# DNA damage tolerance through the modification of replication clamp during Caenorhabditis elegans development

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# 博 士 論 文

DNA damage tolerance through the modification of
replication clamp during *Caenorhabditis elegans*
development

(線虫 *Caenorhabditis elegans* の発生過程における
複製クランプの分子修飾を介した DNA 損傷トレランス)

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東北大学大学院生命科学研究科

生態システム生命科学専攻

邵 震華

# Contents

# I.   Summery

# Summery

While duplication of the genome must be faithfully carried out in proliferating cells, DNA damage potentially, stall DNA replication machineries and causes cell death or genomic instability. To avoid these catastrophic events, when DNA damage is encountered by the replication machinery, DNA damage bypass mechanism provides multiple pathways to tolerate various types of replication blocking damage. Replicative bypass of DNA lesions occurs via translesion synthesis (TLS) by specialized damage-tolerant DNA polymerases. In the other hand, template switch (TS), which makes use of information of the newly synthesized sister chromatid, provides an error-free damage avoidance. In eukaryotes, these DNA damage bypass pathways largely depends on ubiquitination of the replication sliding clamp; proliferating cell nuclear antigen (PCNA). In response to DNA damage, mono-ubiquitination of PCNA at a conserved lysine 164 (K164) localize damage tolerant polymerases (such as polη, polκ, polι etc.) for TLS, whereas poly-ubiquitination triggers TS pathway. Specific E2-E3 enzyme complexes are required to link ubiquitin to PCNA-K164 (Rad6-Rad18 for mono- and Ubc-13-Mms2 for poly-ubiquitination). To date, the role of DNA damage bypass pathways during development of multicellular organisms and its regulation have not been explored. In this study, using Caenorhabditis elegans, I investigated whether PCNA

ubiquitination dependent damage bypass is uniformly functional during different developmental stages. In addition, to examine in which stages of development checkpoint pathways contribute to damage bypass, I investigated how these pathways are coordinated with DNA damage checkpoint activation, which also plays a role in earlier development.

**Experimental approaches**

To abrogate PCNA ubiquitination (PCNA-ub) in C. elegans, I generated the *pcn-1(K165R)* mutated *C. elegans*, in which the lysine residue (corresponds to K164 in yeast, mice, human, etc.) was substituted via CRISPR/Cas9 system. Using this mutant worm, I examined activity of DNA damage bypass at various stages during *C. elegans* development. In particular, following exposure to UV, influence of DNA damage bypass pathways to hatching rate of eggs, developmental variation of larva and progression of meiotic phases were monitored. In addition, I also examined genetic relationship of *pcn-1(K165R)* and mutation in a component of 9-1-1 complex Hus1, which causes the defect in checkpoint activation.

**Conclusion**

To determine the DNA damage sensitivity of this mutant, I first tested the hatching ability in the presence of UV or X-ray. Results show that substitution of PCNA K165 results in significantly UV induced failure in

*C. elegans* early embryo. In the other hand, functional defect of 9-1-1 complex in *hus-1 (op241)* worms did not affect hatchability. In *C. elegans* early embryogenesis stages, cells do not enables time for activation of checkpoint and repair of DNA lesion, thus damage bypass pathway should be a crucial for maintaining rapid cell division in the presence DNA replication blocking damages.

To determine the role of PCNA-ub in larval developmental stages and assess the effect of UV damages on the progression of *C. elegans* larval development from L1 to L4, the wildtype N2, *hus-1(op241)*, and *pcn-1(K165R)* worms were synchronized to the L1 stage and immediately exposed to 20 J/m2 UV irradiation. Interestingly, a partially but considerably larval arrest appeared in *pcn-1(K165R):hus-1(op241)* double mutant even without treatment to induce replication blockage, but this phenotype was not observed in either of the single mutants. As the cell number does not changes after L1 stage of C. elegans, the checkpoint is not expected to be functional at this stage. However, following continuous DNA synthesis and nuclear division results in polyploidy during larval development. Altogether, damage bypass but not checkpoint found to be important for larval development in the presence of UV damage. In contrast, during unperturbed condition, PCNA-ub and checkpoint

activation play complimentary roles to maintain normal development in *C. elegans.*

Given that the defect on PCNA-ub leads to considerably UV sensitive phenotypes in mitotic phases and larval developmental stages, I next investigated meiosis phases in worms deficient for PCNA-ub. Results show embryos early lethal at first 6 hours which zygote to early diakinesis were damaged. While UV damaged pachytene cells that processing meiosis I matured to the embryo at 6 to 12 hours. In addition, consistent with high levels of embryonic lethality due UV-induced damage, *pcn-1(K165R)* mutants display an array of chromosomal abnormalities such as poorly condensed chromosomes, integrated chromosomes and univalents (7–12 stained bodies). These results indicate that PCNA-ub contributes to maintaining chromosome stability during meiosis. As a hypothesis, I reason that the chromosomal defect arise from the failure of cross-over induced spontaneous DSB repair in meiosis I stages.

As a conclusion, in C. elegans, the PCNA-ub dependent DNA damage tolerance pathway is functionalised during all developmental stages in the presence of DNA synthesis blocking damage. Furthermore, these pathways cooperate with checkpoint system may maintain genome stability during post-mitotic processes in unperturbed condition.

# II.  Introduction

# Introduction

In order to transmit the genetic code accurately from one generation to the next, duplication of the genome must be faithfully carried out in all DNA proliferation events. In these processes, DNA damages from both endogenous (reactive oxygen species, enzymatic reaction, etc.) and exogenous (UV, ionizing radiation, chemical agent, etc.) sources can result in different types of DNA defection. Some base lesions induce the replicative polymerases to incorporate incorrect nucleotides, generating point mutations. However, most lesions inhibit DNA replication directly by blocking the polymerase reaction [1]. Their activity needs to be tightly regulated [2], [3]. To remove these DNA replicating blocking lesions from duplication events, organisms are equipped with various specialized tolerance and repair mechanisms [4]. Such as nucleotide excision repair (NER) and base excision repair (BER) can remove DNA lesion before replication events [5]–[7]. However, it is inevitable that some lesions cannot be faithfully repaired and are thus continuously presented to the replication machinery [8]. The remaining DNA lesion sites can potentially induce prolonged replication stalling and/or replication fork collapse. In addition, during rapid cell division or rapid DNA duplicating processes such as embryogenesis, only a relatively limited time window is available for repair processes [9]. In these cases, one of the choice for cells to

overcome DNA replication blocking damages is called post-replication repair (PRR). Actually, PRRs are damage tolerant pathways that make cells available to bypass or overcome lesions during replication [10]. The DNA damage bypass pathways act as a "last insurance" to ensure the completion replication when cells are exposed to DNA damage resources.

