A protocol for genetic analysis at different stages of the nuclear division cycle in *Neurospora crassa*

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Abstract
The filamentous fungus *Neurospora crassa* is an organism that contains multiple nuclei in the asexual conidia and hyphae. Since the nuclei of dormant conidia are arrested at various points in the nuclear division cycle, it has been difficult to analyze drug sensitivity at the specific point of the cycle in *N. crassa*. In this study, we have established a useful method for analysis at different stages of the nuclear division cycle in *N. crassa*. This assay will be a reference for researchers to use the synchronized culture in other diverse analyses.

Introduction
The filamentous fungus *Neurospora crassa* is an organism that contains multiple nuclei in the asexual conidia and hyphae. Moreover, the process of mycelial growth from the germination of conidia differs from that of general cell division. In filamentous fungi, the mycelium grows by apical elongation, and organelles such as nuclei and mitochondria move fluidly within it (Harris 2001). In addition, the division of each nucleus during growth is asynchronous (Serna and Stadler 1978). These features have made it challenging to analyze cells at different stages of the nuclear division cycle on a synchronized culture, as has been established for other organisms.

The asynchronous nuclear division can be seen from the point at which dormant conidia germinate (Serna and Stadler 1978). Serna *et al*. developed two hypotheses to explain the unsynchronized nuclear division. The first is a model in which each nucleus in the conidia is arrested at a particular stage of the cycle, but the restart of the cycle is different in each nucleus, resulting in a lag. The second model is that even if the cycles of all conidia resume simultaneously with the resumption of growth, they are not synchronized because they are at different stages at the point of the arrest. Serna *et al*. tested this hypothesis by analyzing temperature-sensitive mutants in which the nuclear division cycle was inhibited at specific stages (Serna and Stadler 1978). The results supported the latter hypothesis and showed that the nuclei of dormant conidia are arrested at different stages.

Picolinic acid is a known metal chelator with a structure similar to nicotinic acid, which is a precursor in the biosynthesis of NAD^+. Analysis of rat kidney cells revealed that picolinic acid reversibly inhibits cell division at the G1 phase through an unidentified mechanism (Fernandez-Pol *et al*. 1977). Similarly, in *N. crassa*, the nuclei in the mycelia and conidia can be arrested at the G1 phase by treatment with picolinic acid. Furthermore, it is possible to synchronize the nuclear division cycle by release from picolinic acid treatment (Martegani *et al*. 1980). Using this method, the doubling time of the nuclei in mycelia was measured and estimated to be about 100 min (G1 phase 20 min, S phase 30 min, G2 phase 40 min, M phase 10

min) in minimal medium. More recently, it has been shown through flow cytometry that picolinic acid is potentially useful for nuclear division synchronization in *N. crassa* (Lobo *et al*. 2007). While multiple hypotheses have been proposed to explain how picolinic acid synchronizes nuclear division (Fernandez-Pol *et al*. 1977; Martegani 1981), the synchronization mechanism remains unclear.

In our previous study, it was suggested that MUS-38, the homologue of XPF in *N. crassa*, is not only involved in nucleotide excision repair (NER), which mainly functions in G1 phase as a replication-uncoupled pathway, but also in replication-coupled interstrand crosslink (ICL) repair (Tsukada *et al*. 2023). This has not been reported in budding or fission yeasts belonging to the same phylum, suggesting that *N. crassa* may conserve ICL repair mechanisms similar to higher eukaryotes rather than yeasts. Importantly, the involvement of XPF in the Fanconi anaemia (FA) pathway, which is a replication-coupled ICL repair system, was thought to exist only in higher eukaryotes. However, our data suggests that a similar function of the FA pathway may exist in *N. crassa*. In this study, to verify this possibility, we established an experimental method that synchronizes the nuclear division cycle of *N. crassa* using picolinic acid to measure drug sensitivity at the G1 and S phases, respectively. Using this method, sensitivity tests with an ICL-inducing drug showed that the *Δmus-43* strain, which is specifically deficient in NER, significantly increased sensitivity at the G1 phase, whereas the *Δmus-38* strain showed high sensitivity in both the S phase and G1 phase. These results indicate that MUS-38 is additionally involved in replication-coupled ICL repair in an NER-independent manner.

In this study, by refining the methods from a former report (Martegani *et al*. 1980), we have established a useful method for analysis of different stages of the nuclear division cycle in *N. crassa*. This assay can serve as a reference for researchers to use synchronized cultures in diverse analyses.

Materials and Methods

Strains, media, and genetic manipulations in *N. crassa.* *Neurospora crassa* strains used in this study are listed in Table 1. TSKD055 was used for the wild-type strain (Tsukada et al., 2023). The *Δmus-38* and *Δmus-43* strains were created in our previous study (Tsukada *et al*. 2023). The *Δmus-52* strain was obtained from Fungal Genetics Stock Center (FGSC). The *Δmus-11* strain from FGSC was crossed with TSKD055 to obtain a homokaryon. The homokaryotic *Δmus-11* strain (TSKD089-1) was used for experiments. General genetic manipulations were carried out as described (Davis and de Serres 1970). Vogel's minimal medium (Vogel's salts with 1.2% sucrose and 1.2% agar) was used for general culture. Glycerol complete medium (Vogel's salts with 0.25% yeast extract, 0.1% casamino acid, 0.5% malt extract, 1% vitamin stock, 1% glycerol, and 1.2% agar) was used for harvesting conidia. Colony formation medium (Vogel's salts with 1% sorbose, 0.05% glucose, 0.05% fructose, and 1.2% agar) was used for measurements of cell survival and selections of transformants.

