Investigating functional links between Condensin and DNA replication in the fission yeast *S. pombe*

A thesis

Submitted to

Indian Institute of Science Education and Research Pune

In partial fulfilment of the requirements for the

BS-MS Dual Degree Programme

By

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April 2019

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Certificate

This is to certify that this dissertation entitled “Investigating functional links between Condensin and DNA replication in the fission yeast *S. pombe*” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER) Pune, represents original research carried out by Apoorva S Sankar at Laboratory of biology and modelling of the cell (LBMC), under the supervision of Dr. Pascal Bernard, during the academic year 2019-2020.

Apoorva S Sankar

Dr. Pascal Bernard

Declaration

I hereby declare that all the matter embodied in the report entitled “Investigating functional links between Condensin and DNA replication in the fission yeast *S. pombe*” are the results of the investigations carried out by me at the Laborartory of biology and modelling of the cell (LBMC), under the supervision of Dr. Pascal Bernard, and the same has not been submitted anywhere else for any other degree.

Apoorva S Sankar

Dr. Pascal Bernard

Abstract

Condensin is a universal genome organiser with diverse roles throughout the cell cycle. While its role in mitotic chromosome condensation has been well characterized, its role during the remaining cell cycle is still unexplored. Studies have shown that condensin is active and functional during interphase. It remains unclear however, whether condensin is involved in DNA replication and/or DNA damage repair and how this function is achieved. In this study, I have used the fission yeast *Schizosaccharomyces pombe* to show that condensin is involved in the maintenance of DNA integrity during replication stress. We propose that condensin is involved in DNA checkpoint pathways and function as a link between DNA replication and mitotic condensation.

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Acknowledgment

I convey my sincerest gratitude to Dr. Pascal Bernard for his valuable guidance and advice during this period. I would like to thank the entire team chromatin dynamics and chromosome architecture for their support and advice both regarding my project and on how to survive in France. I would like to thank IISER Pune and ENS Lyon for providing me with this opportunity to work at LBMC and all the members of LBMC for their support. I express my sincere gratitude to Isabelle Servais who has continued to help me and made sure everything was in order be it the documents necessary for my internship or my stay or my life in Lyon. I thank Dr Sanjeev Galande for his guidance and input to the project. I am forever grateful to IISER Pune for all the lessons and opportunities it has given me. Lastly, I would like to thank Goury, Jiffin and Sanika for their support and help in completing my project and not letting me lose hope.

Introduction

Condensins are pentameric protein complexes known for their role in chromosome assembly. They belong to the conserved family of SMC (Structural Maintenance of Chromosomes) complexes which also include cohesin, SMC5/6 and bacterial SMCs (Hirota et al. 2004; Toselli-Mollereau et al. 2016; Hirano 2005; Kakui et al. 2017; Hirano 2012; Hirano 2016).

Condensins are the key architects in folding, segregating and compacting DNA into chromosomes during mitosis. It has been shown in vitro that purified budding yeast condensin is able to bind to naked DNA and extrude loops in an ATP hydrolysis dependent manner (Ganji et al.2018). More recently studies by Kim et al and Baumann (Baumann, 2020; Kim et al., 2019) have shown that cohesin, another member of the SMC family also compacts DNA via loop extrusion, suggesting that loop extrusion is a common mechanism by which SMC complexes shape the genome (Sedeño Cacciatore and Rowland, 2019, Baxter et al., 2019; Goloborodko et al., 2016; Marko et al., 2019; Nuebler et al., 2018)

Current models propose that condensin binds to DNA during early mitosis, initiating looping of DNA by extrusion leading to the formation of cylindrical chromatids with radial loops that will then undergo further compaction and organisation (Gibcus et al. 2018) (fig.1). What remains unclear, however, are the mechanisms by which condensin achieves its function in a crowded and changing chromatin environment.

The condensin complex consists of two long (50 nm) ATPase SMC subunits, a Kleisin subunit and two additional subunits. There are two types of condensin in eukaryotes, namely, Condensin I and Condensin II. They share the same pair of SMC subunits; SMC2 and SMC4 and have different non-SMC subunits. CAP-D2, CAP-G, and CAP-H for condensin I, and CAP-D3, CAP-G2, and CAP-H2 for

condensin II (fig.1). The two condensins differ in their localization throughout the cell cycle, with condensin II being nuclear throughout the cell cycle as opposed to condensin I which is largely cytosolic and has access to chromosomes only during mitosis (Hirota et al. 2004). Condensin I and II massively associate with chromatin during mitosis, but do not co-localize along chromosome axes (Ono 2003,Walther 2018). The non-SMC regulatory subunits play a key role in determining the spatio-temporal localization of condensin complexes.

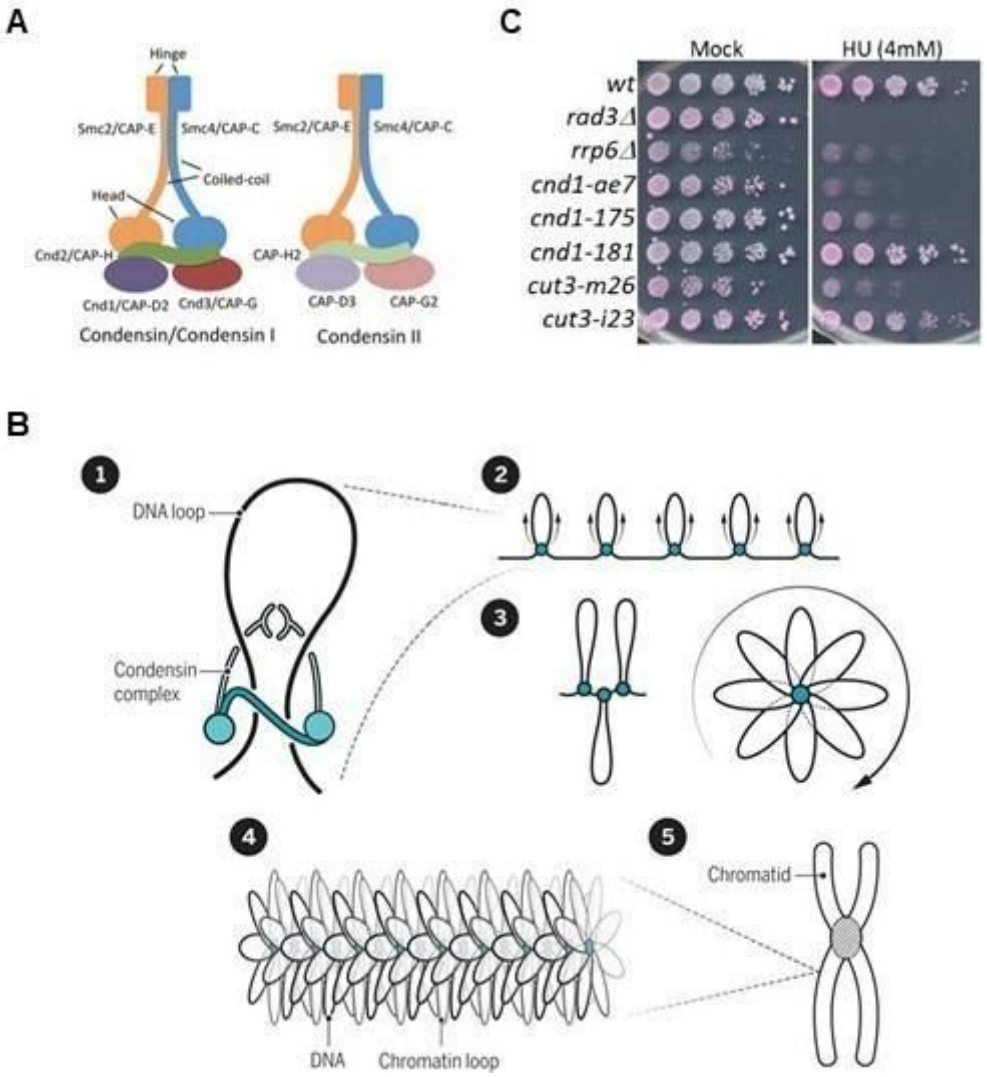


Figure 1 : *Chromosome condensation driven by condensin* (A) *Condensin complexes.* Structure of condensin I and II. The names of fission yeast condensin complexes are written before the / in condensin I (Figure adapted from Uchiyama and Fukui 2015) . (B) *Condensin brings about DNA compaction via loop extrusion .* Scheme of current model of DNA compaction by condensin (adapted from Nasmyth 2017) See text for details.(C) *Some condensin mutants are HU sensitive.* Serial dilution of cells have been made on indicated media and incubated for 3 days at 30ºC. Cells lacking the sensor kinase rad3 was used as control.

Apart from its role during mitosis, studies have shown that condensin is active and functional during mitosis. Studies by Heale et al have shown that condensin I interacts with the DNA nick sensor PARP1 in mammalian cells and is involved in DNA single stranded break and base excision repair (Heale et al. 2006; Kong et al. 2011). Studies in drosophila have also shown that condensin I is involved in DNA damage repair and that condensin II is involved in the organisation and compartmentalisation of the genome during interphase (Bauer et al. 2012; Dej et al. 2004) . Furthermore, studies have shown that condensin II is active during S phase in human cells and is required to separate sister chromatids. Adding mild replicative stress to condensin II depleted cells also increases the frequency of chromosome segregation defects (Ono et al. 2013).

Fission yeast *Schizosaccharomyces pombe* is an excellent model organism with a short life span, genetic tractability and chromatin structure comparable with mammals. *S. pombe* possesses a single condensin complex structurally similar to condensin I (Sutani et al 1999). Studies by Aono et al have shown in *pombe* that a mutation in the cnd2 subunit of condensin causes the cell to be sensitive to replication stress and DNA damage (Aono et al.2002).They have also shown that deleting any of the subunits of condensin affects cds1 activity upon HU adjunction.

How condensins ensure this wide range of seemingly unrelated interphase and mitotic functions is unclear. A possibility is that specificity is conferred by co-factors.To identify condensin co-factors, the hosting lab TAP tagged the Cut3 subunit, pulled down the associated proteins, and identified them by mass spectrometry. This approach identified all 5 subunits of the DNA replication factor C complex (Fig.2). RFC is a ring-shaped complex that loads the sliding clamp PCNA on DNA in the context of the initiation of DNA replication during S phase (Mossi and Hübscher 1998). The interaction between RFC and condensin might provide a molecular basis for as yet characterized functional interactions with DNA replication.