In eukaryotes, DNA damage bypass pathways are known to be efficiently activated by ubiquitination of the replication clamp proliferation cell nuclear antigen (PCNA). Monoubiquitination of PCNA induces efficiently translesion synthesis (TLS) via specific damage tolerant DNA polymerases (Such as polη, polκ, polι etc.) [11]–[13]. When replication fork block at lesion sites, these TLS polymerases have been shown to replace the stalled replicative DNA polymerases (normally polδ & polε) via a mechanism of PCNA monoubiquitination at conserved lysine 164 (K164) by the E2/E3 enzyme complex Rad6/Rad18. Monoubiquitinated PCNA has an increased affinity for TLS polymerases, thus potentially helping to recruit these polymerases to stalled forks and bypass the lesion site by incorporating correct/incorrect bases on the opposite strand [11], [14], [15]. Remarkably, almost all TLS polymerases lack proofreading domain and share a conserved active site, which is usually different from high-fidelity polymerases [16]–[18]. So TLS allows for overcoming of DNA lesion but also results in a reduction of fidelity [12], [19], [20]. On the other hand,

polyubiquitination of PCNA by other E2/E3 (Ubc-13/Mms2) triggers an error-free pathway called template switch (TS). This TS pathway bypass DNA lesion by exchange the template to the sister strand [11], [21]. Both TLS and TS pathways need the ubiquitination on the K164 site of PCNA. Mutational inactivation of these motifs abolishes TLS in yeast and prevents damage-induced association of the mutated polymerases with PCNA in mammalian cells [13], [22]–[28]

In humans, the defection of TLS polymerases results in Xeroderma Pigmentosum Variant complementation (XPV), a syndrome that is associated with a high predisposition towards developing skin cancers [1], [29]–[31]. Thus, the mechanisms that how PCNA ubiquitination-dependent DNA damage bypass works in-vivo should be important to further understand this issue. To date, in vivo study on PCNAK164R mutant mice shows a reduction of mutations at template A/T in B cells [32]. Study on *Xenopus* shows that the E3 ligase RAD18 inhibits checkpoint in the early embryo, indicates cooperation between checkpoint system and damage bypass [33], [34]. However, the role of DNA damage bypass pathways during the development of multicellular organisms and its regulation has not been explored.

In this study, nematode *Caenorhabditis elegans* was used as a Metazoan

model to examine the DNA bypass. I investigated whether PCNA ubiquitination-dependent damage bypass is uniformly functional during different developmental stages and how these pathways are coordinated with DNA damage checkpoint activation, which also plays a role in earlier development.

# III. Experimental procedures

# Experimental procedures

## 1. *C. elegans* genetics and culture

All strains were cultured according to standard methods as described in Brenner [35]. Wild-type nematodes were maintained following a standard protocol: maintained on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 and stored at 20 °C [36]. All strains were maintained at 20°C under standard conditions. Experiments were performed at 20°C. To synchronize worm stage, about 50 gravid hermaphrodites were picked onto a fresh plate and allowed to lay eggs for 2 h before being removed. Alternatively, synchronized gravid hermaphrodites and their previously hatched progeny were washed from a plate, leaving laid embryos on the solid media. The synchronized worms were used in each experiment assay.

The N2 Bristol strain obtained from Caenorhabditis Genetics Center (CGC) was used as the wild-type background. The mutant strain *hus-1(op241)*, which has a substitution in the coding exon, also obtained from CGC. I generated the *pcn-1 (K165R)* mutant via CRISPR/Cas9 system as described by Arribere and Frokjaser [37], [38]. The gRNA, ssODN, and oligoes used in this study were listed (Table. 1). The *pcn-1(K165R): hus-*

*1(op241)* was generated by mating.

## 2. Construction of His-tagged PCNA worm strain

His-tag was designed as 16-His on upstream of PCNA N-terminal start codon with GGSG linker. His-PCNA sequence was infusion with miniMos translocate plasmid (PCFJ1662). The detail procedures were listed (Fig. 5) and the detail method was described by C. Frokjaer-Jensen [38].

## 3. RNA interference

Feeding RNAi experiments were performed at either 20°C or 25°C as described in [39]. The *ubc-1*, *polh-1* RNAi clone was verified from Ahringer's RNAi library. The bacteria HT115 carrying the empty pL4440 vector was used as control RNAi. The effectiveness of RNAi was examined by assaying the expression of the transcript being depleted in three individual worms subjected to RNAi by feeding. Expression of the eef-2 transcript was used as a control.

## 4. Hatching rate assay

Synchronized young adult worms were picked onto each fresh plate at

20 °C after UV exposure. During the egg-laying period, 4 to 6 nematodes were transferred or removed after indicated time in each experiment. The number of the total eggs laid and unhatched eggs were counted. The tests were independently performed 3–5 times. Approximately 80–200 eggs were scored in each experiment.

## 5. Larval development assay.

Method used as described by Daitoku [40]. Synchronized first-stage larvae (L1) (at least 100 worms were tested) were exposed to UV (20 J/m2) on NGM solid plates seeded with bacterial OP50. The larvae at each stage (L1&L2, L3, and L4) were counted at 72 hours after UV exposure.

## 6. Western blot analysis

Total protein was extracted from young adults of cultured N2, *pcn-1(K165R), pcn-1(K108R)* and *pcn-1(K201R)* strains with/without UV exposure and subjected to western blot analysis. *C. elegans* rabbit-anti-PCNA polyclonal antibody (source?), against which site of PCNA, was used as 1st antibody.  The 2nd antibody is xxx. Proteins were stacking on 8% polyacrylamide gels and separated on 15% polyacrylamide gels with SDS 10%, then blotted onto a PVDF membrane (Immobilon-P, Millipore

Corporation). The membranes were treated for 1h at room temperature with indicated 1$^{st}$ antibody and probed with 2$^{nd}$ antibody. Signals were detected with a Pierce™ ECL Western Blotting Substrate (Chemical substrate, ThermoFisher Scientific) using provided protocol. Membrane images were analysis with ImageJ software (NIH).

# 7. *C. elegans* gonad staining and microscopy assay (detail protocol)

Gonad dissections

1. Pick adults to an "unseeded" plate. Alternatively, wash worms off plate with phosphate buffered saline (PBS) or M9 and spin in a clinical centrifuge (3000 rpm) for 1 minute to pellet worms. Aspirate supernatant carefully.

2. Resuspend worms or pick worms into 1-2 ml of PBS or M9 containing 0.2 mM Levamisole or 10–25 mM sodium azide (NaN3) (this will paralyze worms) and transfer to the well of a depression slide.

3. As paralysis sets in, begin cutting off heads at level of pharynx or in the middle of worms: Place single worm between two 25-gauge syringe needles and decapitate by moving needles in a scissors motion. The release from the internal hydrostatic pressure of the worm should result in at least one gonad arm extruding completely.

4. Remove excess liquid with a drawn-out Pasteur pipette.

Fixation

Fix in 3-4 ml of 3% formaldehyde or 3.5% glutaraldehyde in 0.1 M K2HPO4 (pH 7.2) for 30min to 2 hours. After fixation, transfer to a 10 ml glass conical tube, add a few of PBST and spin 1 min in clinical centrifuge (3000 rpm). Remove supernatant, wash 1x in PBST, and then post-fix in 4 ml of ¬20C methanol for 5 min. Fill tube with PBST, spin, and wash 1x in 4 ml PBST.

DAPI/Hoechst 33342 staining

1. After last rinse of fixation protocol (above) pellet worms by either spinning in clinical centrifuge or simply let worms settle by gravity to bottom of glass tube or just in on the slide glass.

Tube method: Aspirate off supernatant and add 1 ml or less of DAPI/Hoechst 33342 solution (1:1000 dilution of stock in PBS).

Slide glass: Prepare a new slide glass and drop 0.1ml of DAPI/Hoechst 33342 solution. (0.1ml of PBS + 1μl of 1/10 DAPI/Hoechst 33342 stock of pre-diluted 1:1000 of stock in PBS)

Stain for 30min to 1hrs in the dark, keep wet.