Construction of vector for creating HA-tagged PCNA. Plasmid pTSKD6 (Tsukada *et al*. 2020) was modified for the substitution of *pcna* (Figure 1). The upstream region, coding region, *bar* (bialaphos resistance gene), and downstream region were connected by In-Fusion (Takara Bio). The HA tag at the N-terminal region was attached by inverse PCR: primers designed to attach the HA sequence were used to perform inverse PCR on a sufficiently diluted plasmid as a template. The PCR products were then treated with *Dpn*I to digest the template plasmid. The digested DNA was phenol-chloroform extracted and precipitated, resuspended in [7.5 µl distilled

water, 1 µl ATP (final concentration of 10 mM), 1 µl Buffer A (Thermo Fisher Scientific), 0.5 µl T4 Polynucleotide Kinase (Thermo Fisher Scientific)], and incubated for 1 hour at 37°C. The enzyme was inactivated by treatment at 75°C for 10 min, and 5 µl was ligated by T4 DNA ligase (NIPPON GENE) and introduced into *E. coli* DH5α. The complete plasmid was verified by DNA sequencing using 3130 Genetic Analyzer (Thermo Fisher Scientific) and named pTSKD47. Primers for the construction of the plasmid are listed in Table 2.

Transformation of *N. crassa.* Electroporation was employed for transformation with slight modifications as previously described (Margolin *et al*. 1997; Ninomiya *et al*. 2004). Conidia collected from the *Δmus-52* strain underwent three washes with 1 M sorbitol. After washing, over 300 ng of linearized plasmid was mixed with a 40 µl conidial suspension and subjected to a 5-minute cooling period on ice. The chilled mixture of conidia and plasmid was then transferred to an electroporation cuvette (2 mm gap width). The electroporation was conducted using an ECM 630 Electroporation System (BTX) with settings of 1.5 kV, 200 Ω, and 50 µF. Following electroporation, 1 ml of Vogel's minimal liquid medium was added to the cuvette. The resulting solution was gently mixed by pipetting and relocated to a fresh microtube for a 2–hour incubation period at 30°C. The treated conidial suspension was combined with colony formation medium containing 200 µg/ml bialaphos before being poured into a 15 cm diameter Petri dish. Following an incubation period of 2–3 days at 30°C, the resulting transformants were selected. The accuracy of gene targeting was confirmed by PCR. The strains correctly targeted were crossed with wild-type strain (TSKD055) to exclude mutation of *mus-52*.

Treatment with picolinic acid. A conidial suspension (2×10^8 conidia/ml) was treated with 25 mM of picolinic acid in Vogel's minimal liquid medium in a sterilized Erlenmeyer flask or centrifuge tube. The conidial suspension was incubated with shaking for 2 hours at room temperature and then transferred to a 50 ml centrifuge tube and spun out (1,600 g, 2 min). The supernatant was removed, and the conidial pellet was resuspended in 40 ml phosphate buffer (0.067 M, pH 7.0). The resuspended conidia were washed and spun out at 1,600 g for 2 min. To completely remove picolinic acid, conidia were washed twice. The washed conidia were resuspended with Vogel's minimal liquid medium at a final concentration of 2×10^8 conidia/ml and incubated with shaking at room temperature to restart growth. The concentration of conidia and their growth was measured by spectrophotometry (450 nm; SHIMADZU, UV-1800). The average absorbance values were calculated from three measurements.

Measurement of DNA content. A 50 µl aliquot of the conidial suspension was mixed with 200 µl of quartz sand and 500 µl of isolation buffer [1% *N*-lauroylsarcosine in TE (pH 8.0)] in a screw cap tube. The conidia were crushed with a Micro Smash™ (TOMY, MS-100) set at 5,500 rpm for 100 sec at 4°C. The tube was centrifuged for removal of foam, then 300 µl of 7.5 M ammonium acetate was added and the tube was cooled on ice for at least 10 minutes. After centrifugation, 500 µl of the supernatant was transferred to a new microtube, and 500 µl of phenol/chloroform/isoamyl alcohol was added. After centrifugation, 300 µl of the supernatant was transferred to a new microtube, to which 750 µl of 99.5% ethanol was added. The tube was then cooled at −80°C for at least 10 minutes. Centrifugation was performed again, and the supernatant was removed, to which 1,000 µl of 70% ethanol was added, followed by gentle mixing and centrifugation. The supernatant was discarded and the pellet was dried. The precipitate was dissolved in TE (pH 8.0). RNase A (final concentration of 20 µg/ml) was added

to the solution, which was then incubated at 37°C for 30 min. Ethanol precipitation was then used to remove digested RNA before DNA concentration was measured. A NanoDrop One^{C} (ThermoFisher) was used for measurements, and the average of two samples was calculated as the measured value.

