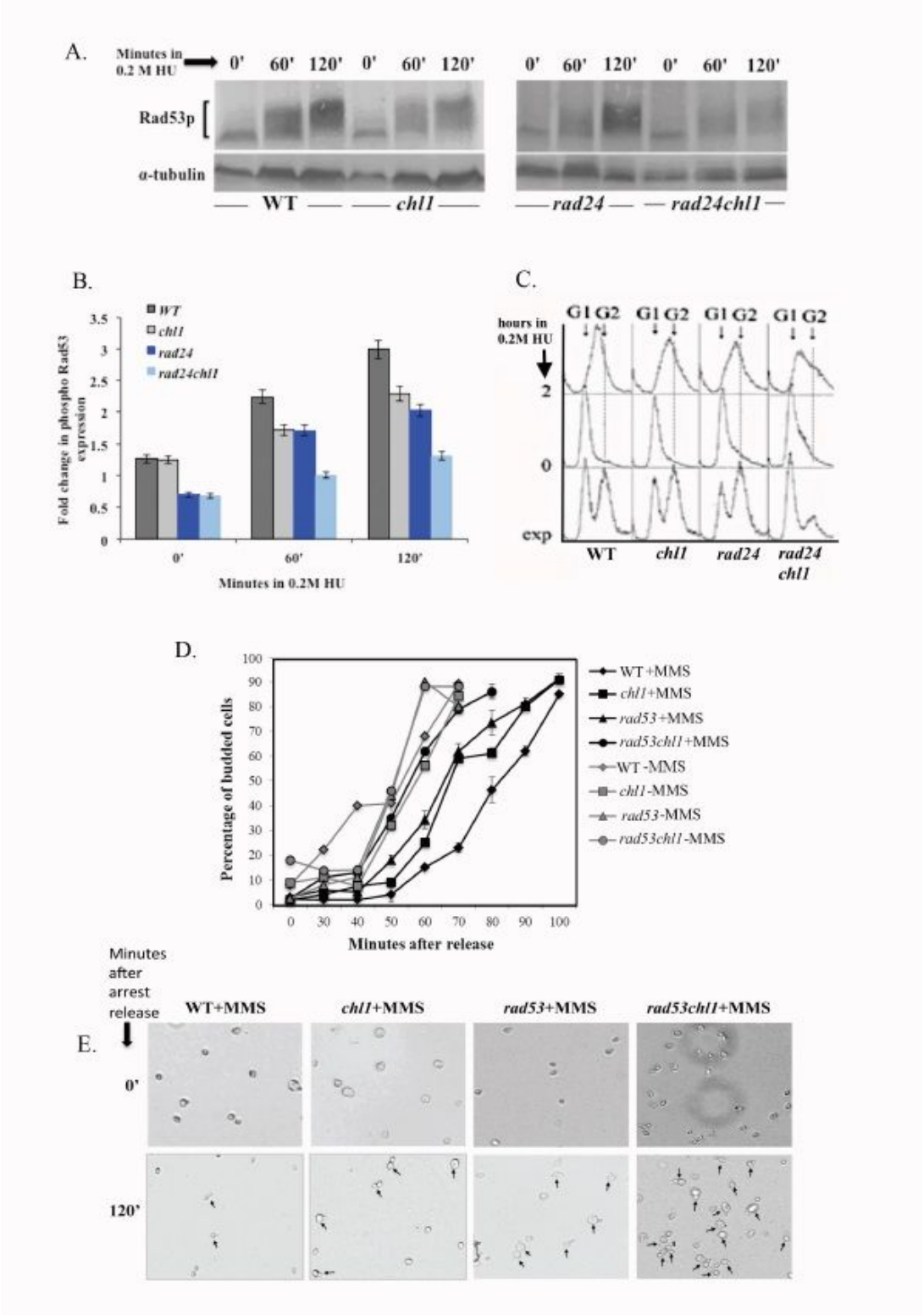

Figure 5

Chl1p plays role in dual mode of arrest upon DNA damage in G1/S phase of the cell cycle. A. rad24chl1 cells are more compromised in Rad53p phosphorylation compared to chl1 cells in response to HU treatment in G1/S-phase. Wild-type (699) and mutant cells 699Dchl1 (chl1), SL3 (rad24), SL3Dchl1 (rad24 chl1) cells were arrested in G1 phase and released in fresh YEPD medium containing 0.2M HU at 30°C. Rad53p phosphorylation was detected by western blot analysis of proteins extracted from aliquots