2. Transfer worms onto the slide glass for Microscopy with 3-6μl (Depend on glass size) DABCO in each hole. Set cover glass and seal.

3. Slides can be stored at 4C for a week or more, particularly if sealed with nail polish around the periphery of the coverslip.

Microscopy assay

All the microscopy check was taken by Olympus xxx.

Materials

PBS: Dilute to 1x from 10x stock.

10x PBS: 80 g NaCl, 2 g KCl, 6.1 g anhydrous $Na_2HPO_4$, 2 g $KH_2PO_4$, $H_2O$ to 1 liter. Autoclave and store at room temperature.

PBST: 1x PBS with 0.1% Tween 20

3% formaldehyde/0.1 M $K_2HPO_4$(pH 7.2): Prepared from sealed ampoules of 16% EM grade formaldehyde. Freeze any excess.

3.5% glutaraldehyde/0.1 M $K_2HPO_4$(pH 7.2): Prepared from sealed ampoules of 70% EM grade glutaraldehyde.

DAPI/Hoechst 33342: Make stock solution by dissolving powder in 70% ethanol to a concentration of 100 µg/ml. To use, dilute stock in buffer 1:1000 (final concentration is 100 ng/ml).

DABCO: 1% 1,4-diazobicyclo [2,2,2]-octane (DABCO) in 90% glycerol in PBS. Store at -20 good for years.

Methanol: 100% stock kept at ¬-20C

# IV. Results

# Result

## 1. The Ubiquitination of PCNA is crucial for progeny hatching in the presence of DNA replication blocking damages.

The ubiquitin-dependent DNA damage bypass in *Saccharomyces cerevisae* depends on the E2-E3 enzyme complex that links the ubiquitin to lysine 164 (K164) of PCNA. To investigate the roles of PCNA ubiquitination in *C. elegans*, I aligned the *C. elegans* pcn-1 amino acid sequences with other six species from yeast to human. (Fig.1a)　As there is one amino acid shift in the PCNA sequence of *C. elegans*, I modified the lysine 165 (K165) into arginine via the CRISPR/Cas9 system (Fig. 1b). Thus, ubiquitin cannot conjugate to PCNA in this *pcn-1(K165R)* mutant worm strain.

To determine the DNA damage sensitivity of this mutant, I first tested the hatching ability in the presence of UV that caused DNA lesion damage or X-ray that induced not only double strand break (DSB), but also a large amount of single strand lesions. After UV or X-ray exposure, I observed severed does dependent hatching rate reduction of *pcn-1(K165R)* eggs laid in first 2 hours (Fig. 2).

Until now, in *C. elegans*, less mutant strains are significantly UV sensitivity.

Remarkably, even with a very low dose of UV exposure ($10J/m^2$), the hatchability of *pcn-1(K165R)* strain was about 30% whereas it was about 80% of wildtype worms. As I know the UV induced DNA lesion will stick the replication machine during DNA proliferation, these data show that modify the K165 of PCNA cause significantly UV induced defection in *C. elegans* eggs, indicating that the ubiquitination of PCNA is necessary for maintain hatchability when exposed to DNA replication blocking damages.

## 2. Chemical evidence for UV induced specific PCNA ubiquitination

I considered finding some directly chemical evidences that PCNA ubiquitination cannot take place in the *pcn-1(K165)* mutant. However, unfortunately, additional modified PCNA bonds appeared in mutant worms and it was not UV dependent in our western-blot assay (Fig. 3). Therefore, I considered that whether there are other ubiquitin conjugation targets in *C. elegans* PCNA. In order to figure out this issue, I blasted *C. elegans* PCNA amino acid sequences with other seven species from yeast to human. After mapped all possible lysine on the 3D model of human PCNA, I distinguished the position of lysine108 and lysine201 are very close to lysine165 in *C. elegans* PCNA. (Fig. 4a). I generated pcn-1(K108R) and

pcn-1(K201R) mutant via CRISPR/Cas9, but unluckily both of these two mutants did not show UV induced defect on embryo and larval development. UV dependent ubiquitination of PCNA was also not detected (Fig. 4b, c).

I another way, I wondered whether the modified band upon PCNA were exactly ubiquitination bands or not. To figure it out, I designed a His-tagged PCNA plasmid and made this additional His-tagged PCNA translocated into a random location of the genome *C. elegans* via microinjection (Fig. 5). Finally, I got the mutation which His-PCNA sequence was detected by sequencing (Fig. 6a). However, unfortunately, the results of western blotting using anti-PCNA or anti-His showed that His tag did not express (Fig. 6b).

Above all, I failed to display the chemical evidence of PCNA ubiquitination. The experimental methods in *C, elegans* should be further improved in my plan.

## 3. UV-induced Egg Lethality Is Independent of Checkpoint but Involves the DNA replication bypass.

Since I did not discover the direct evidence of PCNA modification in *C. elegans*, next I decided to characterize which pathway was involved in this UV sensitivity in *pcn-1(K165R)* mutant. It is well known that the ubiquitination of PCNA will lead to translesion synthesis (TLS) or template switch pathway, which is considered to bypass the DNA lesion damages during replication. These bypasses need unique E2-E3 enzyme complex to conjugate ubiquitin onto the specific lysine of PCNA. *C. elegans* possesses ubc-2 gene, which is an orthologue of TLS E2 rad6 and polh-1 encodes a specific polymerase that is homologs with human TLS polη. In this experiment, synchronized wildtype N2 and *pcn-1(K165R)* worms were feed with control bacteria or *ubc-1*, *polh-1* siRNA bacteria. Then Young adult worms were exposed to mild UV irradiation, and the hatching rate of the first 8 hours was counted. As expected, knockdown either *ubc-2* or *polh-1* significantly decrease the UV tolerance of wild-type worms. However, the UV tolerance with/without silenced *ubc-2* or *polh-1* remained almost same level in *pcn-1(K165R)* worms (Fig. 7). These epistatic results indicated that the K165R modification in PCNA defects the TLS replication bypass thus the UV tolerance of mutant worms significantly decreased.

In addition, I wondered that whether this defect on UV tolerance in K165R mutant worms related with the DNA damage checkpoint. The Hus-1 is a

component protein of DNA-bound 9-1-1 complex that can facilitate ATR-mediated phosphorylation and activation of Chk1, a protein kinase that regulates S-phase progression, G2/M arrest, and replication fork stabilization. I used a *hus-1(op241)*.mutant as a partially checkpoint defect model in this study because it showed normal hatchability (Fig. 2). With even very low dose of X-ray irradiation, the hatching rate of both *pcn-1(K165R)* and *hus-1(op241)* mutant significantly decreased compared with wildtype N2 (Fig. 2b). Remarkably, hatchability of *hus-1(op241)* remained almost same level with wildtype after UV exposure while the hatchability of *pcn-1(K165R)* mutant eggs appeared heavily dose dependent reduction.

To further investigate this issue, *pcn-1(K165R):hus-1(op241)* double mutant was generated via mating. Although this mutant has a severe defect on producing embryos, I tried to accumulate a large number of eggs (over 100 eggs per experiment) to exam whether hatchability change with/without UV exposure. In opposite from the larval development, the hatching rate of unperturbed double mutant embryos remained almost same level compared with wildtype and every single mutant. After UV exposure, the hatchability of double mutant was similar to *pcn-1(K165R)* single mutant embryos (Fig. 8).