Measurement of number of nuclei. To a microtube containing 1 ml of 99.5% ethanol, 50 µl of conidial suspension was added and fixed for 30 min at room temperature. After 20 min of centrifugation, the supernatant was discarded, and the pellet was dried. The pellet was resuspended in 10 µl of 50 µg/ml Hoechst 33342 (Nacalai Tesque) diluted in PBS buffer and treated for 30 min at 37°C. A 5 µl aliquot was prepared for observation using a fluorescence microscope (Olympus BX51) to measure the number of nuclei per conidia. At least 100 conidia per sample were examined.

Western blotting. Conidial suspensions were transferred to 15 ml centrifuge tubes and spun out at 1,600 g for 5 min. Total cell protein was then extracted in protein isolation buffer [50 mM HEPES buffer, pH 7.6, 10% glycerol, 137 mM NaCl, complete protease inhibitor cocktail (Roche Diagnostics)] by homogenization with a Micro Smash (MS-100; 5,500 rpm, 100 sec, 4°C). The extracts were electrophoresed in 10% SDS-PAGE gels and transferred to a PVDF membrane. The membrane was blocked with blocking buffer [5% skim milk in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.68 mM KCl and 0.1% Tween 20) buffer] for 1 hour at room temperature. The membrane was then probed with an anti-HA antibody (1/20,000, Proteintech) in 10 ml blocking buffer for 1 hour at room temperature. After washing with TBST buffer three times, the membrane was probed with an HRP-conjugated anti-mouse IgG antibody (1/10,000, Promega) in 10 ml blocking buffer for 1 hour at room temperature. Probed membranes were washed with TBST buffer three times before treatment with chemiluminescent substrate (EzWestLumi One, ATTO). Blots were imaged with a ChemiDoc XRS system (Bio-Rad).

Cell survival assay. After picolinic acid treatment of conidial suspensions as described above, the suspensions were divided in half for G1 and S phase treatment, respectively. For the G1 phase treatment group, the suspension was divided into two 15 ml centrifuge tubes of 2 ml each. Then, 1,2,7,8-Diepoxyoctane (DEO) was added to one of them to reach a final concentration of 100 mM, and the tubes were incubated at room temperature for 30 min with shaking. Then, the tubes were centrifuged (1,600 g, 2 min) to remove the supernatant and washed twice with 10 ml phosphate buffer. The conidia were appropriately diluted and spread on 16 ml of colony formation medium. For the S phase treatment group, picolinic acid-treated conidia were washed as described above and resuspended in Vogel's minimal liquid medium at a final concentration of 2×10^8 conidia/ml. Conidial suspension (2 ml) was added to two 15 ml centrifuge tubes and incubated for 1.5 h, followed by DEO treatment in the same way as described for the G1 phase treatment group. Each plate was incubated in an incubator at 30°C for two days, and the growing colonies were counted. The average of the two plates was determined, and the survival rates from the DEO-untreated control and the ratio of the survival at the G1 and S phases were calculated.

Results and Discussion

Establishment of experimental procedure of picolinic acid treatment

Picolinic acid arrests nuclei of *N. crassa* at the G1 phase. The nuclear division cycle can be synchronized by releasing from picolinic acid treatment (Martegani *et al*. 1980). However, this

method has not been applied to phenotypic analyses such as drug sensitivity tests. Therefore, based on previous studies, the timing of progression from the G1 phase to the S phase was verified in the present study by adjusting the concentration of the conidia, picolinic acid concentration, and incubation time.

The experiment is schematically shown in Figure 2A (see Materials and Methods for details). A conidial suspension of the wild type was prepared in a minimal liquid medium, to which picolinic acid was added, and then the culture was shaken for two hours. The conidia were then washed thoroughly. Growth was confirmed by measuring the absorbance of the culture, which showed an increase after about two hours of incubation without picolinic acid compared to the culture in the presence of picolinic acid (Figure 2B). In the earlier study, picolinic acid treatment concentrations of 9 mM in Vogel's minimal medium were used for conidia (Martegani *et al*. 1980), but growth did not stop at 9 mM under the conditions of the present study (data not shown). This may be due to the extremely high concentration of conidia used in the present study (2×10^8 conidia/ml). Treatment of conidia with concentrations of picolinic acid higher than 25 mM did not restart growth after releasing from treatment (data not shown). To prepare sufficient quantities of 2×10^8 conidia/ml suspension, it is necessary to grow a large quantity of hyphae. It may be possible to scale down, if necessary, but care should be taken as cell concentration may affect the rate of growth. It is known that dormant conidia are arrested at various points of the nuclear division cycle (Martegani *et al*. 1980). Therefore, in the earlier study, proliferation was observed for approximately one hour from the start of picolinic acid treatment until all nuclei were arrested at the G1 phase (Martegani *et al*. 1980). However, this was not clearly confirmed in the present study. This could also be due to the slower initiation of germination and growth of the conidia when they were cultured at high concentrations.