Protein	Function	Seq. cov. (%)
Cnd2	Condensin non-SMC	82.5
Cnd3	Condensin non-SMC	78.7
Cnd1	Condensin non-SMC	63.5
Cut14	Condensin SMC2	84.6
Cut3	Condensin SMC4	81.3
Bir1	CPC	41.8
Pic1	CPC	45.6
Clp1	CDC14 phosphatase	60.25
Cdc2	CDK1	13.8
Pmt3	SUMO	42.7
Rfc3	DNA replication factor C complex	36.8
Rfc4	DNA replication factor C complex	36.3
Rfc1	DNA replication factor C complex	35.3
Rfc5	DNA replication factor C complex	34.4
Rfc2	DNA replication factor C complex	33.8
Dbp2	RNA helicase homolog DDX5	72.5
Sum3	RNA helicase homolog Belle/DDX3	70.1
Rrp6	3'-5' ribonuclease	24.6

Figure 2 : *RFC identified as potential binding partner of condensin* Condensin binding partners were identified using proteomic approach in cycling fission yeast cells.

To investigate this further, my hosting lab has screened condensin mutants for hypersensitivity to sub-lethal concentrations of Hydroxyurea (HU), a toxin that inhibits the ribonucleotide reductase (RNR), an enzyme that reduces ribonucleotides into deoxyribonucleotides. HU inhibits DNA replication by starving the replication forks of dNTPs by reversibly inhibiting RNR (Young and Hodas 1964; Singh and Xu 2016; Koç et al. 2004).

Condensin being essential for life, all mutations are hypomorphic or conditional point mutations. Out of 11 condensin mutants tested, three were identified as

hypersensitive to a sub-lethal dose of HU: cnd1-ae7, cnd1-175 and cut3-m26 (fig.1). These mutants were first made to identify the functional partners of condensin and were all selected for exhibiting chromosome missegregation (Robellet et al. 2014).

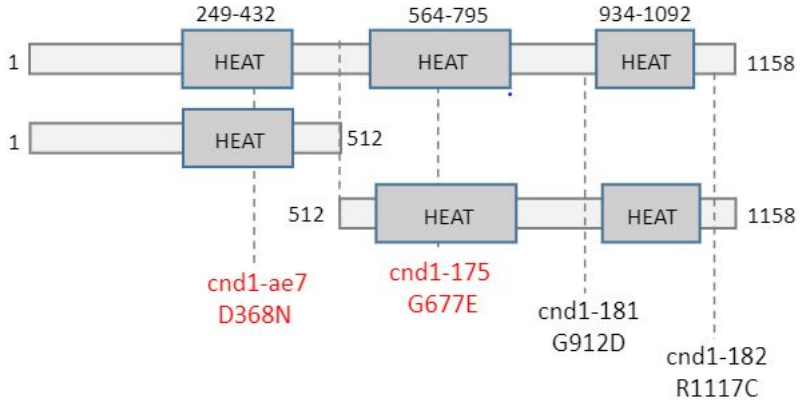


Figure 3 : ***Pictorial representation of major mutations used in the project*** The figure represents the cnd1 subunit of the *S.pombe* condensin complex and the dotted lines indicate the positions where point mutations have been made. The mutations shown in red are HU hypersensitive.

In this project we have tried to investigate the links between condensin and DNA replication using the model system *Schizosaccharomyces pombe*. *pombe* has a single type of condensin complex that is structurally similar to condensin I. We have made use of three HU hypersensitive mutants (cnd1-ae7,cnd1-175, cnd1-181) and two non-HU hypersensitive mutants (cnd1-181, cut3-477). We have tried to characterize and compare the response of the two kinds of mutants (HU hypersensitive and non hypersensitive) to HU at varying levels. We have also tried to determine the integrity of the DNA replication checkpoint in these mutants.

In this manuscript, I provide evidence that condensin is functionally linked to the DNA integrity complex and discuss a possible model based on my results on how condensin could be functioning during the interphase to ensure genome integrity.

Results

HU causes delay in cell cycle progression

In order to study the effects of HU on the wild type cell cycle kinetics, cells were treated with varying concentrations of HU (1mM, 2 mM, 3 mM and 4 mM). The cell density of the cultures as well as the average DNA content of the cells were measured every 30 minutes for 4 hours post HU adjunction. It was seen that the cells show a delay in growth even at 1mM HU concentration. As shown by the FACS analysis, the cells are seen to accumulate at the 1C stage after adding HU indicative of the slowing down in the progression of the cells through S phase from which they are seen to revert back to having a majority of 2C cells at rates inversely proportional to the concentration of HU added. At 4mM HU concentration, the majority of the cell population remains with 1C even 240 minutes post adjunction. This retardation is also reflected in the cell densities which are considerably lower than the control with the 4mM HU culture having only about half the cell density of the control after 4 hours (fig.4).

Thus, 4mM HU delays replication and, as a consequence, slows down cell cycle progression.

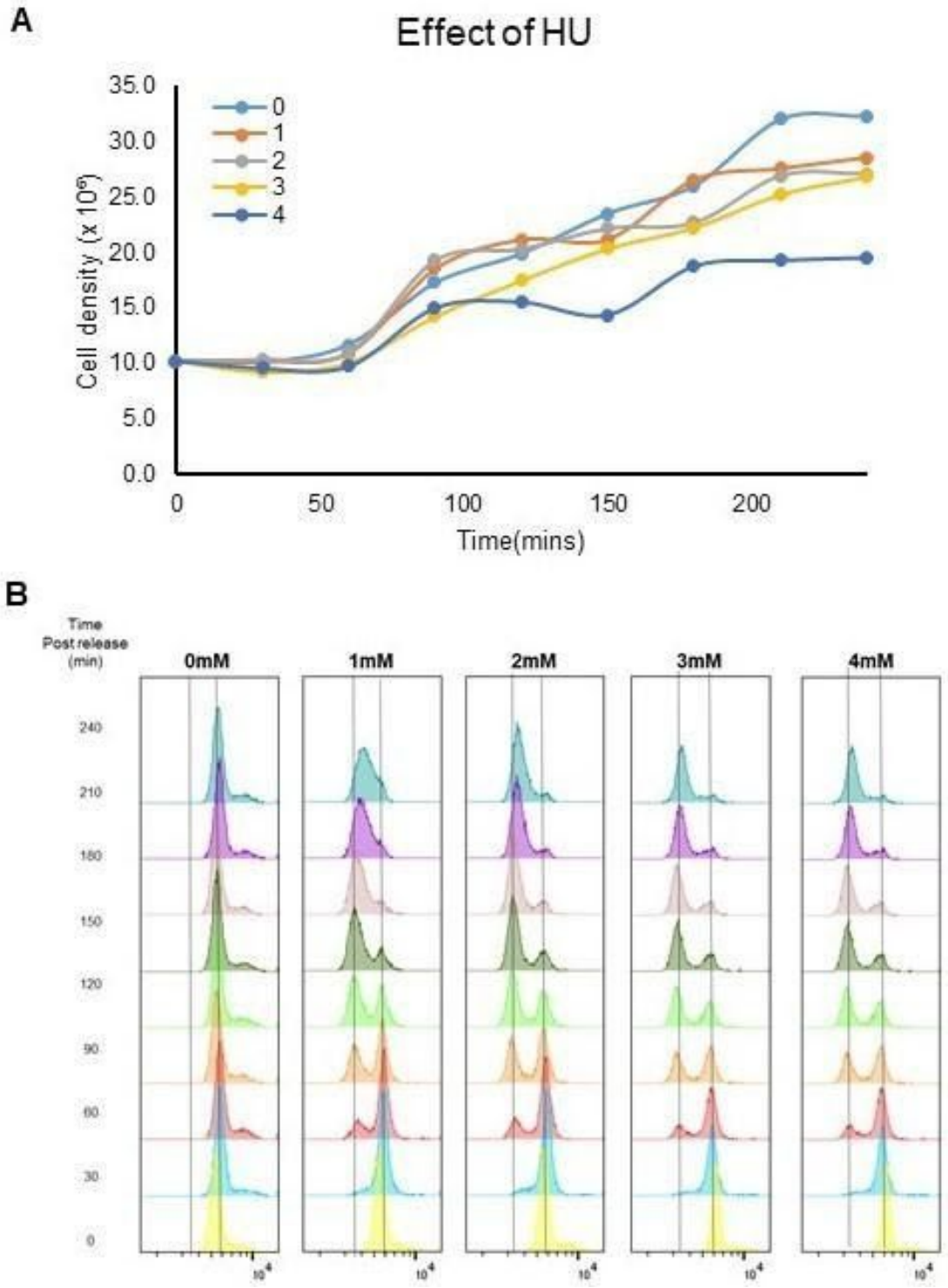


Figure 4 : *HU delays cell cycle progression* : (A-B) Wild type fission yeast cells were treated with varying concentrations of HU and the cell density and DNA content was measured over time. (A) Cell density of the cultures were calculated with the help of a Thoma cell every 30mins for 4 hours post treatment. (B) Cells were fixed in

EtOH and processed for FACS every 30mins. ~20,000 cells were measured for each time point. (N=1)

Treatment with HU increases the frequency of chromosome missegregation specifically in HU hypersensitive condensin mutants

Condensins play an integral role in chromosome condensation. It has been shown that mutations in condensin cause segregation defects. The main phenotype seen when condensin is impaired is the formation of a chromosome bridge in anaphase, where chromosome arms are unable to resolve after centromeres have reached opposite poles.