As a conclusion, the DNA replication bypass but not DNA damage

checkpoint is crucial for damage tolerance in the embryogenesis processes in *C. elegans*.

## 4. UV induces early embryo mitosis arrest in the absent of PCNA ubiquitination.

Having confirmed that ubiquitin-dependent replication bypass is essential to overcome DNA replication blocking damages in embryo, I thought to investigate how the defect on *pcn-1(K165R)* mutant affected embryogenesis after UV exposure. I therefore carefully check the cell division from the 2-cells stage to over 8-cells stage. Young adult worms of N2, *pcn-1(K165R)* and *hus-1(op241)* were exposed to 35J/m2 of UV irradiation, then they were dissected in order to release the embryos. Our initial hypothesis is that UV induced DNA lesion blocking the replication machine so that the mitosis should slow down when the replication bypass was inhibited. To figure it out, I took several photos of early 2-cell stage embryos in each strain to observe the early embryo cell division. Intriguingly, both of the *hus-1(op241)* and *pcn-1(K165R)* embryos that successfully divided into 4 cells after 20 minutes and shared almost same speed of cell division with wildtype N2 in the absence/presence of UV exposure. However, high frequency of obviously cell cycle arrest phenotype appeared in K165R embryos (Fig. 9). Moreover, I counted 50

embryos of each strain in the 2-cell stage. Two hours after UV exposure, about 20% of N2 and *hus-1(op241)* embryo arrested at 2 or 4 cell stage while it was almost 80% of *pcn-1(K165R)* strain (Fig. 10).

As we know, in *C. elegans*, rapidly mitosis with fast cell cycle threaded the whole embryogenesis processes. Taken together, these results reveal that the PCNA ubiquitination-dependent replication bypass but not DNA damage checkpoint maintain rapidly cell cycle to overcome DNA synthesis blocking damages.

## 5. PCNA ubiquitination is essential for UV tolerance during larval development.

In *C. elegans* larval stages, the somatic tissues development are composed of proliferative DNA during polyploidy and nuclear division processes. Since the heavily UV induced defect on embryogenesis in the absence of replication bypass, I next focus on larval development because TLS is accompanied by DNA replication. In this study, I used the method described by Daitoku [40] to assess the effect of UV damages on the progression of *C. elegans* larval development from L1 to L4. (Fig. 11a). In this assay, the wildtype N2, *hus-1(op241)*, and *pcn-1(K165R)* worms were synchronized to the L1 stage and immediately exposed to 20 J/m2 UV irradiation. After 72 hours, the developmental stages of the corresponding

UV exposed worms were categorized as L1/L2, L3, and L4. I found that UV irradiation gave rise to a considerable and an almost complete L1/L2 arrest in the *pcn-1(K165R)* but not *hus-1(op241)* mutant (Fig. 11b).

In addition, almost same level of larval arrest appeared when knock down the TLS polymerase *polh-1* using siRNA, and it was epistatic with *pcn-1(K165R)* mutant (Fig. 11b), These results suggest that PCNA ubiquitination-dependent DNA replication bypass rather than DNA damage checkpoint contributes to the resistance of UV-induced DNA damage in the larval development.

## 6. The ubiquitination of PCNA is crucial for unperturbed larval development in the checkpoint deficient background.

Our data showed that the DNA damage checkpoint is not essential in both embryogenesis and larval developmental stages in the absence/presence of DNA synthesis blocking damages.

Surprisingly, a partially but considerably larval arrest appeared in *pcn-1(K165R):hus-1(op241)* double mutant even without any DNA synthetic stress, but either of the single mutants did not observe this phenotype (Fig.12a). In addition, I observed a high ratio of abnormal morphology of

the developing vulva that protruding vulva (Pvl) appeared (Fig. 12b). Moreover, in these Pvl animals, observed defection on germline formation always accompanied with the abnormal vulva that these worms could not produce the next generation. I also tested the effect of knockdown other checkpoint component using siRNA. Results show similar defect on unperturbed larval development with knockdown all components of 9-1-1 complex and chk-1(Fig.13). In addition, not only larval development, but also germline formation was arrest in the double mutant (Fig. 14).

Taken together, my results indicate that PCNA ubiquitination-dependent pathway cooperate with DNA damage checkpoint is essential to maintain normal development in *C, elegans*.

## 7. PCNA ubiquitination inhibits chromosome instability during meiosis

Due to the defection on PCNA ubiquitination leads to considerably UV sensitive phenotypes in mitotic phases and larval developmental stages, next we, therefore, investigated the meiosis phases of PCNA ubiquitination defected mutation. Firstly, I performed a time-course of hatchability in the presence of UV damage. Young adult worms of wild-type N2 and *pcn-1(K165R)* were exposed to 35J/m2 UV irradiation and were moved to new plates every 2 hours. The hatching rate of wildtype eggs that laid in 0-2 and

2-4 hours was about 40%-50% while the *pcn-1(K165R)* mutant was 10%-20%. However, the hatchability of wildtype recovered from 4-6 hours to 10-12hours then finally up to about 100%. Remarkably, the hatching rate of PCNA mutant embryos still at a very low level that below 40%. In this assay (Fig. 15), UV irradiation damaged embryos and whole germline of young adult worms. The embryos produced at first 6 hours are range from zygote to early Diakinesis areas that meiosis I nearly finished. UV damaged pachytene cells that processing meiosis I matured to the embryo at 6 to 12 hours. Therefore, this data indicates that the ubiquitination of PCNA plays important roles also in meiotic stages.

To further investigate what happened to the meiotic-original dead eggs, I stained wild type and *pcn-1(K165R)* worms with Hoechst 33342 to labeling total DNA and quantified the number of Hoechst-stained bodies in the most-mature diakinesis-arrested oocytes after UV exposure. In case of wildtype, 6 Hoechst-stained indicating bivalents could be clearly distinguished. Consistent with high levels of embryonic lethality, *pcn-1(K165R)* mutants display an array of chromosomal abnormalities that include poorly condensed chromosomes, integrated chromosomes and univalents (7–12 stained bodies) or a combination of them all (Fig. 16a). As expected, abnormal chromosomes appeared in oocytes and the numbers reduced during the time after UV exposure that might relate with the

recovering of hatchability. However, these chromosomal variances also appeared even without UV exposure in *pcn-1(K165R)* oocytes (Fig. 16b). Taken together, these results indicate that the ubiquitination of PCNA contributes to maintaining chromosome stability during meiosis.

# V. Discussion

# Discussion

Here I constructed *the pcn-1(K165R)* mutation using *C. elegans*, which is prohibited site-specific modifications of PCNAK165 (PCNAK164 in yeast, mice, human etc.) required for PCNA-dependent DNA damage bypass. I demonstrate that this mutation results in UV-sensitive phenotypes during embryogenesis (mitotic phases), meiotic phases of *C. elegans*. This is consistent with the role of PCNA ubiquitination in DNA damage tolerance, which has been characterized in various organisms [1]. In post-mitotic stages, the TLS Polη deficient mice do not appear any considerably spontaneous/UV-induced development delay at least in the first year. [41] However, K165R worms also appear severe UV induced development retardation. As we know, the division of somatic cells occur across the developmental stage of mice, so the checkpoint system may work to overcome DNA damage and adjust cell cycle. However, the cell number would not change after L1 stage of *C. elegans* thus checkpoint cannot work. However, polyploidy and cell nuclear division are needed for larval development [42]. So the damaged DNA duplication cannot be rescued by the checkpoint system thus replication bypass should be a crucial pathway in this situation.