In order to confirm the synchronization of the nuclear division cycle, we sampled the culture after picolinic acid removal over time and measured the amount of DNA (Figure 2C) and the number of nuclei per conidia (Figure 2D). The amount of DNA did not increase much until one hour after the removal of picolinic acid, but a sharp rise was observed from 1 to 2 hours after removal. Then, it stopped the increase from 2 to 3 hours, and showed a sharp rise again from 3 to 4 hours. In a cell population where the nuclear division cycle is synchronized, it is expected that a plot of DNA amount and the number of nuclei will show a sharp rise and a plateau on both sides, so we believe that cell division is synchronized under this experimental condition. This pattern in the graph was not observed in similar experiments using unsynchronized cells (Serna and Stadler 1978). In terms of the number of nuclei per conidia, an increase was detected from about 2.5 hours after the removal of picolinic acid. That is, the nuclei that have finished the S phase are proceeding to the G2 phase and the M phase, and cell division is occurring. Therefore, we detected that there is a delay in the increase in the number of nuclei compared to the increase in DNA amount. If all nuclei had been synchronized, it is expected that the amount of DNA should double at the end of the S phase, but this increase was not confirmed under this experimental condition (Figure 2C). Also, the number of nuclei per conidia stagnated at about 1.4 times at 3.5 hours later (Figure 2D). It is possible that a certain number of nuclei are completely stopped in the G1 phase by picolinic acid. An alternative explanation is that the restart of nuclear division cycles may not occur simultaneously upon the removal of picolinic acid. This discrepancy may be related to the inherent variation in the number of nuclei present within the *N. crassa* macroconidia, leading to some heterogeneity in the response to picolinic acid treatment.

Neurospora crassa forms two types of conidia: relatively large, orange macroconidia and small, green-brown microconidia (Ramesh 1999). The macroconidia commonly used in experiments do not contain a uniform number of nuclei (about 1 to 4), so it may be difficult to align and analyze them in a completely uniform cell population. On the other hand, since microconidia are mononuclear, more accurate analysis may be possible. However, the amount of microconidia is significantly less than that of macroconidia, it is difficult to collect enough amounts, and the germination rate is extremely low (Ramesh 1999), so it could be challenging to use microconidia for phenotypic analysis.

Based on these data, we were able to synchronize the nuclear division cycle by adding 25 mM of picolinic acid to a conidia suspension of 2×10^8 cells/ml prepared in minimal liquid medium and incubating it at room temperature for 2 hours followed by release from picolinic acid treatment. The experiments described below were performed under these conditions.

Analysis of HA-PCNA expression

Proliferating cell nuclear antigen (PCNA) acts as a clamp molecule that connects double-stranded DNA and DNA polymerase during DNA replication. It is known that its expression rises from late G1 to S phase (Bravo *et al*. 1987; Sasaki *et al*. 1993; Kisielewska *et al*. 2005; Bártová *et al*. 2017). Therefore, assuming that PCNA expression in *N. crassa* is similar to most other organisms (rising from late G1 to S and falling thereafter), we used HA-tagged PCNA (HA-PCNA) to estimate the timing of S phase in our synchronized cultures.

We performed the picolinic acid treatment using the method described above, sampled the treated conidial suspension during the recovery period, and analyzed the expression of N-terminally-tagged HA-PCNA by western blotting (Figure 3). The expression of HA-PCNA started to increase about 1 hour after picolinic acid removal, peaked around 2 to 2.5 hours, and then decreased. As DNA levels increase in S phase due to DNA replication, this result roughly correlates with the actual plot of measured DNA quantity (Figure 2C). Thus, the synchronized cell division cycle progresses from G1 phase to S phase around 1 to 1.5 hours after picolinic acid removal.

In previous studies, the duplication time of nuclei in hyphae cultured in minimal liquid medium was estimated to be approximately 100 minutes, of which the S phase was about 30 minutes (Martegani *et al*. 1980). However, in this study, the expression of HA-PCNA increased over approximately 1.5 hours from 1 hour to 2.5 hours after picolinic acid removal, suggesting that the cell division cycle might progress at a relatively slow speed under our conditions. Or, because we used conidia before germination, they may show a different nuclear division cycle pattern compared to post-germination hyphal nuclei.

In this study, we did not measure the actual synchronization rate, so it is unclear what proportion of nuclei shift to S phase with the same timing. Therefore, we cannot exclude the possibility that time differences in progression to S phase may occur due to conidium size or number of nuclei. Because the numbers of nuclei in macroconidia are random, it may not be possible to make it completely uniform, and it may be necessary to devise ways to collect conidia of the same size, perhaps by filtration. Moreover, to measure the synchronization rate, observing nuclear division directly by live imaging could be useful.

In conclusion, we have shown that the proportion of S phase nuclei becomes highest between 1 and 2.5 hours after picolinic acid removal when the nuclear division cycle is synchronized by our experimental system.