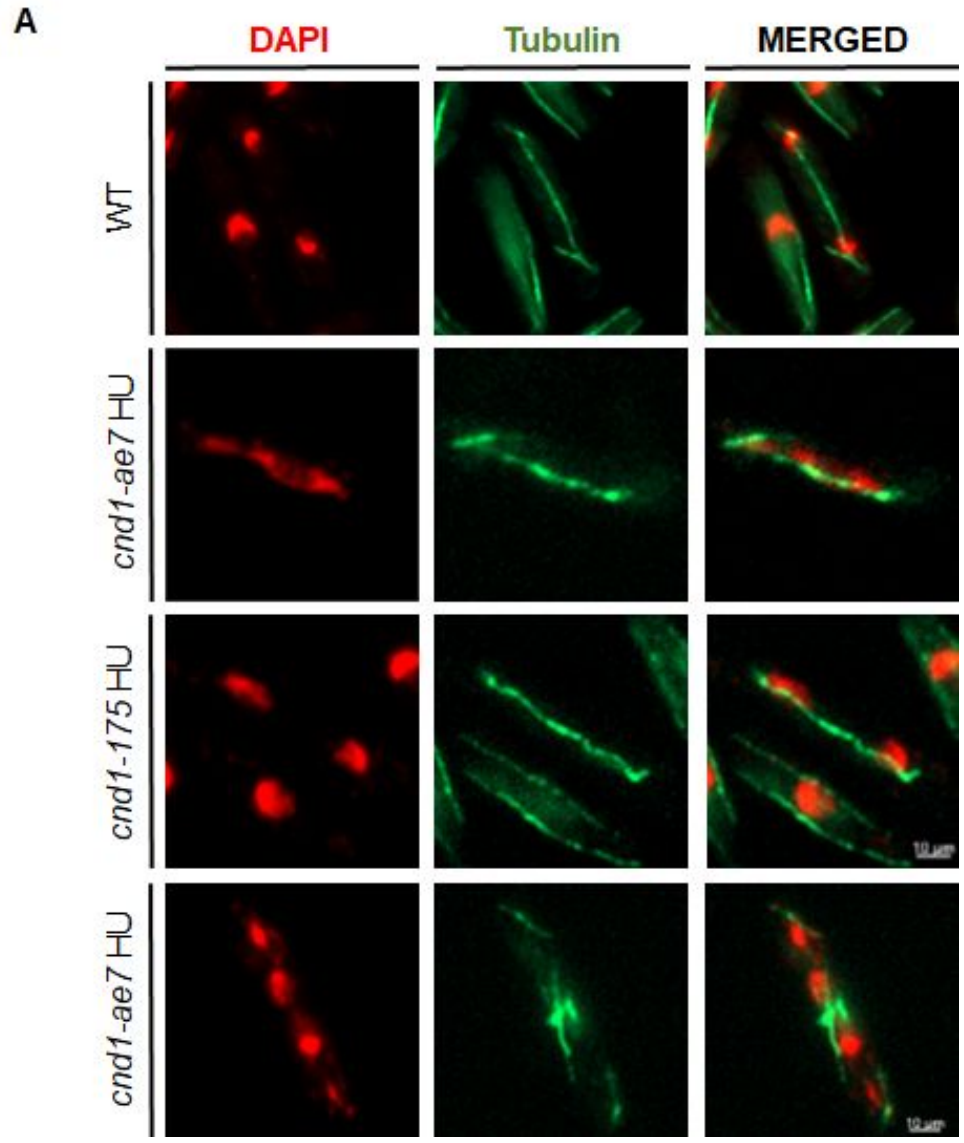
To examine whether HU affects mitotic chromosome segregation by condensin, cells mutants were treated with HU and chromosome segregation during anaphase was assessed (fig.5)

Exponentially growing cell cultures were split in two, one half was treated with HU 4mM for one generation (cells went through S phase in the presence of HU) and the other half left untreated. Cells were collected and processed for cytological analysis of chromosome segregation.

Wild-type cells exhibited no segregation defect, regardless of HU treatment. All condensin mutants exhibit missegregation during anaphase in non-HU condition, consistent with the fact that condensin is impaired. Remarkably, HU-hypersensitive cells show an increased frequency in missegregation upon HU treatment. This phenotype was specific to HU-sensitive mutants and unrelated to the penetrance/severity of the mutation since cut3-m26 and cut3-477 both had shown similar levels of missegregation in non HU cultures and only the HU sensitive cut3-m26 mutant showed an increase in the level of missegregation upon treating

with HU (Fig. 6B). Hence, this increase is a highly specific mitotic phenotype of HU hypersensitive mutants.

To assess the responsiveness of the DNA replication checkpoint we scored the percentage of late mitotic cells. If the DNA replication checkpoint was functional, the cell cycle would slow down upon HU adjunction due to the triggering of the checkpoint in response to the lack of dNTPs. This would result in a lower percentage of cells being in late mitosis as most cells would still be in their S phase/early mitosis.Upon calculating the percentage of late mitotic cells in the population, it was seen that there was no change in the frequency of late mitotic cells in HU hypersensitive populations upon HU treatment. On the other hand, wild type and non hypersensitive mutants showed a decrease in the frequency upon adding HU which could be an indication of the cell cycle being slowed down in response to stress (fig.5C).


A
DAPI
Tubulin
MERGED
WT
cnd1-ae7 HU
cnd1-175 HU
cnd1-ae7 HU

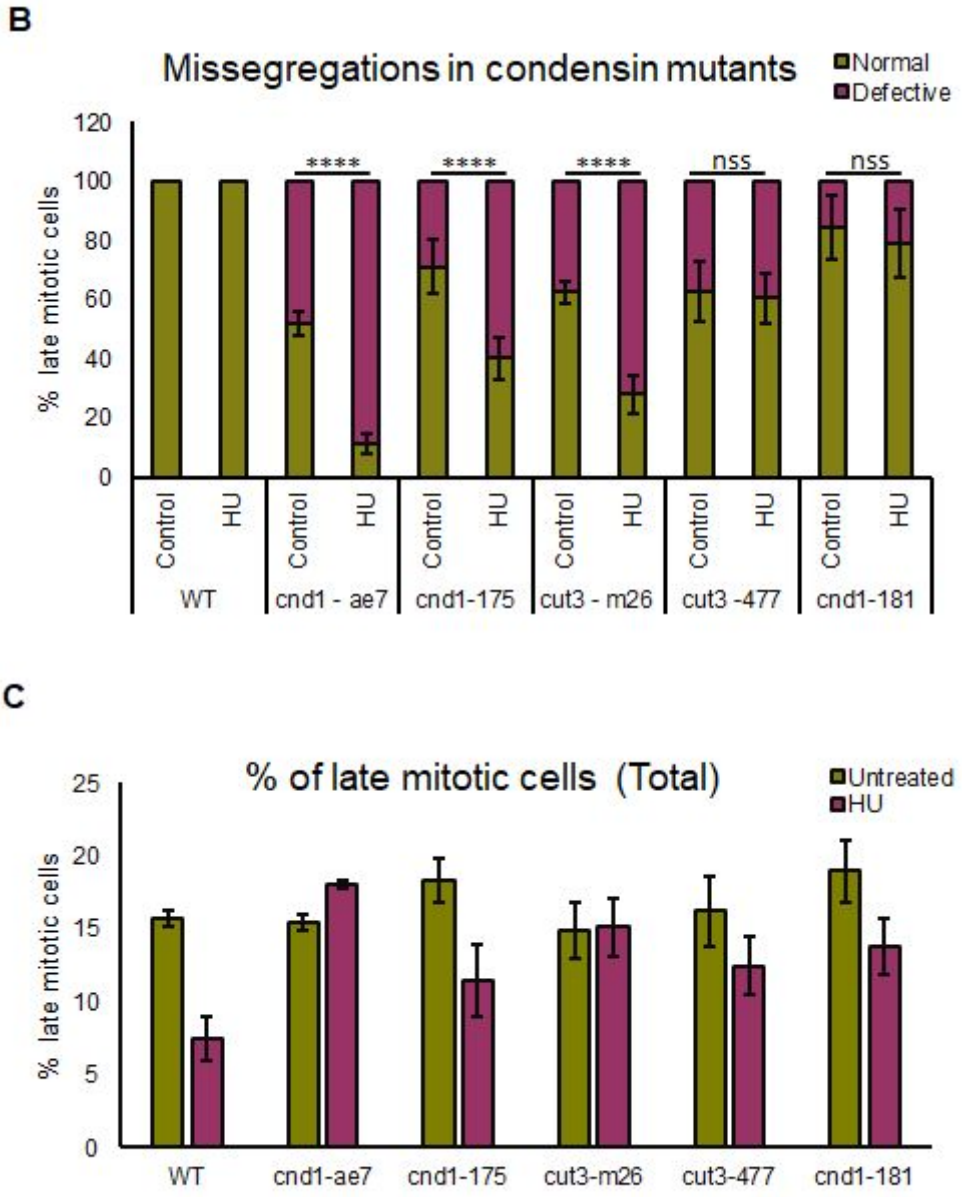


Figure 5 : *HU increases chromosome bridges in HU hypersensitive mutants* (A-C) Chromosome segregation in Fission yeast cells. Fission yeast cells were grown exponentially at 30C and treated with 4mM HU for 3 hours and then processed for immunofluorescence against a-tubulin. DNA was stained with DAPI. A: Segregation phenotypes seen in WT and mutants. The top panel shows wild type segregation while the bottom panels show the various phenotypes of missegregations that were observed upon treating condensin mutants with HU. B:

Frequency of chromosome bridges in cells with mitotic spindle length >5um was calculated. Significance of data was calculated by using two tailed Fisher's exact test. C: % of late mitotic cells in the population. All cells in the population were counted with cells exhibiting mitotic spindle length >5um considered as late mitotic cells.

Cell cycle analysis of condensin mutants' response to HU

It was seen that wild type cells respond to low (4mM) dose of HU by slowing down their cell cycle (fig.4). In order to test whether condensin mutants responded in the same way and if they did, to visualise where the delay was occuring, I synchronised the cells in the G2/M transition stage and released the cells in the absence or presence of 4mM HU.

The cells used in this experiment were cells that had cdc-2as as their sole cdk1 kinase. Cdc-2as is an analogue sensitive version of cdk1 which is unable to function in the presence of the drug 3BrPP1. As a result, cells are unable to enter into mitosis and accumulate in the G2/M transition stage. This block is reversible and can be removed by washing out the drug.

Upon releasing the cells in 4mM HU, it was seen that strains reached their first mitosis around 20 minutes post release. The second mitosis occurred around t = 120 mins in non-HU conditions. Upon addition of HU, no second mitosis was seen even at 180 mins post release in all strains (Fig.4). Since no second mitosis was observed regardless of the presence of a condensin mutation, I am unable to conclude the effect of HU on condensin mutants using this method. This is because there is no way to quantify the delay in the control strain and hence we cannot compare or say that the condensin mutants are equally delayed.