of cells removed at indicated times, using antibodies directed against the Rad53 protein. B. Quantification of Rad53p expression in wild type and chl1 cells. The intensity of the phosphorylated bands of Rad53 in Wild-type (699) and mutant cells 699Dchl1 (chl1), SL3 (rad24), SL3Dchl1 (rad24 chl1) cells in western blots were quantified using Image J software. The values of the Rad53p intensities were normalized with corresponding intensities of alpha-Tubulin to normalize the protein loading in different samples. C. G1/S-phase progression of single mutants, double mutant and wild-type cells in the presence of HU. DNA content of cells in (A) was measured by flow cytometry. Arrows indicate G1 and G2 DNA contents. D. Chl1p acts independently of Rad53p at G1/S after DNA damage. Wild92 type (699) and mutant cells 699Dchl1 (chl1), SL7 (rad53) and SL7Δchl1 (rad53 chl1) were grown to exponential phase and follow through same experimental 93 procedures as done in 4A. The graph represents the percentage of bud emergence in WT, chl1, rad53 and rad53chl1 cells at different time intervals after release from G1 arrest and 0.1% MMS Treatment simultaneously.The black filled symbols are given for cells treated with MMS, the grey filled symbols indicates the absence of MMS. Data shown are averages of values obtained from three independent experiments and the deviations from the mean are shown as error bars. E. Budding of mutant and wild-type cells after MMS treatment. The bright fields of WT and mutant cells from (D) at 40X resolution show the budded cells in different cultures after 2 hours release from MMS treatment. The budded cells are indicated with arrows.

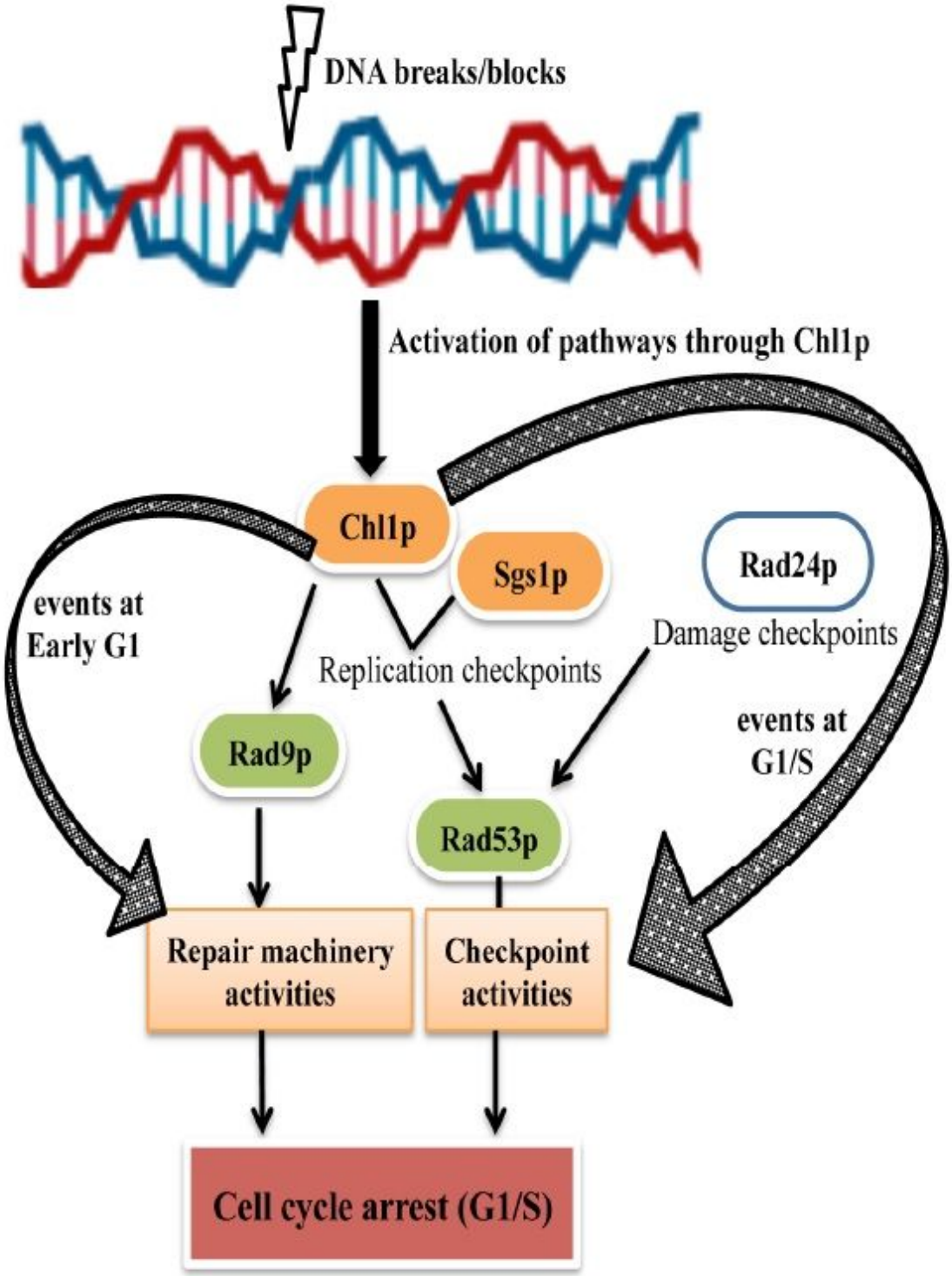

Figure 6

Chl1p plays a role as a checkpoint at G1/S phase, which leads to Rad53 activation and prevents bulk DNA synthesis. Apart from its checkpoint function at G1/S phase, it plays some additional function, may be the repair function independent of Rad53p and in synchrony with Rad9p to regulate the budding kinetics following insult to the genetic material

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