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**Discussion**

Until now, there rarely exist worm strains that sensitive to a low dose of UV exposure in mitotic phases. The severe UV sensitive mutation such as a DNA interstrand cross-link (ICL) repair defective mutation *dog-1*, can endure UV irradiation at 50 J/m$^2$ [43]–[45]. Remarkably, our *pcn-1(K165R)* mutant is sensitive to a very low dose of UV (less than 20 J/m2) in embryogenesis phases. During rapid cell division stages in the early embryogenesis that immediately after fertilization of mature oocytes, I found that the embryo division and survival depend on the PCNA-ub involved DNA damage bypass but not DNA damage checkpoint, the TLS components seem not to interacted with checkpoint system, however, the E3 ligase RAD18 inhibits checkpoint in Xenopus early embryos [34]. This result coincides with a study that checkpoint response to DNA damage is actively silenced in embryos [46]. In addition, there are some studies indicate that NER is important in response to specific DNA damages during mitotic phases [45], [47]. However, there is a study consist that embryo survival is determined by TLS factors and not by NER [48]. In almost all major animal phyla, the embryonic cell cycles tend to be extremely fast [46], [48]. Noticeably, the early cycles of *C. elegans* last only 10–40 min. In this case, our results indicate that there should be a strict timing of DNA synthesis, which cannot "wait" for repair processes to be taken place because the rapid cycling allows no time for lesion repair. Therefore, lesion bypass but not NER or other checkpoint related repaid

pathways might be the only feasible option to maintain embryogenesis in the presence of replication blocking damages.

Our present results imply abnormal somatic cells development and vulva formation appeared in *pcn-1(K165R):hus-1(op241)* double mutant worm even in unperturbed condition. One possibility is that defect of the checkpoint will accumulate errors in the genome. Some errors will block replication machinery if lack of TLS to bypass mutation sites thus severe developmental retardation appear. Another possibility may be these defect not rely on the bypass function of PCNA-ub. In fission yeast, the ubiquitination of PCNA increases the proportion of chromatin-associated PCNA during unperturbed replication. The ubiquitination of PCNA in K164 increases the Polδ association with PCNA thus prolong the chromatin association with replication proteins to allow efficient completion of Okazaki fragment synthesis by mediation gap filling [49], [50]. In addition, HUS1 is required for genome stability under non-stressed conditions in Leishmania [51]. These reports suggest that both PCNA-ub and Hus-1 contribute to maintaining genome stability under unperturbed condition. Therefore, PCNA-ub together with Hus-1 may play some synergistic roles to maintain genome stability during post-mitotic developmental processes in *C. elegans*. The other possibility may involve in the functional overlap between DNA damage bypass and checkpoint

pathways. As ATR-dependent phosphorylation of polη is necessary to restore normal survival and post-replication repair after UV damage in human xeroderma pigmentosum variant fibroblasts, and require Polη binding to ubiquitilyted PCNA [52]. In addition, the Chk1 which locates in the downstream of 9-1-1 complex, tend to stabilize Claspin thus regulates the binding of ubiquitin ligase Rad18 to chromatin [53]. Thus, the developmental retardation in this double mutant may result from the loss of these interactions between DNA damage checkpoint and replication bypass.

It has been known the inherited cancer-propensity syndrome xeroderma pigmentosum variant results from error-prone TLS of UV induced lesion of DNA. Moreover, the tumor suppressor p53 can promote PCNA monoubiquitination via transcriptional induction of Polη [54]. Our study shows in the meiosis I phase, abnormal, low condense or univalent chromosomes appear in mature oocytes and increase frequency in the presence of UV irradiation, indicates that PCNA-ub mediated DNA damage bypass contributes to at least from crossovers (COs) generation to meiosis I division. As I know, faithful segregation of chromosomes during the first meiotic division depends on recombination between homologous chromosomes, resulting in the generation of COs after double-strand break (DSB) events [55]. The DSBs induction and repair are crucial to this

process. These DSBs can be repaired via homologous recombination (HR) or the error-prone nonhomologous end joining (NHEJ) pathway. Therefore, in this case, I consider that PCNA-ub depends on DNA damage bypass may contribute to these DSB repair events because DNA synthesis is needed in either HR or NHEJ process in *C. elegans*. In the *pcn-1(K165R)* mutant, DSBs generated in COs formation processes cannot be efficiently repaired, so meiosis I division failed that abnormal chromosomes appear in late oocytes. Lack of damage bypass may defect DSB repair, to further investigate mutation with Spo-11, a DSB inducer during COs formation. These chromosomal defects in this study are similar to tumor p53 (CEP-1) defective *C. elegans* mutation [55]. So it suggests that PCNA-ub may play similar roles like p53 to maintain genome stability during gametogenesis, thus, this study may contribute to better understand of chromosomal instability related tumor formation.