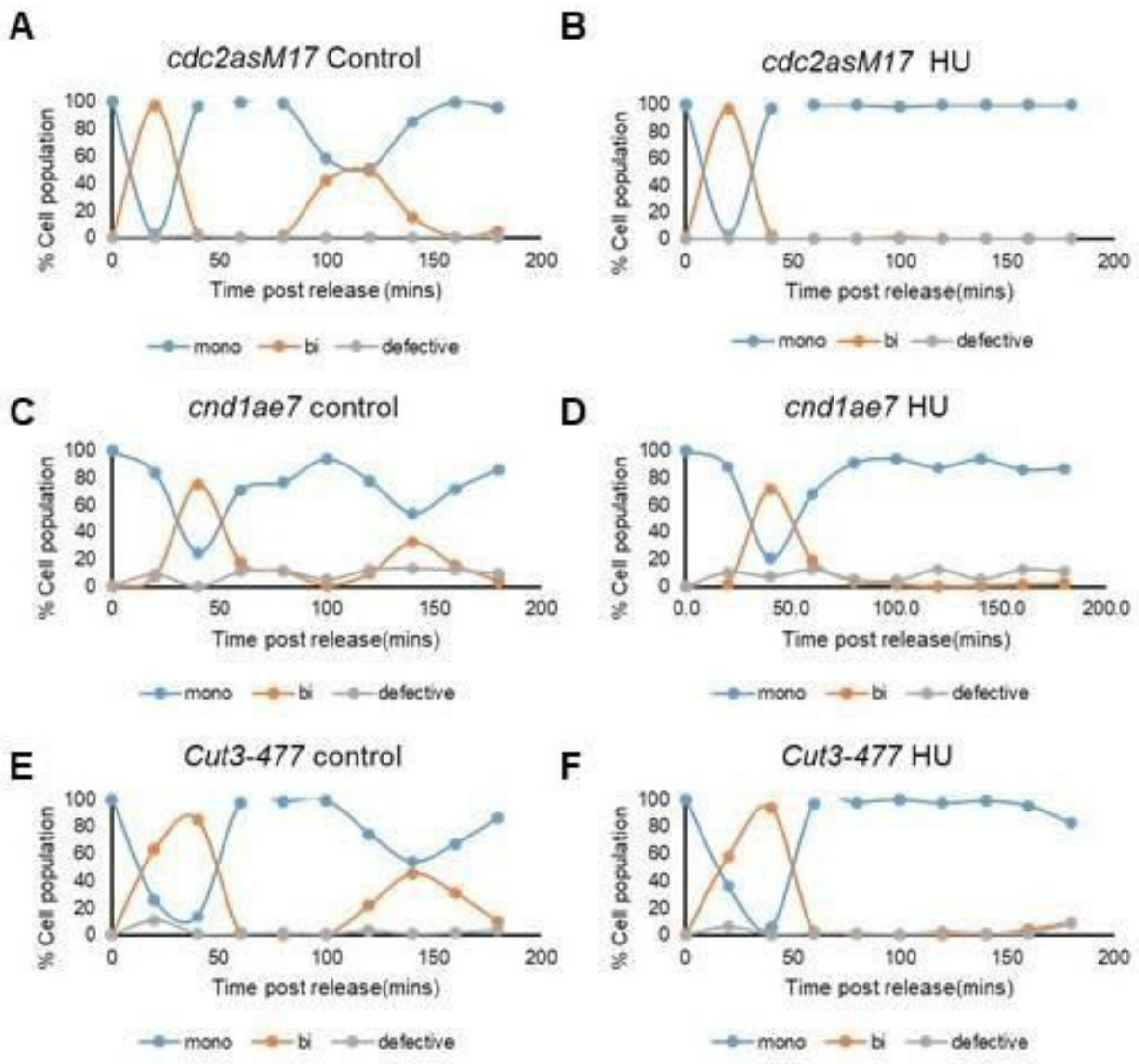


Figure 6 : *HU delays cell cycle progression* (A-F) Fission yeast cells were synchronised in G2/M transition and allowed to proceed through the cell cycle in media +/- 4 mM HU. Cells were fixed using EtOH every 20mins post treatment. The proportion of cells exhibiting one/two/defective nuclei were calculated by staining with DAPI and visualizing under the microscope.

Condensin mutants are able to respond to and recover from HU stress

Cells respond to high levels of HU (>15mM), by arresting their cell cycle in the S phase. This arrest occurs due to the activation of the DNA replication checkpoint which senses the shortage of dNTPs surrounding the replication fork and halts the fork to prevent faulty replication. Cells lacking a functional checkpoint would be unable to detect this stress and would go through the cell cycle leading to a catastrophic mitosis. The replication stress caused by HU is reversible and can be removed by washing out the drug.

The ability of condensin mutants (1) to arrest their cell cycle in response to high HU and (2) to resume cell cycle progression upon HU removal was studied by treating them with high (20mM) HU and then measuring their septation index and average DNA content every 20 mins for the next 120 minutes (fig.7).

In order to ensure the integrity of DNA while going through the cell cycle, there are several checks in place with at least two different pathways functional during the S phase, namely the replication and damage checkpoints. The DNA replication checkpoint ensures the completion of DNA replication before entering mitosis while the other checks for the integrity of the DNA; i.e, whether or not the DNA is damaged. Rad3 is one of the six rad proteins that form the DNA integrity checkpoint (DIC) and operates both these pathways. Lack of this protein leads to the cells having a non-functional DIC and hence being blind to DNA damage and replication stress. Upon encountering faulty DNA the rad proteins activate either the DNA damage or replication checkpoint by phosphorylating specific kinases downstream. Cds1 and chk1 are two such kinases that get phosphorylated in a rad3 dependent

manner to cause S phase arrest. Of these, cds1 is activated in response to both DNA damage and DNA replication (only during S phase) and is necessary for the cells to tolerate replication stress by HU. chk1 kinase is phosphorylated in response to DNA damage and is known to get phosphorylated in cells lacking cds1 kinase upon HU treatment (Lindsay et al.1998) (fig.). Cells lacking rad3 were used as a negative control.

The wild type cells were able to arrest their cell cycle upon HU adjunction as seen by the accumulation of cells with 1C DNA content and the reduction of septation index from around 18% in cycling cells to 1.47%. As time progressed, it was seen that the peak of average DNA content slowly started shifting from 1C to 2C indicating that the cells have recovered and are progressing through their S phase. This shift started at around 30 minutes post release and the peak reached 2C around 100 minutes post release. Concurrently, the septation index slowly started increasing around 120 minutes post release indicating cells undergoing division.

On the other hand, rad3 deletion mutants which have a non-functional checkpoint was unable to arrest themselves upon HU adjunction. This resulted in them entering mitosis with unreplicated DNA resulting in cells with less than 1C DNA content (data not shown). The septation index in these cells increased from around 8% in cycling cells to around 50% indicative of cells which is a phenotype shown by these mutants upon HU adjunction due to failure to divide properly (Xu et al. 2016).

The FACS analysis showed that all condensin mutants studied accumulated with a 1C peak upon adjunction of 20 mM HU, and the septation indexes dropped to less than 5% indicating that the cells were able to arrest their cell cycle.

Upon release from HU, the HU hypersensitive mutant cnd1-ae7 and the non-hypersensitive mutant cut3-477 were able to recover faster than the wild type. The HU hypersensitive cut3-m26 mutant, however, recovered with kinetics similar to the wild type**.** This is supplemented by the septation index measurements which

concur with the FACScan observations. The septation index is very low just after the release and starts to increase at t = 120 post release. The kinetics of the condensin mutants are very different from that of a DNA integrity checkpoint impaired mutant (rad3D).

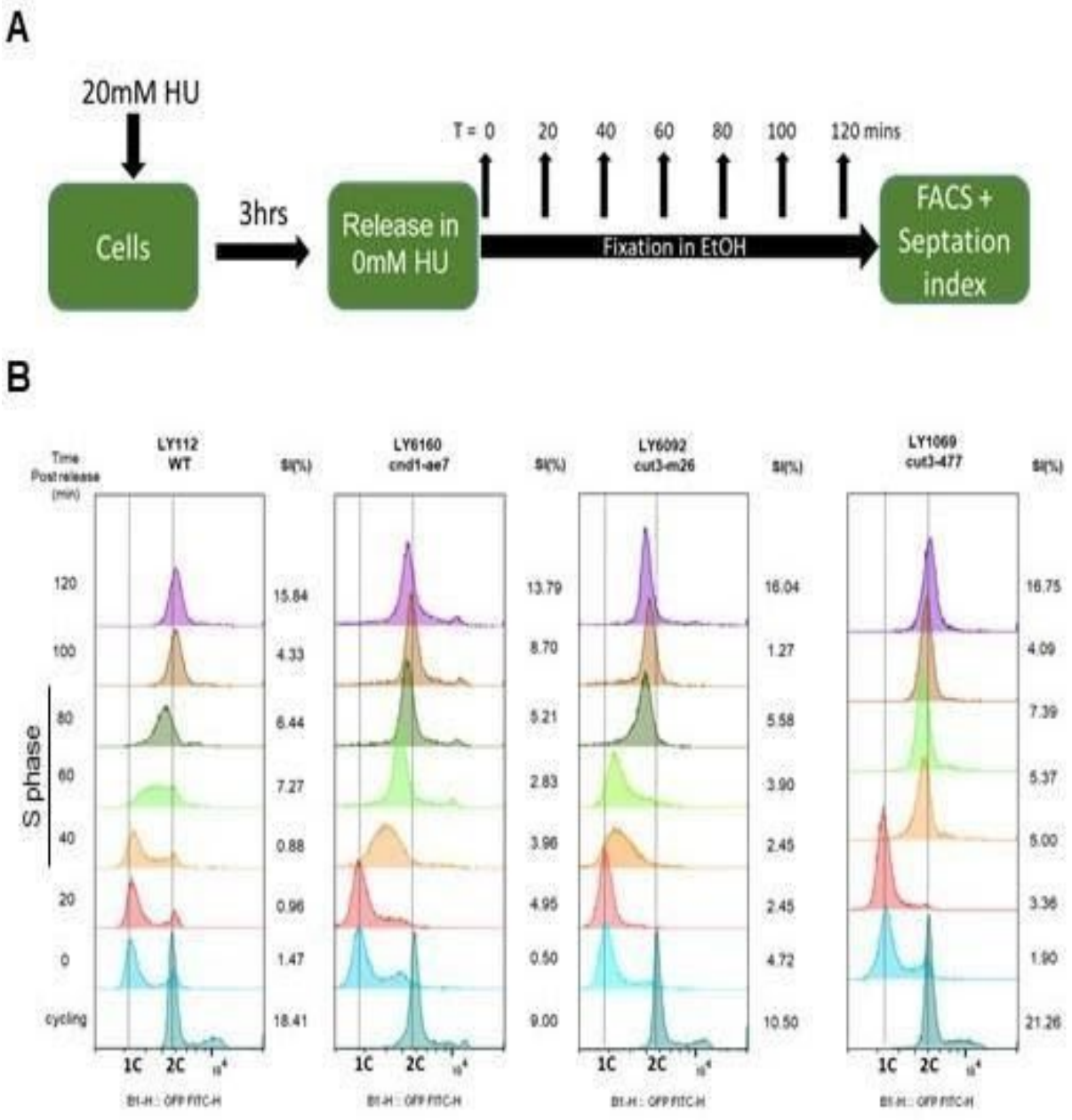


Figure 7: ***Condensin mutants are able to recover from HU block*** (A) Scheme of experiment to visualize recovery of condensin mutants from HU arrest (B) FACS analysis showing average DNA content of condensin as a function of time after release from HU block. Cells were fixed in EtOH and processed for FACS every 20mins. ~20,000 cells were measured for each time point. (N=2)

Since the condensin mutants are able to arrest their cell cycle in response to high doses of HU, this property was used to synchronise cells in their early S phase to study their response to low (4mM) doses of HU. As seen previously, wild type cells delay their cell cycle in response to the stress. In this case, this delay is visualised as a delay in recovering from the block and the shift of the DNA content peak from 1C to 2C. Here, the shift started at around 80 minutes post release and had not reached 2C even 180 minutes post release (fig.8). In this case too, the rad3 deletion mutant was unable to recognise the stress and went through cell division resulting in a noticeable spread of less than 1C DNA.

As in the previous experiment, the cnd1-ae7 and cut3-477 mutants showed faster recovery while the cut3-m26 showed wild type kinetics. The faster recovery is especially noticeable here as the peak started shifting from 1C to 2C at around 60 minutes post release for the two mutants (fig.8).