Measuring drug sensitivity at different nuclear division cycles in N. crassa

Below, we demonstrate the practical utility of our established method. It has been suggested that MUS-38, the homologue of XPF in *N. crassa*, is involved in replication-coupled ICL repair (Tsukada *et al*. 2023). To test this possibility, we treated conidia with DEO, an ICL inducing agent, during G1 and S phase and compared their sensitivities. We believe that the KO strains of genes primarily functioning in replication-coupled repair should show higher DEO-sensitivity during S phase than during G1 phase. The experiment is schematically shown in Figure 4A. We treated the conidia with picolinic acid for 2 hours under the same conditions as before, and we immediately added DEO (final concentration 100 mM) and processed it for 30 minutes for the G1 phase treatment group. For the S phase treatment group, we performed the recovery incubation for 1.5 hours after picolinic acid removal and then similarly performed the DEO treatment. We conducted sensitivity tests under these two conditions. Figure 4B shows the survival rate at each cycle, and Figure 4C shows the ratio of S phase to G1 phase survival. In the wild-type strain, there was not much difference in DEO sensitivity between S phase and G1 phase, but the *Δmus-43* strain showed over 20 times higher sensitivity in G1 phase compared to S phase. This is consistent with previous findings that XPA (mammalian homolog of MUS-43) functions mainly in replication-uncoupled ICL repair by being involved in NER (Seol *et al*. 2018). NER is also partially involved downstream of the FA pathway, which is a replication-coupled ICL repair system, and it is thought to ultimately remove residual damaged nucleotides after replication has completed (Mouw and D'Andrea 2014). However, the *Δmus-43* strain demonstrated significantly higher sensitivity in the G1 phase rather than S phase, suggesting that the repair activity of NER in *N. crassa* primarily occurs in the G1 phase, as in other organisms. On the other hand, although the *Δmus-38* strain showed significantly higher sensitivity in both S phase and G1 phase than the *Δmus-43* strain, it showed slightly higher sensitivity in the S phase. The *Δmus-11* strain also showed high sensitivity in the S phase. MUS-11, a RAD52 homologue, functions in S/G2 phase when sister chromatids are available, by being involved in homologous recombination, which is also a key mechanism participating downstream of the FA pathway (Sakuraba *et al*. 2000; Seol *et al*. 2018). With these results, we have shown that MUS-38 functions in replication-coupled ICL repair during S phase, similarly to MUS-11. Furthermore, the *Δmus-38* strain shows higher sensitivity than the *Δmus-43* and *Δmus-11* strain, and the difference between the G1 phase and S phase was smaller than these strains (Figure 4C). From these results, we believe that MUS-38 functions in both NER, which primarily handles repair in the replication-uncoupled pathway, and in the replication-coupled pathway that is independent of NER.

This study's established method of survival rate measurement involves a temporary drug treatment at specific stages of the nuclear division cycle. However, it is important to note that it does not directly evaluate the activity of each gene at each stage, as the conidia are immediately spread on agar medium for culture after washing out the drug. In other words, it is unclear at which stage (during drug treatment or during culture on agar medium) the effects of the treated drug result in cell death. Therefore, comparing survival rates between strains is not straightforward. However, by standardizing the survival rate at G1 phase by performing drug treatment under two conditions, as done in this study, it is possible to evaluate the relative activity of each gene product at G1 and S phase.

In this study, an experimental system was established by using a wild-type strain to confirm synchronization of the nuclear division cycle. However, in mutant strains showing growth delay, the timing of S phase progression after G1 phase arrest may be delayed compared

to the wild type. Therefore, in strains showing obvious growth abnormalities, it may be necessary to consider the timing of the start of drug treatment. However, even though the *Δmus-11* strain used in this study showed slightly slower colony growth on colony formation medium compared to other strains (data not shown), it exhibited similar growth in liquid medium to the wild-type strain after picolinic acid removal, according to the experimental scale established in this study (Figure 4D). If the progression of the nuclear division cycle is slow and the start of S phase is after 1.5 hours from picolinic acid removal, we expect that the difference in survival rate would be about the same as in G1 phase. However, DEO sensitivity in S phase of the *Δmus-11* strain was about 10 times higher than in G1 phase (Figure 4C). From these results, we believe that even in strains with some growth delay, the effect in S phase can be analyzed with a 30–minute drug treatment because the duration of S phase is sufficiently long. This is a significant advantage for *N. crassa*, which cannot easily confirm the nuclear division cycle. However, picolinic acid has been suggested to inhibit gene transcription by affecting RNA polyadenylation (Martegani 1981). Therefore, mutant strains with genes involved in RNA processing may exhibit different mechanisms of G1 phase arrest and subsequent synchronization by picolinic acid.

In conclusion, we established an experimental system to synchronize the nuclear division cycle of *N. crassa* using picolinic acid and measure drug sensitivity in G1 and S phases. As a result of sensitivity analysis with DEO, the *Δmus-38* strain showed higher sensitivity in S phase compared to the *Δmus-43* strain. With these results, we have shown that MUS-38 is involved in NER-independent replication-coupled ICL repair in addition to NER.

Limitations of Study

In this study, we synchronized the nuclear division cycle by treating a high concentration of 2×10^8 conidia/ml with picolinic acid. While this offers the advantage of enabling analyses that require a large number of cells, it is also possible that the high concentration of conidia changes various aspects such as gene expression. Researchers can investigate these concerns as appropriate for the specific objectives of their experiments. Second, picolinic acid reportedly does not change the level of NAD (Fernandez-Pol *et al*. 1977), however, because this method may impact its metabolism, it is expected that various facets of cellular biology may be altered by picolinic acid treatment. This should be considered when interpreting the effects that are presumed to be a result of synchronization. In addition, we measured drug sensitivity with a fixed concentration, however, measuring dose-dependency could be a significant consideration for conducting a more detailed analysis.