Nevertheless, our study distinguished several separate roles of PCNA-ub during mitotic, meiotic and post-mitotic stages in multicellular animal *C. elegans* and deepened understanding of DNA damage bypass pathways in vivo during development.
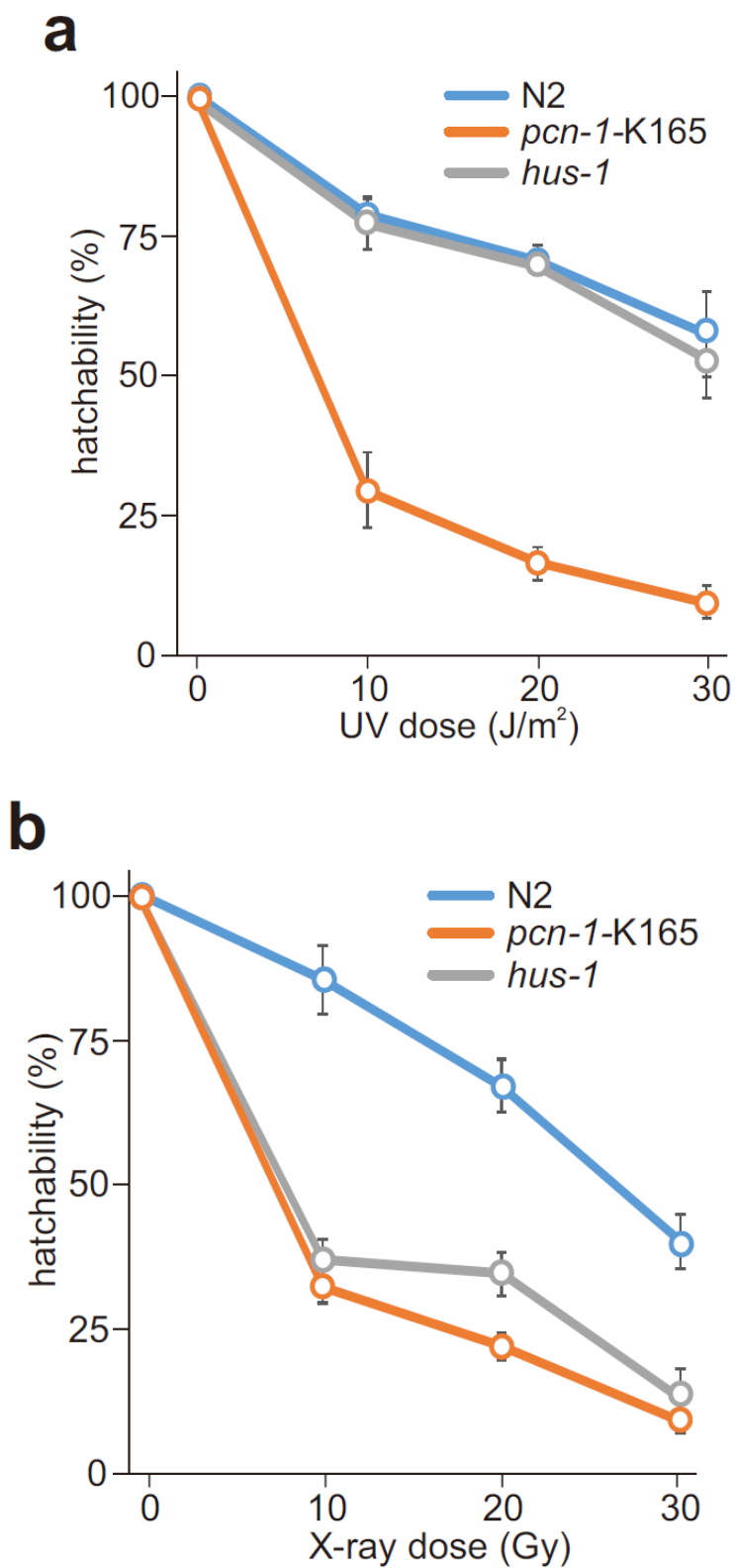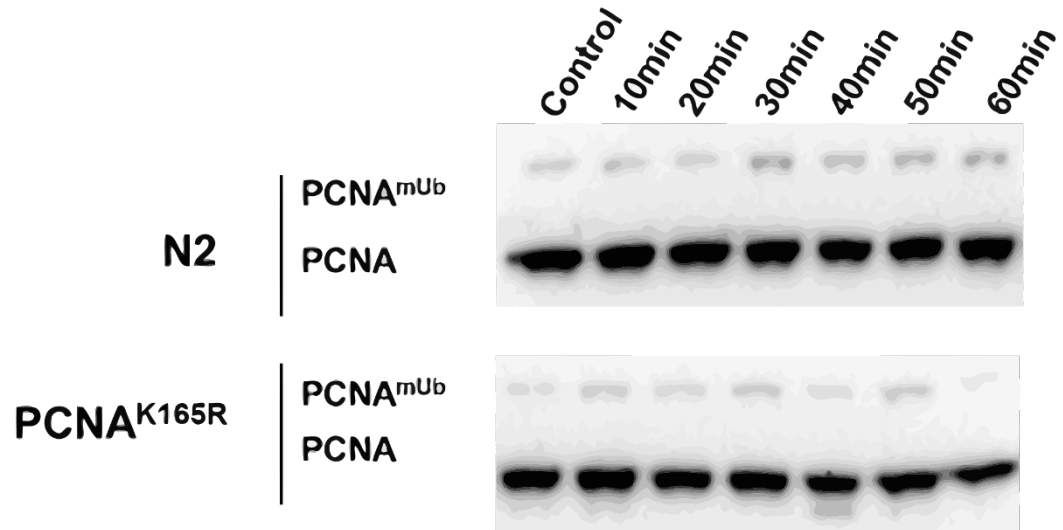
# VI. Figures

# Figure. 1

a

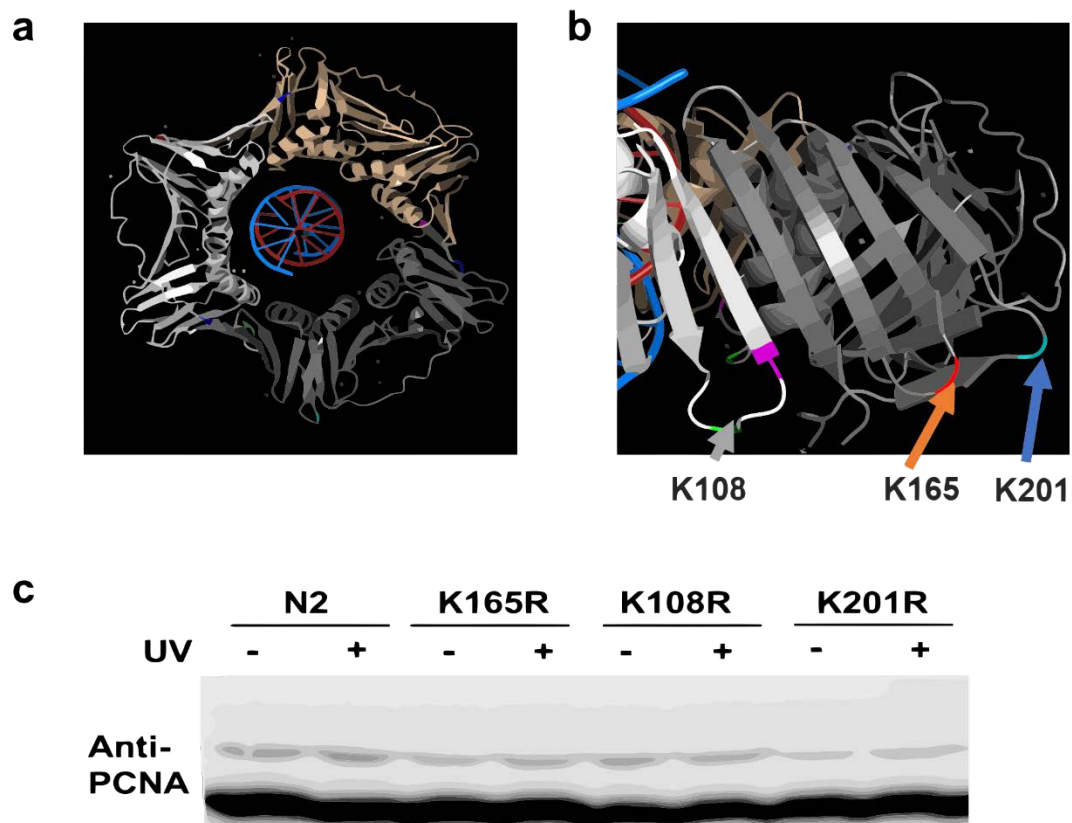| | | | | | | | | | | | | | | 164 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. cerevisiae* | 152 | S | Q | L | S | D | S | I | N | I | M | I | T | K | E | T | I |
| *S. pombe* | 152 | * | T | L | S | D | S | V | * | I | * | * | * | K | E | G | V |
| *C. elegans* | 153 | S | T | F | S | D | S | * | N | I | * | I | T | K | * | G | I |
| *Drosophila* | 152 | * | Q | F | S | * | S | V | V | I | M | I | T | K | E | G | V |
| *Xenopus* | 152 | S | Q | I | G | D | A | V | V | I | M | I | A | K | D | G | V |
| *Mouse* | 152 | S | H | I | G | D | A | V | V | I | M | I | A | K | * | G | V |
| *Human* | 152 | S | H | I | G | D | A | V | V | I | M | I | A | K | D | G | V |

b

Co-injection marker gRNA
dpy-10 target seq
U6 prom. tracrRNA

Donor ssDNA

Target gRNA
target seq
U6 prom. tracrRNA

Donor ssDNA

Cas9 expression Plasmid (pDD162, Dickinson et. al. 2013 )
eft-3 promoter Cas9 ORF tbb-2 3'UTR