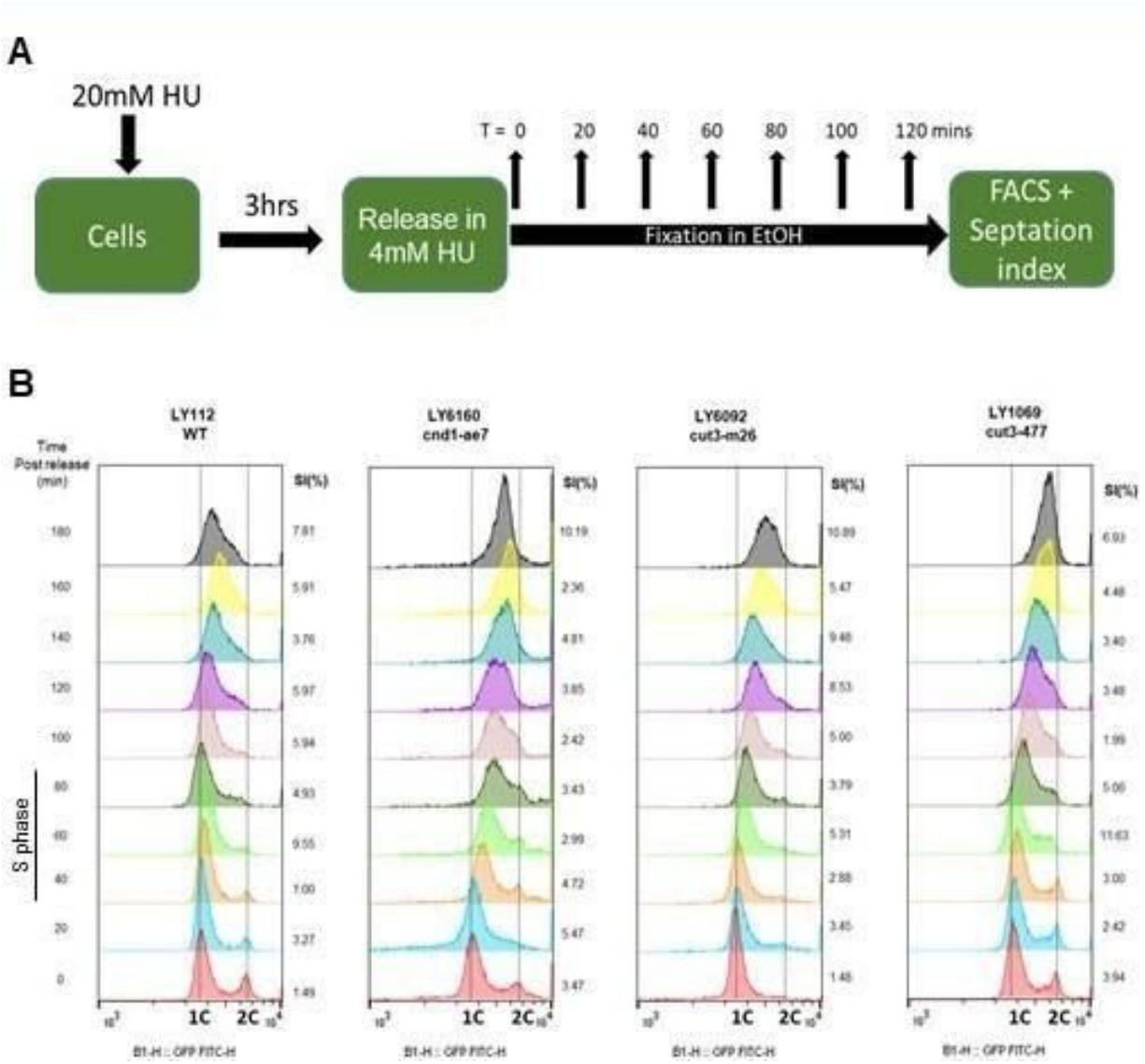


Figure 8: ***Condensin mutants are able to respond to low levels of HU*** (A) Scheme of experiment to visualize response of condensin mutants to low concentration of HU (4mM) (B) FACS analysis showing average DNA content of condensin as a function of time after release from HU block into 4mM HU. Cells were fixed in EtOH and processed for FACS every 20mins. ~20,000 cells were measured for each time point. (N=1)

Cds1 gets phosphorylated in condensin mutants

Cds1 is a protein kinase that gets phosphorylated in a rad dependant manner in response to DNA replication checkpoint activation (fig.9).It is known that in cells with a functional DNA replication checkpoint, cds1 gets phosphorylated upon S phase arrest by HU and this phosphorylation can be detected as an upper shift in the cds1 band by western blot (Lindsay et al. 1998).

In order to check if the DNA replication checkpoints are functional in the condensin mutants, the cds1 phosphorylation was checked by western blotting after S phase arrest. It was seen that out of the condensin mutants tested, cut3-m26 and cut3-477 showed a clear upper shift in S phase arrested cells indicating cds1 phosphorylation; but the shift was not so clear in the cnd1-ae7 mutant. The DNA replication checkpoint non-functional rad3D mutant shows no upper shift. (fig.9)

We can conclude that the cut3-m26 and the cut3-477 mutants have a functional DNA replication checkpoint while this may not be so clear in the case of the cnd1-ae7 mutant.

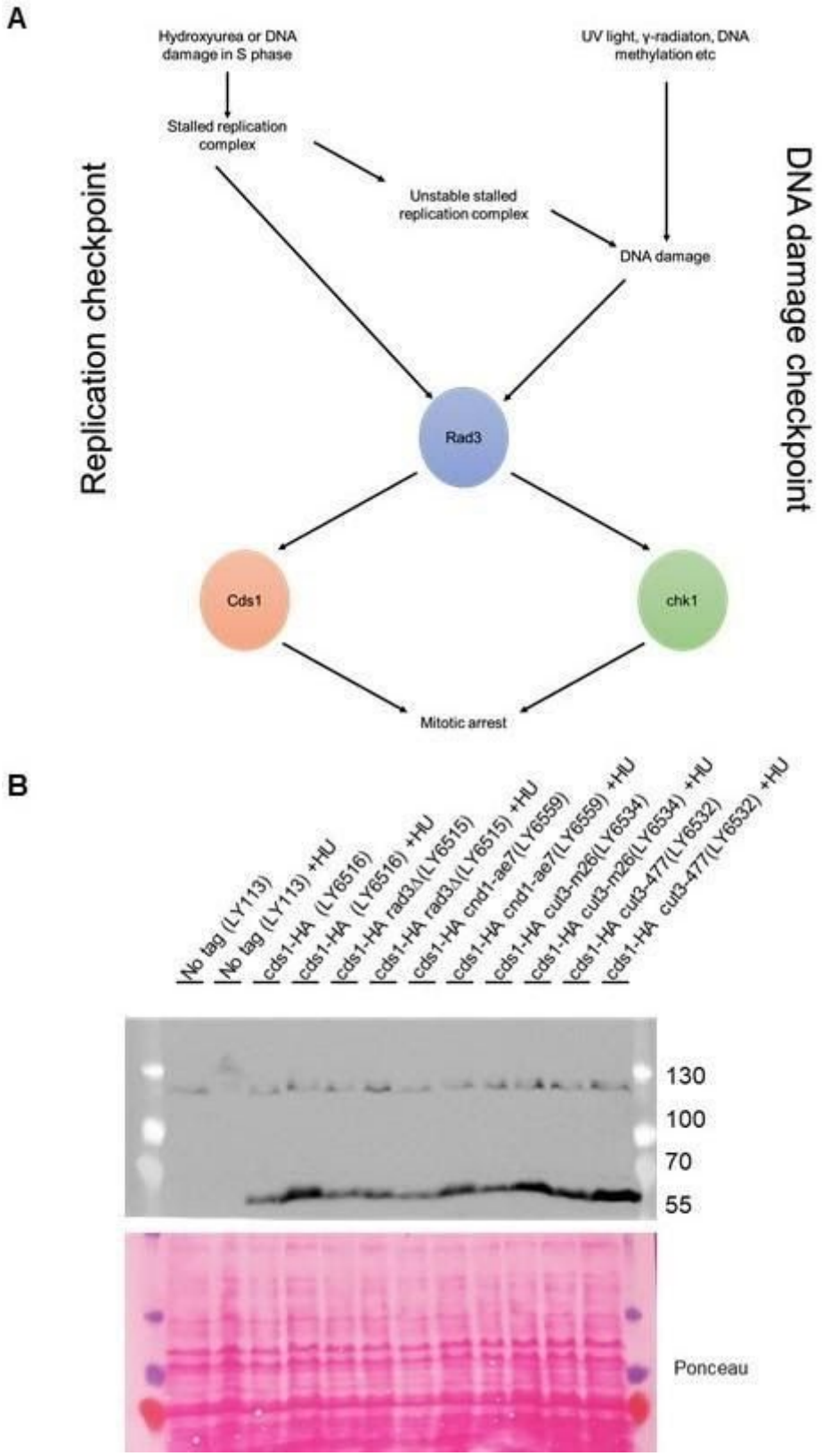


Figure 9: ***Checking cds1 phosphorylation in condensin mutants*** (A) a simplified map of the DNA integrity checkpoint in fission yeast *s.pombe.* (B) *cds1*

phosphorylation in condensin mutants. Cells were treated with 20mM HU for 3 hours. (N=2)

Interactions between rfc and condensin

The lab has previously identified RFC as a potential binding partner of condensin.To test if there were any genetic links between RFC and condensin, double mutants having a RFC mutation rfc3-1 and a condensin mutation (cnd1ae7 or cut3-477) was serially diluted and plated on YES+A+Phloxin plates and kept at 28℃, 30℃, 32℃, 34℃ and 36℃.

The rfc3 subunit of the RFC is known to be involved in the DNA integrity checkpoint. The rfc3-1 mutation is a point mutation that causes a change in the amino acid from arginine to tryptophan in the conserved domain of box VIII of the protein.The rfc3-1 mutation exhibits defects in the replication and DNA damage checkpoints. Furthermore, the mutant is hypersensitive to HU making it an excellent candidate for checking genetic interactions between condensin and rfc3.