Data and Resource Availability

Strains FGSC 15968 and FGSC 16079 can be obtained from the Fungal Genetics Stock Center. All other strains and resources constructed for this study are available upon request.

Competing Interests

The authors declare no competing interests.

Author Contributions

K.T. performed all the experiments and wrote the manuscript. S.H. and S.T. commented on the manuscript. S.T. supervised the work. All authors discussed the data and approved the submitted manuscript. All authors reviewed and approved the final revised manuscript for publication.

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Literature Cited

Bártová, E., J. Suchánková, S. Legartová, B. Malyšková, M. Hornáček *et al*., 2017 PCNA is recruited to irradiated chromatin in late S-phase and is most pronounced in G2 phase of the cell cycle. Protoplasma 254: 2035–2043. https://doi.org/10.1007/s00709-017-1076-1

Bravo, R., R. Frank, P.A. Blundell and H. Macdonald-ravo, 1987 Cyclin/PCNA is the auxiliary protein of DNA polymerase-α. Nature 326: 515–517.

Davis, R.H., and F.J. de Serres, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. 17: 79–143. https://doi.org/10.1016/0076-6879(71)17168-6

Fernandez-Pol, J.A., V.H. Bono and G.S. Johnson, 1977 Control of growth by picolinic acid: Differential response of normal and transformed cells. Proc. Natl. Acad. Sci. U. S. A. 74: 2889–2893. https://doi.org/10.1073/pnas.74.7.2889

Harris, S.D., 2001. Septum formation in *Aspergillus nidulans*. Curr. Opin. Microbiol. 4: 736–739.

Kisielewska, J., P. Lu and M. Whitaker, 2005 GFP-PCNA as an S-phase marker in embryos during the first and subsequent cell cycles. Biol. Cell 97: 221–229. https://doi.org/10.1042/bc20040093

Lobo, D.S., I.B. Pereira, L. Fragel-Madeira, L.N. Medeiros, L.M. Cabral *et al*., 2007 Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. Biochemistry 46: 987–996. https://doi.org/10.1021/bi061441j

Margolin, B.S., M. Freitag and E.U. Selker, 1997 Improved plasmids for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet. Rep. 44. https://doi.org/10.4148/1941-4765.1281

Martegani, E., 1981 Inhibition of RNA synthesis in *Neurospora crassa* hyphae treated with picolinic acid. Eur. J. Biochem. 121: 71–76. https://doi.org/10.1111/j.1432-1033.1981.tb06431.x

Martegani, E., M. Levi, F. Trezzi and L. Alberghina, 1980 Nuclear division cycle in *Neurospora crassa* hyphae under different growth conditions. J. Bacteriol. 142: 268–275.

Mouw, K.W., and A.D. D'Andrea, 2014 Crosstalk between the nucleotide excision repair and Fanconi anemia/BRCA pathways. DNA Repair (Amst). 19: 130–134. https://doi.org/10.1016/j.dnarep.2014.03.019

Ninomiya, Y., K. Suzuki, C. Ishii and H. Inoue, 2004 Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc. Natl. Acad. Sci. U. S. A. 101: 12248–12253. https://doi.org/10.1073/pnas.0402780101

Ramesh, M., 1999. Microconidia of *Neurospora crassa*. Fungal Genet. Biol. 26: 1–18.

Sakuraba, Y., A.L. Schroeder, C. Ishii and H. Inoue, 2000 A *Neurospora* double-strand-break repair gene, *mus-11*, encodes a *RAD52* homologue and is inducible by mutagens. Mol. Gen. Genet. 264: 392–401. https://doi.org/10.1007/s004380000342

Sasaki, K., A. Kurose and Y. Ishida, 1993 Flow cytometric analysis of the expression of PCNA during the cell cycle in hela cells and effects of the inhibition of DNA synthesis on it.

Cytometry 14: 876–882. https://doi.org/10.1002/cyto.990140805
Seol, J.H., C. Holland, X. Li, C. Kim, F. Li *et al*., 2018 Distinct roles of XPF-ERCC1 and Rad1-Rad10-Saw1 in replication-coupled and uncoupled inter-strand crosslink repair. Nat. Commun. 9. https://doi.org/10.1038/s41467-018-04327-0
Serna, L., and D. Stadler, 1978 Nuclear division cycle in germinating conidia of *Neurospora crassa*. J. Bacteriol. 136: 341–351.
Tsukada, K., S. Hatakeyama and S. Tanaka, 2023 DNA interstrand crosslink repair by XPF-ERCC1 homologue confers ultraviolet resistance in *Neurospora crassa*. Fungal Genet. Biol. 164: 103752. https://doi.org/10.1016/j.fgb.2022.103752
Tsukada, K., R. Yoshihara, S. Hatakeyama, A. Ichiishi, and S. Tanaka, 2020 A partial photoreactivation defect phenotype is not due to unrepaired ultraviolet-induced pyrimidine dimers in ultraviolet-sensitive mutants of *Neurospora crassa*. Genes Genet. Syst. 95: 281–289. https://doi.org/10.1266/ggs.20-00022