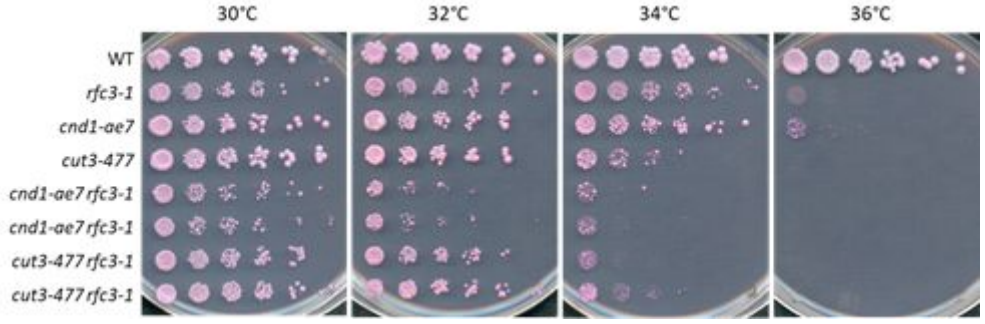


Figure 10: ***HU hypersensitive mutations make rfc mutants sick*** Indicated strains have been grown on YES+A. Serially diluted 1/5 in YES+A, spotted on indicated plates and incubated for 5 days. First drop contains 10^4 cells.

It was seen that the rfc3-1 and cnd1-ae7 single mutants were able to grow in temperatures upto 34℃ while the cut3-477 single mutant was sick at 34℃. All mutants were unable to grow at 36℃. The cnd1-ae7 cut3-1 double mutant was sick

and unable to grow at 32℃ while the cut3-477 rfc3-1 double mutant was able to grow at 32℃ but not at 34℃.

This implies that there could be a checkpoint pathway functioning via condensin that gets disrupted in the HU sensitive cnd1-ae7 mutant but not in the non HU sensitive cut3-477 mutant. This pathway could be redundant in cells possessing functional rfc3, but in the cells possessing the mutant rfc3-1, the disruption of the condensin pathway makes the cells sicker as seen by the rfc3-1 cnd1-ae7 double mutant.

Discussion

Condensin complexes are versatile enzymes whose role in the organisation and compaction of the genome during mitosis are well known. Recent studies indicate that condensin is active and functional during the interphase and might play a role in maintaining the genome integrity during this period. However, this role of condensin is yet to be explored. In this project, I have tried to explore the link between condensin and DNA replication. For this purpose, I have made use of a reversible replication inhibitor hydroxyurea and studied its effect on various condensin mutants. I have shown that certain condensin mutants are able to recover faster from replication stress from hydroxyurea and that HU hypersensitive condensin mutants show an increased frequency of missegregation upon treatment with a low dose of HU. This suggests the existence of a function of condensin in DNA replication and/or DNA integrity maintenance. Upon further characterisation of the mutants, I was able to see that some mutants recovered faster than the wild type. Of these two, one was HU hypersensitive while the other was not. Interestingly, the DNA replication checkpoint was intact in the non HU hypersensitive mutant but not in the Huhs cnd1-ae7. This suggests that the two mutants recover faster due to two different mechanisms. I have also shown that the HU hypersensitive mutation cnd1-ae7 when combined with the replication checkpoint defective rfc3-1 mutation makes the cells sick. This points to the presence of a replication checkpoint pathway dependant on condensin that operates parallel to the rfc3 replication checkpoint pathway.

Taken together, the results indicate these possible hypotheses -
(A) condensin is linked to the DNA integrity checkpoint and different mutations can interfere with the DNA replication checkpoint pathway or the DNA damage checkpoint pathway or both. This impairment would lead to abnormal (damaged/ unreplicated) DNA reaching mitosis where it would cause abnormal segregation.

Condensin might be doing this by acting as a scaffold where the checkpoint kinases can selectively attach and access the DNA.

It has been shown previously that impairing the checkpoint (cds1Δ / chk1Δ) leads to abnormal segregation of chromosomes during mitosis. This hypothesis is in agreement with the studies done by Aono et al where they have shown in s.pombe cells that the cnd2 subunit of condensin is involved in the response to HU induced S phase arrest as well as in DNA damage. They had also shown that all subunits of condensin were required for cds1 activity upon HU adjunction (Aono et al. 2002).

(B) Condensin segregation defects caused by certain condensin mutations makes cells hypersensitive to HU. The segregation defects caused by condensin are propagated into the S phase of the next cell cycle where they would exacerbate the sensitivity of the replication machinery and/or the DNA replication checkpoint to low doses of HU.

Although formally possible, this second hypothesis is hard to reconcile with the observation that not all condensin mutants showing segregation defects exhibit HU sensitivity. This is especially clear when we take the case of cut3-m26 and cut3-477 mutants, both of which show similar levels of segregation defects and yet only the former exhibits HU hypersensitivity.

(C) A third possibility is that condensin plays a role at replication fork during S phase for the proper assembly and segregation of chromosomes in the subsequent mitosis.The fact that cnd1-ae7, cnd1-175 and cut3-m26 exhibit increased mis-segregation upon HU, whereas cnd1-181 and cut3-477 do not, is a genetic evidence for a separation of function. Condensin could play a specific role during S phase for proper chromosome segregation during mitosis, and this role would be specifically impaired by cnd1-ae7, cnd1-175 or cut3-m26.

Genome instability is a driving force in tumorigenesis and cancer. It has been shown that condensin deficiency leads to aneuploidy and increases both the prevalence and aggressiveness of tumors (Martin et al. 2016; Ham et al. 2007; Rawlings et al. 2011; Gosling et al. 2007; Coschi et al. 2014). Currently, the molecular mechanisms underlying the role of condensin in DNA replication is unknown. Our current results indicate that condensin might play a role in the maintenance of structural integrity of the genome during S phase. This could potentially open up a new research avenue in tumorigenesis and cancer biology.

Future plans

The exact mechanism by which condensin is linked to DNA replication still remains unclear. In order to further study the mechanism, I propose that we study the positioning of condensin during S phase by performing ChIP against the Kleisin subunit of condensin in the presence and absence of HU. If condensin was required for the functioning of the checkpoint, we would expect an increase in the concentration of condensin around TSS where most forks would have stalled upon HU adjunction. We could also check for physical or genetic interactions between condensin subunits and the checkpoint proteins such as the rad kinases. This would help us understand how condensin affects the checkpoints.

Materials & Methods

Cell culture and manipulation

Media and molecular genetics methods were as previously described (Moreno et al., 1991). Fission yeast cells were grown in complete YES+A medium. Cell cycle arrest in G2/M was done by adding 3BrPP1 to the growing media to reach a final concentration of 2μM. Treatment with HU was done by adding freshly made stock in ultra-pure water filtered by using a syringe filter (.45μm) to the cultures to obtain desired concentrations. CPT 20mM (500X) stock solution was made in DMSO and directly added to the cultures.

Yeast strains

Strains used in this study are listed below (Table 1). The strains indicated in bold have been made by me for this project.

Strain	Genotype
47	h+ ura4- cut14-3HA-6His-ura4+
112	h+ leu1-32 ura4D18 ade6-210
113	h- leu1-32 ura4D18 ade6-210
692	h- leu1-32 ura4D18 ade6-210 rad3D::ura4+
1069	h- leu1-32 ura4D18 cut3-477
1261	h+ leu1-32 ura4D? ade6-210 cnd1-175 slc175-5
1264	h- leu1-32 ura4D? ade6-210 cnd1-181 slc181-3
4453	h90 leu1-32 ura4D18 ade6-216 s1-131 rfc4-GFP-HA-KanR
4559	h+ leu1-32 ura4D nda3-KM311 rfc4-GFP-HA-KanR cnd2-pK9-HygroR

4578	h- leu1-32 ura4D ade6-216 rfc3.1
4580	h- leu1-32 ura4D ade6-210 cut3-477-NatR rfc3.1
4581	h+ leu1-32 ura4D ade6-210 cut3-477-NatR rfc3.1
4623	h- leu1-32 ura4D nda3-KM311 cnd2-pK9-HygroR
4629	h- leu1-32 elp1-HA3-KanR
4681	h+ leu1-32 ura4D18 cdc2asM17
4945	h- leu1-32 ura4- ade6-210 rad22-GFP-KanR
6092	h- leu1-32 ura4D s1- cut3-m26
6160	h+ leu1-32 ura4- ade6-210 s1- his7- cnd1-ae7
6321	h- leu1-32 ura4D cdc2-asM17 lacI-GFP::his7+
6343	h- leu1-32 ura4D18 cut3-477 cdc2-asM17
6428	h+ leu1-32 ura4- ade6-210 his7- s1- cdc2-asM17 cnd1-ae7
6447	h- leu1-32 ura4- ade6-210 cnd1-ae7 ts
6449	h+ leu1-32 ade6-210 chk1-HA-LEU2
6455	h- asf1.33-myc13-KanR chk1-myc13-HygroR
6456	h- leu1-32 ura4D18 asf1.33-myc13-KanR cds1-3HA-HygroR
6461	h- ura4+-pcn1-mCherry-NatR
6474	h- leu1-32 ura4D18 ade6-210 cut3-477 rad3D::ura4+
6475	h+ leu1-32 ura4D18 ade6-210 cut3-477 rad3D::ura4+
6478	h? leu1-32 ura4D18 ade6-210 cnd1-ae7 rad3D::ura4+
6479	h? leu1-32 ura4D18 ade6-210 cnd1-ae7 rad3D::ura4+
6490	**h**? leu1-32 ura4- ade6-210 cnd1-ae7 rfc3-1
6491	h+ leu1-32 ura4- cnd1-ae7 rfc3-1
6505	h+ leu? ura4D rad3::ura4+ rad22-GFP-KanR
6509	h+ leu1-32 ura4D cnd1-ae7 rad22-GFP-KanR
6513	h+ leu1-32 ura4D cut3-m26 rad22-GFP-KanR

6515	h- leu1-32 ura4D rad3D::ura4+ cds1-HA3-HygroR
6516	h+ leu1-32 ura4D cds1-HA3-HygroR
6517	h- leu1-32 ura4D cds1-HA3-HygroR
6535	h+ leu1-32 ura4D18 cut3-m26 cds1-HA3-HygroR
6536	h+ leu1-32 ura4D18 cnd1-GFP-ura4+ chk1-myc3-Hygro
6537	h- leu1-32 ura4D18 cnd1-GFP-ura4+ chk1-myc3-Hygro
6538	h+ ura4D18 rad3::ura4+ chk1-myc13-HygroR
6539	h- ura4D18 rad3::ura4+ chk1-myc13-HygroR
6554	h+ leu1-32 cut3-m26 ura4+-pcn1-mcherry-NatR
6555	h? cut3-477 ura4+-pcn1-mcherry-NatR
6556	h+ cnd1-ae7 ura4+-pcn1-mcherry-NatR

Table 1 : Fission yeast strains used in this study

Immunoprecipitation

Yeast strains having the required tags were grown in liquid media at 32°C. The culture were treated with 20mM final concentration of HU and incubated for 3 hours in a shaking incubator (200 rpm) at 32°C the cell density of each culture was determined using a Thoma cell and $2x10^8$ cells were fixed by flash freezing with liquid nitrogen. The cells were lysed with either cold acid-washed beads (0.5 mm Sigma) using a Precellys homogenizer or by using the Retsch MM400 system. The lysates were resuspended in 400µL fresh ice-cold lysis buffer (50 mM Hepes-KOH pH 7,5 ; 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0,1 %(w/v) sodium deoxycholate. One tab of protease inhibitor cocktail cOmplete EDTA-free (#05 056489 001) per 10 ml of Lysis buffer) and two replicates were combined for each sample. The lysates were pelleted down and resuspended in PBS-T containing monoclonal α-Pk9 antibodies (Serotec # MCA1360) coupled to dynabeads-protein A

(15µL beads/ 2x10^{8} cells) and incubated on a rotating wheel at 4°C for 40minutes. The beads were resuspended in ice-cold benzonase buffer and split into two. One half was treated with 100units of benzonase for 10 minutes at 4°C. All beads were washed with benzonase and lysis buffers and resuspended in Laemmli buffer (62.5mM Tris-HCL 1.5M pH6.8, 10% glycerol, 3% SDS electrophoresis grade, 0.01% Bromophenol blue) with β-Mercaptoethanol (355mM) and heated 5 min at 98°C. Total extracts and immunoprecipitates were analysed by Western blot.