Table 1 *Neurospora crassa* strains used in this study

Strain	Genotype	Source, reference
TSKD055	*A*	Tsukada et al. (2023)
FGSC 15968	*a mus-52::hph*	FGSC*
FGSC 16079	*a mus-11::hph* (heterokaryon)	FGSC
TSKD089-1	*A mus-11::hph*	This study
TSKD115-10	*A mus-38::bar*	Tsukada et al. (2023)
TSKD269-1	*A mus-43::bar*	Tsukada et al. (2023)
TSKD537-7	*a HA-pcna:bar*	This study

*Fungal Genetics Stock Center

Table 2 Primers used in this study

Primer name	Sequence (5'-3')
pFLAGN1_SP6_F	TATAGTGTCACCTAAATCGTATGTG
pFLAGN1_NotI_R	GGCCGCCACCGCGGTGG
PCNA_up_F	ACCGCGGTGGCGGCCCTATAATCTTACATGGCATC
PCNA_ORF_R	GTCTGATCCGAATTCTTACTCCTCGTCGCCAATC
Bar_F	GAATTCGGATCAGACATGATAAGATACATTGATG
bar_PCNA_R	AGGTAAATGGGGCGTTCAGATCTCGGTGACGGGC
PCNA_dwn_F	ACGCCCCATTTACCTCCAACAAC
PCNA_dwn_R	TTAGGTGACACTATAGATCAATTGGAAATAGTATC
PCNA_HA_F	TTCCAGATTACGCTCTTGAAGCACGGTTGGAGCAG
PCNA_HA_R	CATCGTATGGGTACATTTCTGTTGTTGTGGTAG

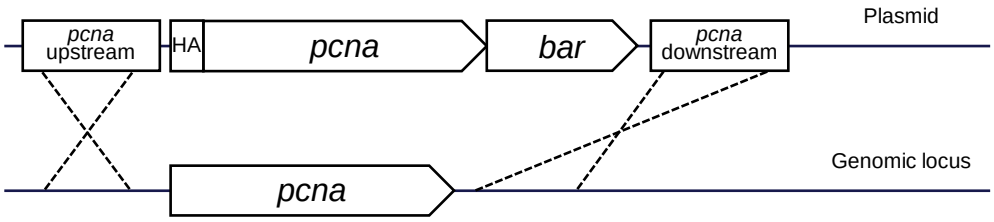

Figure 1. Creation of HA-tagged PCNA. The HA-tagged *pcna* gene was created by substituting the internal gene. The bialaphos resistance gene (*bar*) was inserted after the *pcna* gene for selection of transformants.

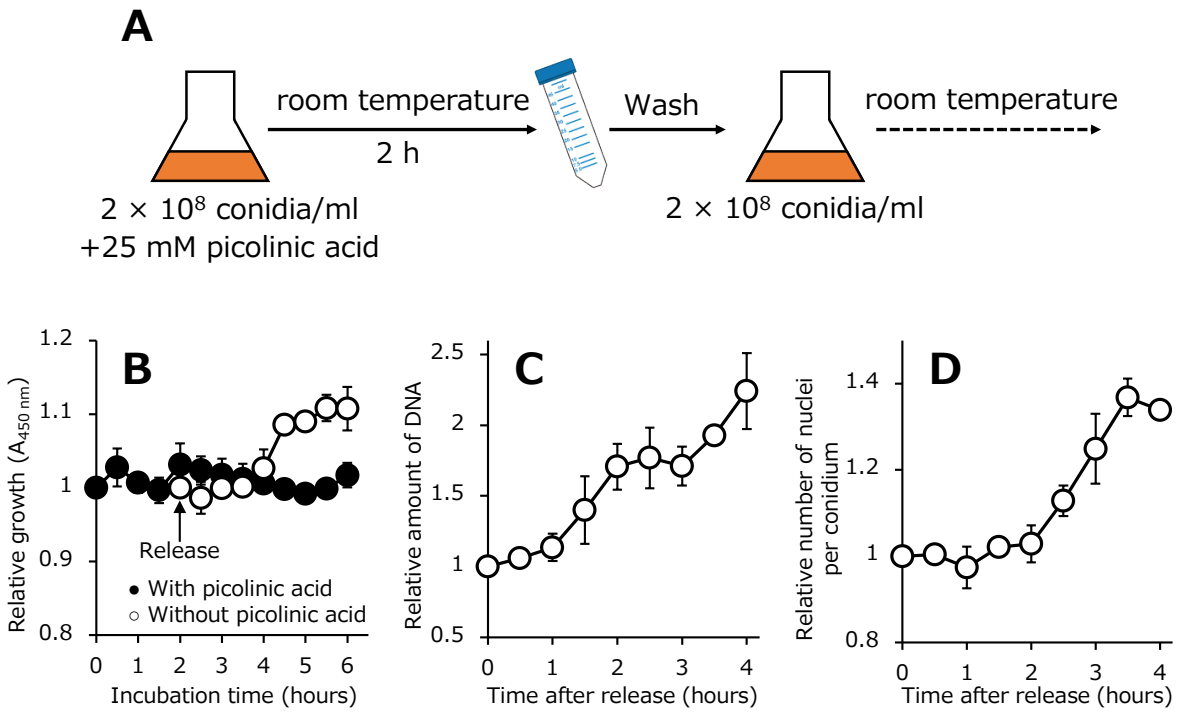

Figure 2. Picolinic acid mediated synchronization of nuclear division cycle. A) Overview of the experiment. B) Verification of proliferation. Conidial suspensions during picolinic acid treatment or during recovery incubation were sampled, and absorbance was measured at 450 nm. C) Measurement of DNA quantity. DNA was extracted from conidia during recovery incubation, and its concentration was measured. D) Measurement of the number of nuclei per conidium. Conidia collected during recovery incubation were stained with Hoechst 33342, and the number of nuclei per conidium was measured. The mean of the relative values, taking the start point of the culture as one, was plotted. Error bars represent standard errors calculated from data from at least three independent experiments.