Immunofluorescence assay

Cells were collected, fixed in 70% EtOH and stored at -20°C. 10^{7} cells were rehydrated in PEM buffer (100 mM PIPES, 1 mM EGTA, 1mM MgSO4, pH 6.9) by giving 3 washes with incubations of 5, 10 and 30 minutes in between on a rotating wheel at RT. Cell wall was digested by treating with zymolyase 100T in PEMS buffer (PEM + 1.2 M sorbitol) for 30 minutes at 37°C and permeabilized by treating with triton X-100 for 2 minutes at RT. The cells were incubated for 30 minutes RT in PEMBAL (PEM, 1% BSA, 100 mM Lysine-HCl, 0.1% Azide) and then incubated overnight at 4°C with 100µL primary antibody 1:50 α-TAT1 in PEMBAL to stain α-tubulin. After incubation, the cells were washed thrice with PEMBAL with 10-15-30 minutes incubations on a rotating wheel at RT in between. The cells were incubated for 2 hours on a rotating wheel at RT with 100µL secondary antibody 1:400 anti-mouse alexa488 in PEMBAL. After incubation, the cells were washed once with 100µL PEMBAL (+ 5 minutes incubation) and twice with 200µL PEM (+ 5/10 minutes incubation) and stained with 0, 5 µg/mL 4', 6-diamidino-2-phenylindole (DAPI) in PEM buffer. The cells were incubated in the dark for 5 minutes. The images were obtained by using the 63X immersion lens of an Axio-Imager Z1 microscope. Mitotic spindles >5µm in length were taken for analysing chromosome missegregation. Anaphase was considered unresolved when the two DNA masses seen by DAPI staining were not fully separated.

Total protein extraction

10^8 cells were collected per sample and resuspended in 200µL ice cold 20% TCA. The cells were lysed with cold acid-washed beads (0.5 mm Sigma) using a Precellys homogenizer. 200µL ice cold 5% TCA was added and the lysate was collected. The protein pellets were dissolved in 100µL 2X sample buffer (0.1M Tris-HCL pH9.5; 20% Glycerol;4% SDS electrophoresis grade;0.2% Bromophenol blue) + β-Mercaptoethanol (715mM) and boiled for 5 minutes at 98°C.Cleared supernatants were recovered after centrifugation and analysed by Western blot.

Western Blotting

Samples were prepared in 2x Laemmli buffer and boiled for 5 minutes at 98°C prior to use. Proteins were separated by electrophoresis through homemade gels (7.5%, 29:1 acrylamide: bis-acrylamide for Co-IP or 10%, 200:1 acrylamide: bis-acrylamide for cds1 phosphorylation validation) 180V for 1h20. Semi-dry protein transfer was performed on 0.2 µm nitrocellulose membranes for 90 minutes. Transfer was validated with Ponceau staining and membranes were blocked in PBS-T 5% non-fat dry milk. Incubation with primary antibodies were done overnight at 4°C using the following antibodies: α-PK9 (Serotec #MCA1360, 1:1000) or α-GFP (JL8 Clontech 632380, 1:1000), α-HA (Roche 12CA5, 1:1000), α-myc (A-14, 1:1000). Incubation with secondary antibodies were done at RT for 40 minutes with α-mouse (Amersham NA931, 1:10000) or α-rabbit secondary antibodies (Amersham NA 49340, 1:10000) and the Femto kit ECL (ThermoFisher West Femto 34096). Blots were imaged using the ChemiDoc apparatus (Bio-Rad).

FACScan

Cells were fixed in 70% EtOH overnight. 10^7 cells were rehydrated by washing thrice with 1mL 50mM NaCitrate with 5 minutes of incubation at RT in between. The cells are resuspended in 1mL 50mM NaCitrate and digested by adding 10µL 10mg/mL

RNase A and incubating in a water bath at 37°C for 2 hours. The samples are split into halves and one is kept at 4°C as backup. The other half is sonicated with 3 pulses of 1 second each at 40% amplitude to break clusters and 0.5mL NaCitrate 50mM + 2µM Sytox green is added to get 1mL cells in 50mM NaCitrate + 1µM Sytox green final.~20,000 cells per Sample are measured using MACSQuant Analyser taking care that a maximum of 1500 cells are analysed per second. The data obtained are then analysed using FlowJo ver 10.6.1.

Septation Index Measurement

Fission yeast cells fixed in EtOH was spread on a glass slide and allowed to dry. Coverslips were mounted over the cells using ~3 µL 0.5mg/mL Calcofluor in PBS buffer and sealed with clear varnish. The cells were imaged using the 63X immersion lens of an Axio-Imager Z1 microscope and the fraction of cells with a complete septa and no invagination was determined by counting at least 200 cells.

Spot assay

Fresh yeast cells were resuspended in liquid YES+A media and made to 3 x 10^6 cells/mL. The cultures were serially diluted fivefold with the help of a microplate and spotted on YES+A+Phloxin plates using a replicator VP407 AH and incubated at the indicated temperatures.

Co-IP

Cells expressing the required tags were grown, treated and 2 x 10^8 cells were fixed using liquid nitrogen per sample per tube. 4 x 10^8 cells were lysed using Retsch MM400 and resuspended in 400µL Lysis buffer + PI (50 mM Hepes-KOH pH 7,5 ; 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0,1 % (w/v) sodium deoxycholate.1mM PMSF + one tab of protease inhibitor cocktail cOmplete EDTA-free (#05 056 489 001) per 10mL) per sample. Lysis efficiency was checked

under the microscope and the crude extracts were clarified by centrifugation. The supernatant for each sample was combined. 15µL of the supernatant was saved as total extract and boiled at 98°C with 5µL 4X Laemmli buffer (62.5mM Tris-HCL 1.5M pH6.8, 10% glycerol, 3% SDS electrophoresis grade (w/v), 0.01% bromophenol blue, 355mM β-mercapto ethanol). The remaining lysates were transferred into tubes containing monoclonal α-Pk9 antibodies (Serotec # MCA1360) coupled to dynabeads-protein A (15 µl/ 2 x 10^8 cells) and incubated for 40 minutes on a rotating wheel at 4°C. The beads + proteins were collected, washed twice with 1mL lysis buffer + PI and once with 0.5mL Benzonase buffer (2mM Mg2+; 50mM Tris pH8; 1% Triton; 0.1mg/mL BSA, cOmplete EDTA 1X final). The samples were resuspended in 0.2mL Benzonase buffer and split into two. One received 100 units of Benzonase while the other was left untreated. Both were kept on ice for 10 minutes and then given two washes with Benzonase buffer and one wash with lysis buffer + PI. The beads were then collected and resuspended in 35 µl 1X Laemmli buffer and boiled for 5 mins at 98°C. Total extracts and immunoprecipitates were then analysed via western blotting.

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Supplementary data

Pilot experiments for checking phosphorylation status of chk1 as an indicator of functional DNA damage checkpoint

Chk1 is a protein kinase that is known to get phosphorylated in response to DNA damage during S phase. This phosphorylation increases the kinase activity which is essential for the maintenance of the checkpoint delaying the mitotic entry of the cell. Phosphorylation of chk1 reduces its mobility in SDS-PAGE which can be visualised as an upper shift in the chk1 bands (Walworth and Bernards 1996, Capasso et al. 2002).