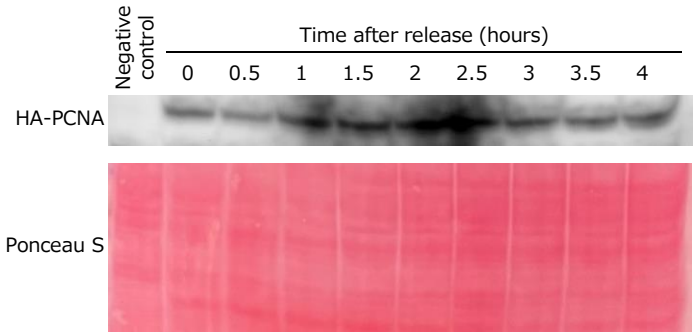

Figure 3. Analysis of HA-PCNA expression levels**.** Protein was extracted from conidia during recovery incubation, and HA-PCNA was detected by western blotting. 100 μg of protein was loaded into each lane. Ponceau S staining is shown as a loading control. This is a representative image of two independent experiments.

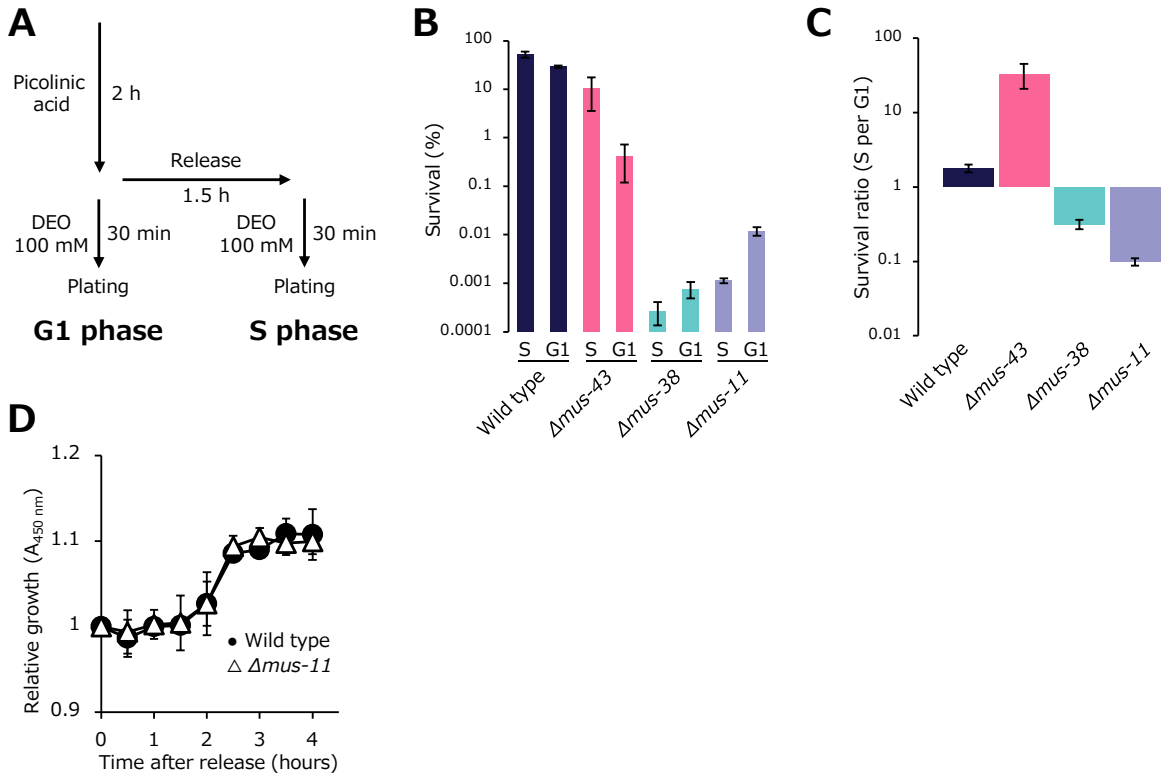

Figure 4. DEO sensitivity in G1 and S phases. A) Overview of the experiment. B) Survival rate upon DEO treatment. DEO was applied to conidia during G1 phase and S phase as shown in A, and spread on medium. After 2 days of incubation, the colonies in each dish were counted, and the survival rate was calculated. C) Ratio of sensitivity in each cycle. Based on the survival rate obtained in each experiment, the ratio of sensitivity in the G1 phase to S phase was calculated. D) Confirmation of proliferation. This assay was conducted as described in Figure 2B. Error bars in each figure represent standard errors calculated from data from at least three independent experiments.