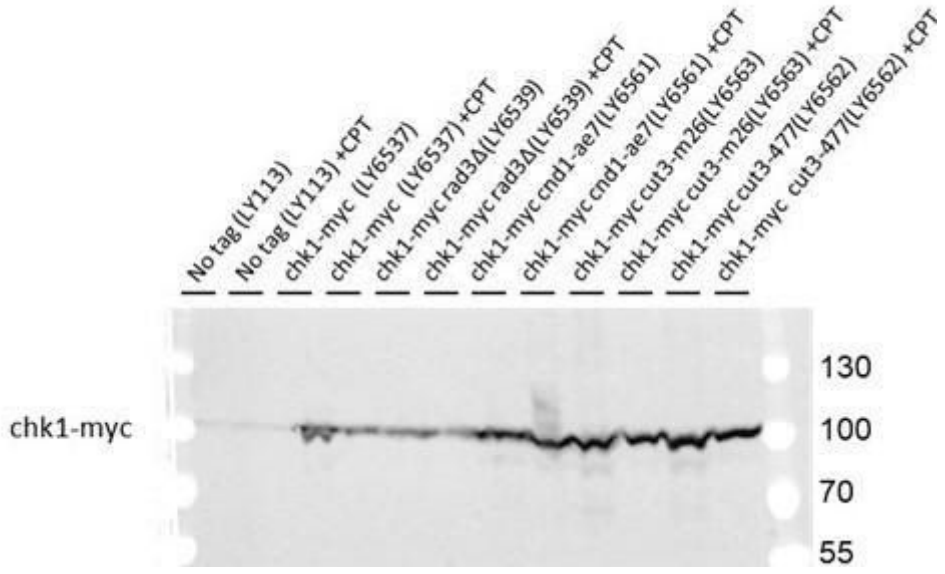


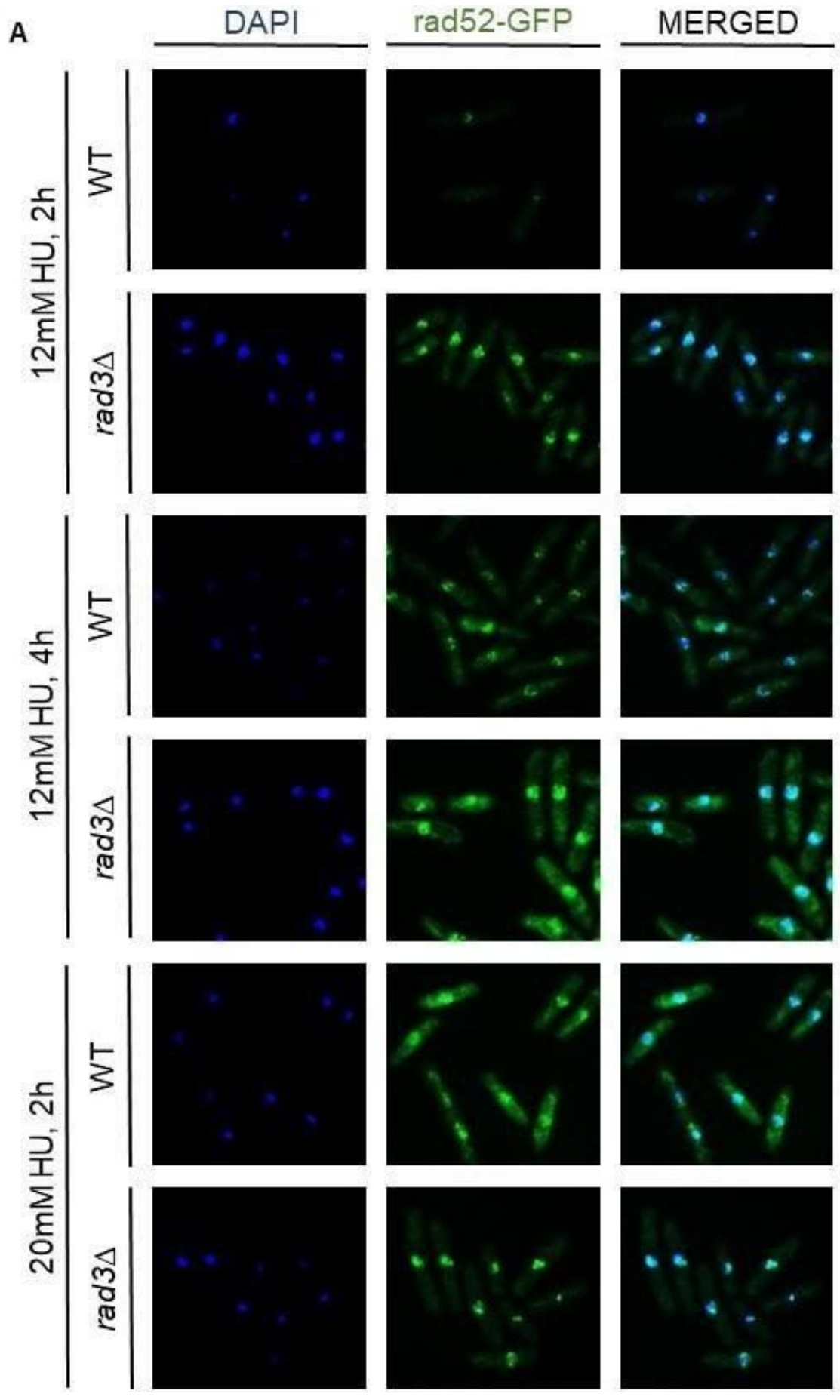
Figure 11: *Pilot experiments to determine chk1 phosphorylation status.(N=1)*

If the DNA damage checkpoint was damaged in the condensin mutants, treatment with camptothecin (CPT) a DNA damaging agent, would cause the phosphorylation of chk1 which could be visualised by western blotting. In order to do the same,

strains of the condensin mutants (cnd1-ae7, cut3-m26, and cut3-477) as well as a checkpoint defective rad3D mutant with myc tagged chk1 was made. The strains were treated with 40μM CPT for 2 hours and tested for chk1 phosphorylation by western blotting. I was unable to see a clear shift in any of the strains including the control and hence was unable to draw any conclusions.

Visualizing Rad52 foci as a measure of DNA replication checkpoint functioning

If the DNA replication checkpoint was non-functional, the replication forks would become unstable and collapse under HU stress, causing DNA damage (fig.). Rad52 is a protein that is involved in the homologous recombination and DNA repair pathway. Rad52 binds to ssDNA forming Rad52-DNA repair foci at the site of DNA damage (Kim et al. 2000). This could be visualised by observing Rad52 foci in the nuclei of such cells. In cells with undamaged DNA, Rad52 would appear as a diffused signal throughout the nuclei whereas in nuclei with damage they would appear as distinct foci (Noguchi et al. 2009).


A
DAPI
rad52-GFP
MERGED
12mM HU, 2h
WT
rad3Δ
12mM HU, 4h
WT
rad3Δ
20mM HU, 2h
WT
rad3Δ

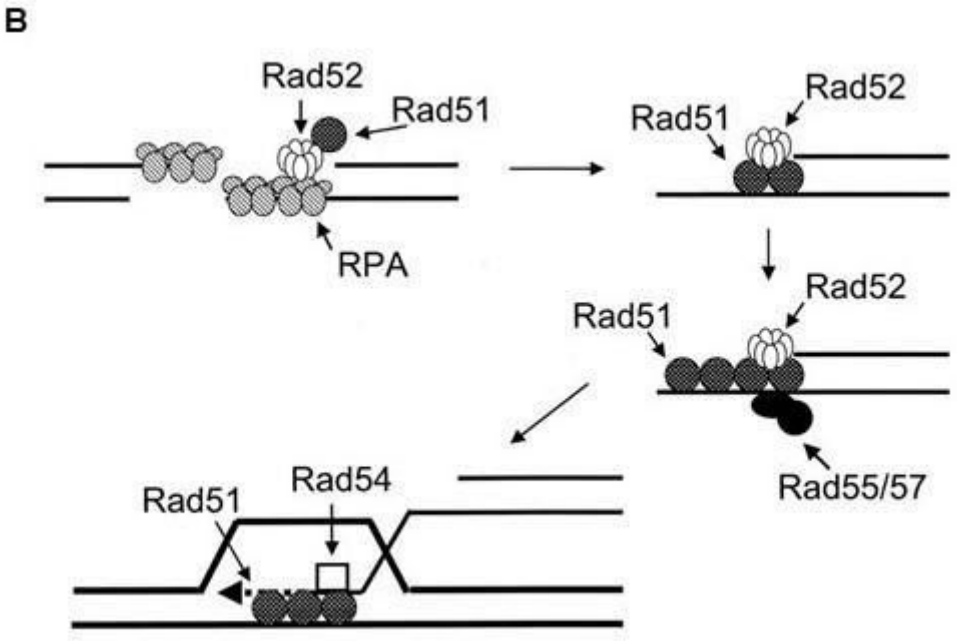


Figure 12: ***Rad52 DNA repair foci visualization*** (A) Standardisation tests done to determine Rad52 foci visualisation conditions. Fission yeast strains were grown exponentially and treated with HU (conditions and strains as indicated) and visualised for Rad52 foci (see text for details). (B) Rad52 DNA repair pathway. Adapted from Symington 2002 (see text for details).

In order to do so, I made strains of the condensin mutants (cnd1-ae7, cut3-m26, cut3-477) as well as a checkpoint defective rad3D mutant with GFP tagged Rad52. In order to objectively measure Rad52 foci levels as a proxy for DNA damage, I had to find a condition (concentration of HU + duration of treatment) where wild type cells exhibited diffused signal while checkpoint non-functional Rad3D mutant displayed distinct foci. I was unable to find such a condition and was hence unable to go forward with the experiment.

Making pcna tagged strains as a measure of DNA replication duration

Proliferating cell nuclear antigen (PCNA) is a toroidal shaped protein that acts as a processivity factor for DNA polymerase δ and ε (Kelman 1997). Since it is a part of the DNA replication machinery, it can be used as a proxy to visualize the DNA replication machinery. In the cell cycle, PCNA tag signal would become visible during early S phase and would die out by the end of it. I could hence use tagged PCNA to study the recovery of the replication forks in condensin mutants from HU stress.

In order to visualize pcna, I made strains of the condensin mutants (cnd1-ae7, cut3-m26, cut3-477) containing mcherry tagged pcna.

Upon visualizing the rate of disappearance of PCNA in wild type and the mutants upon release from a HU block, I expect the rate to be higher in the cnd1-ae7 and cut3-477 mutants as compared to the wild type in concurrence with the FACScan analysis.

Co-IP between rfc and condensin

The hosting lab had identified all subunits of RFC as potential interactors of condensin. Cells expressing condensin subunit cnd2 tagged with pk9 and RFC subunit RFC4 tagged with HA were used to verify the presence of a physical linkage between the two. Cycling cells were fixed and cnd2-pk9 was immunoprecipitated. The IP was then treated with benzonase to digest any DNA present to avoid proteins linked to cnd2 via DNA. The IP was then probed for RFC4 using the HA tag. I could find no RFC4 in the IP. To ensure that the IP was done correctly, the same was done to probe for HA tagged Cut14, a condensin subunit. Since both cut14 and cnd2 are condensin subunits and are physically linked, an IP of cnd2 would contain cut14

which would be visible upon probing and visualization. This was indeed the case, implying that the IP was done correctly and there is no physical link between cnd2 and RFC4 that could be detected by immunoprecipitation.

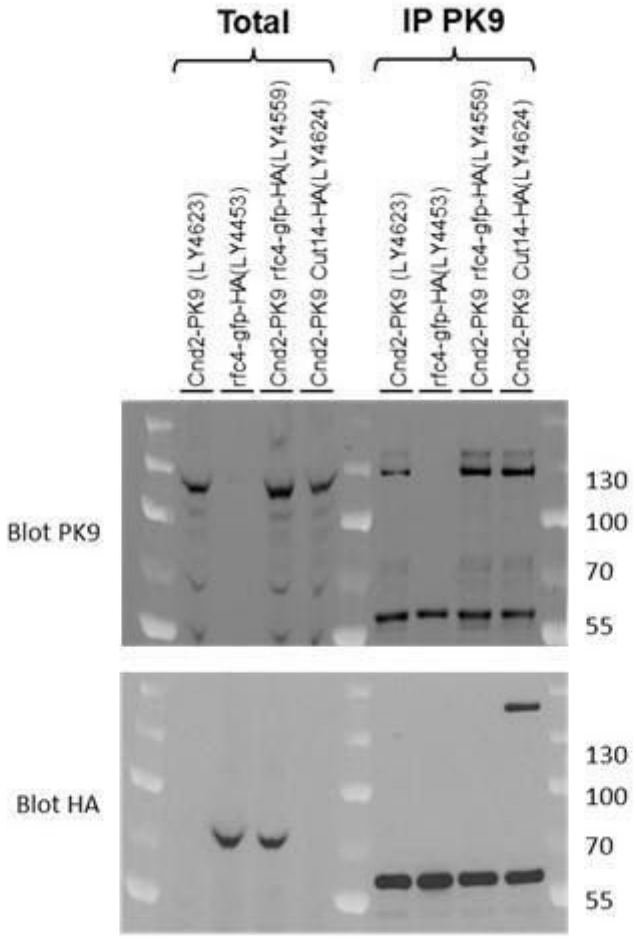


Figure 13 : *No physical interaction between RFC and condensin found.*