Injection

F1 Screen F1 for dumpy or roller type

F2 Transfer single F2 to individual plate

F3 Worm PCR using mix of single F2 and F3

Check with restriction enzyme or sequencing

Homologues mutant  ×  Wildtype N2 ♂

Backcross two times

Homologues/non-dumpy mutant

# Figure. 2

**a**



**b**

# Figure. 3

# Figure. 4

a



b



K108     K165     K201

c

| | N2 | | K165R | | K108R | | K201R | |
|---|---|---|---|---|---|---|---|---|
| UV | − | + | − | + | − | + | − | + |

Anti-
PCNA

# Figure. 5
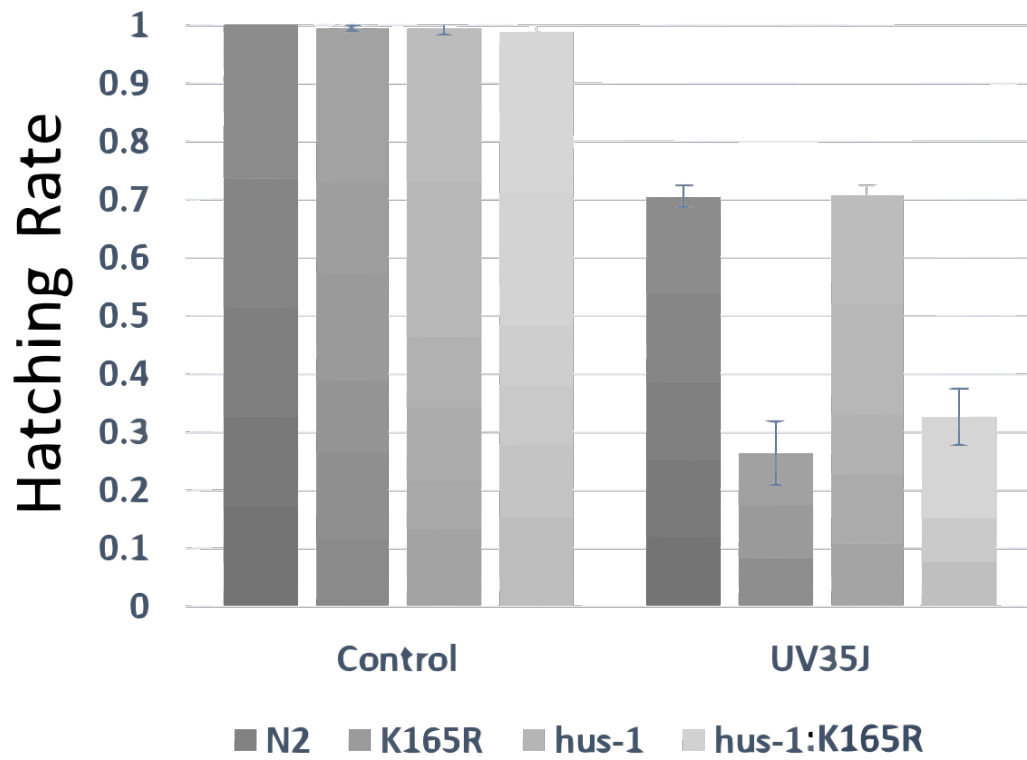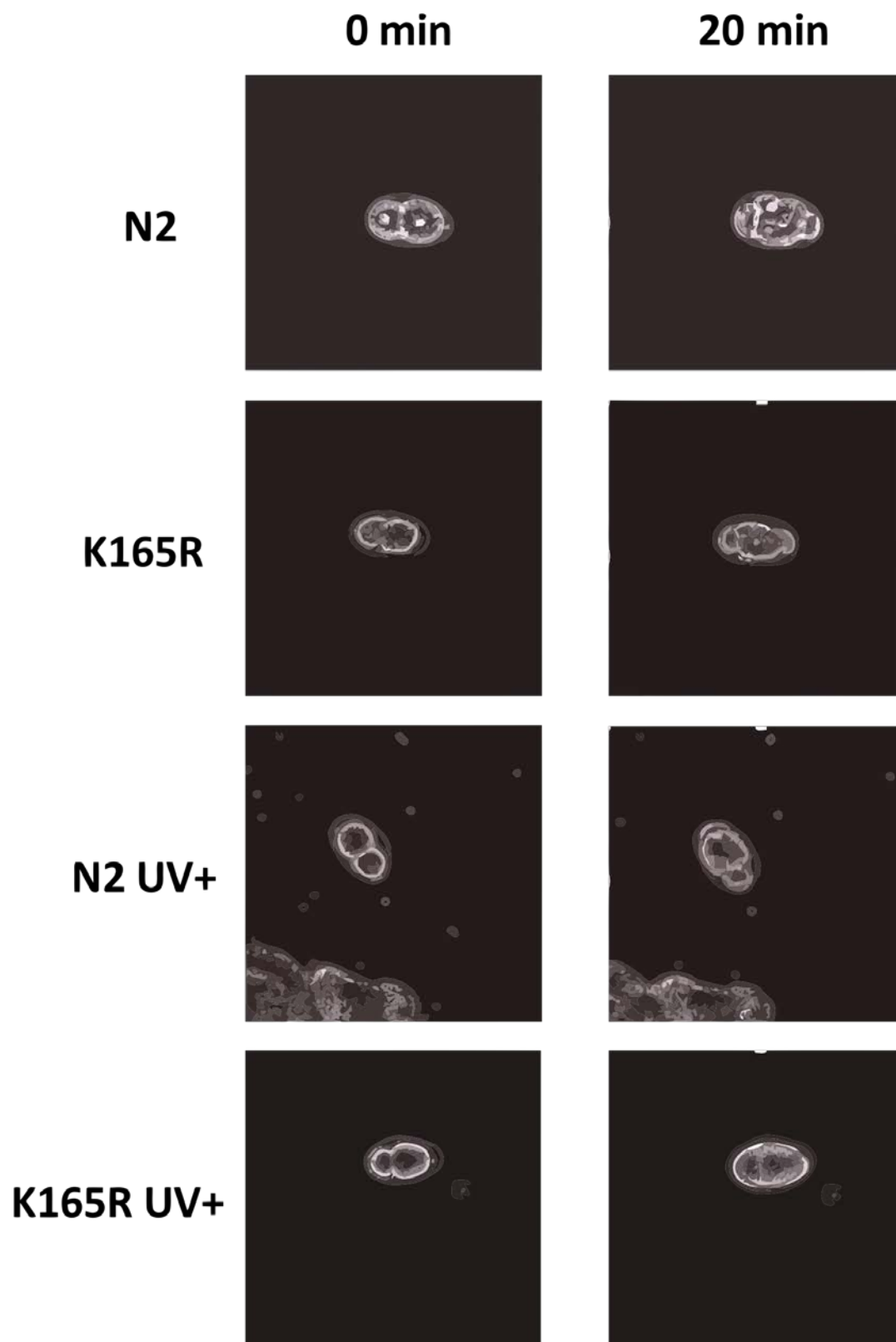


**Induce His-tagged PCNA into worms**
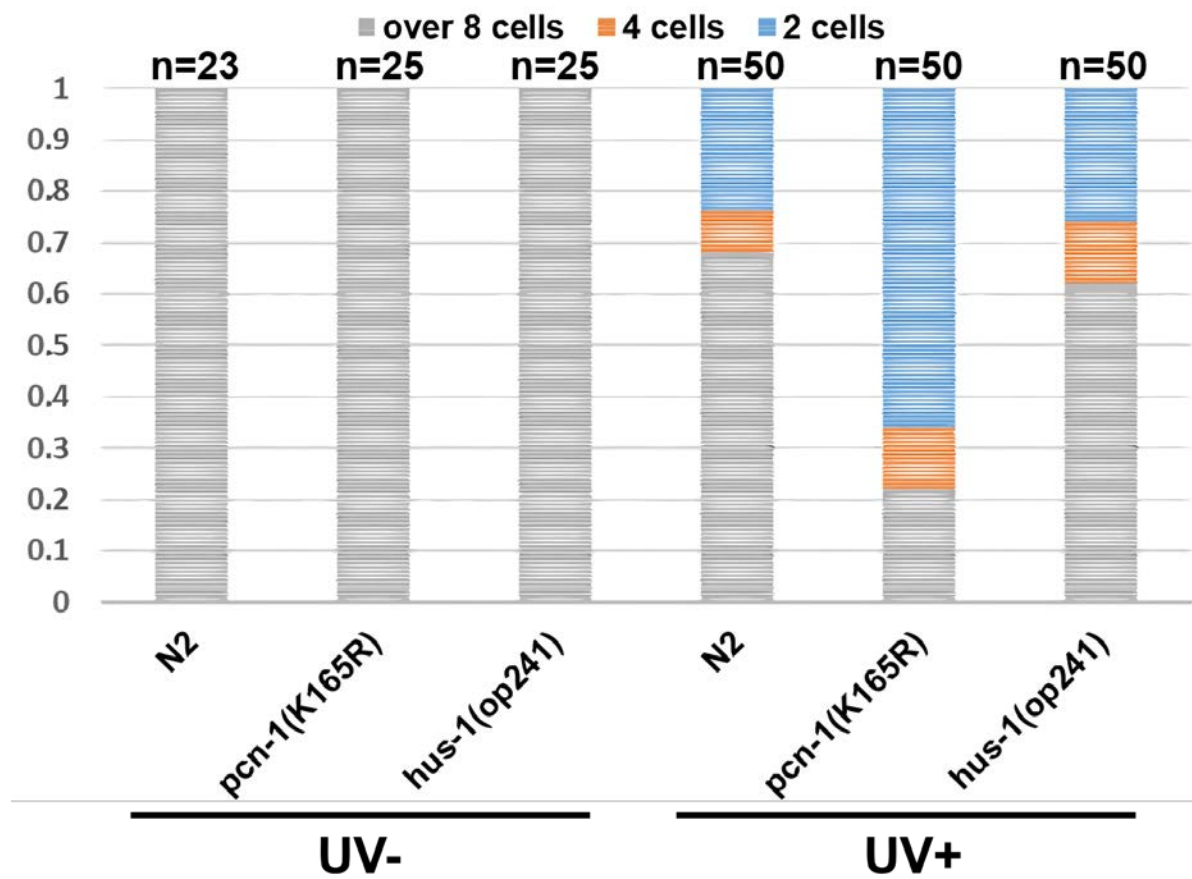
# Figure. 6

# Figure. 7

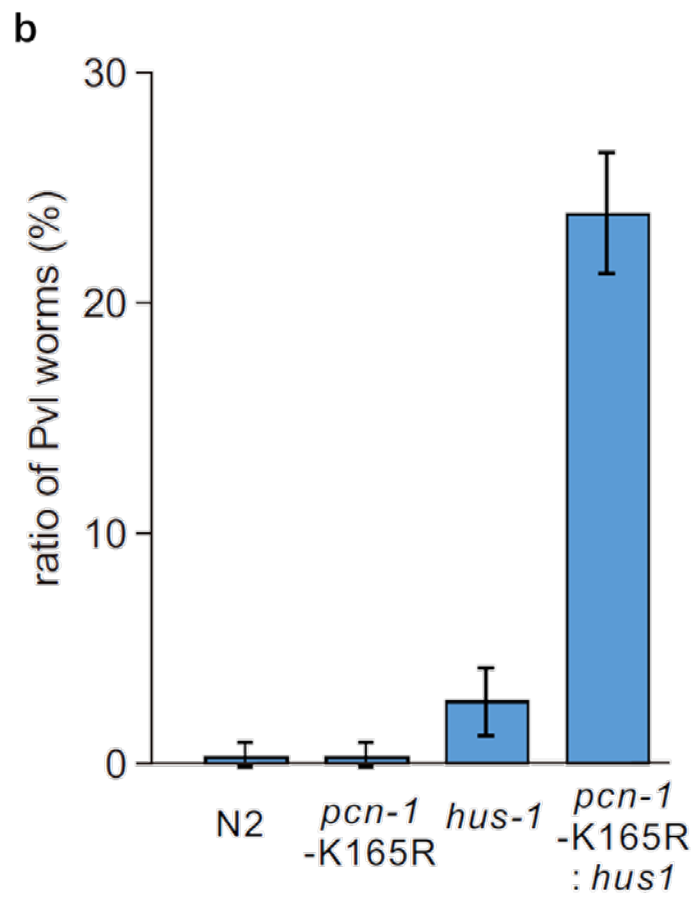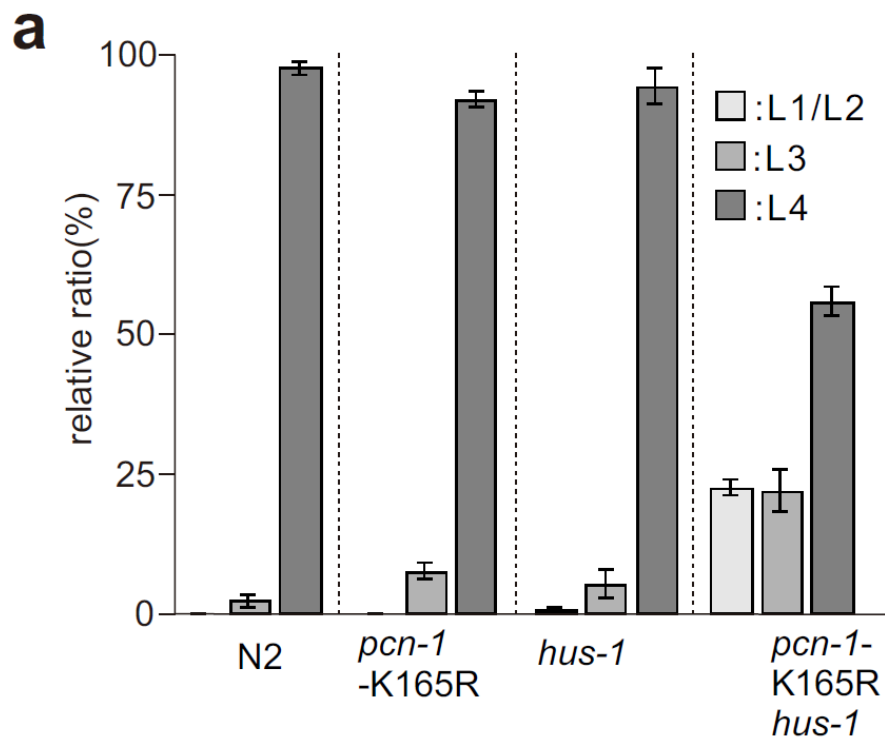# Figure. 8

# Figure. 9

## Figure. 10
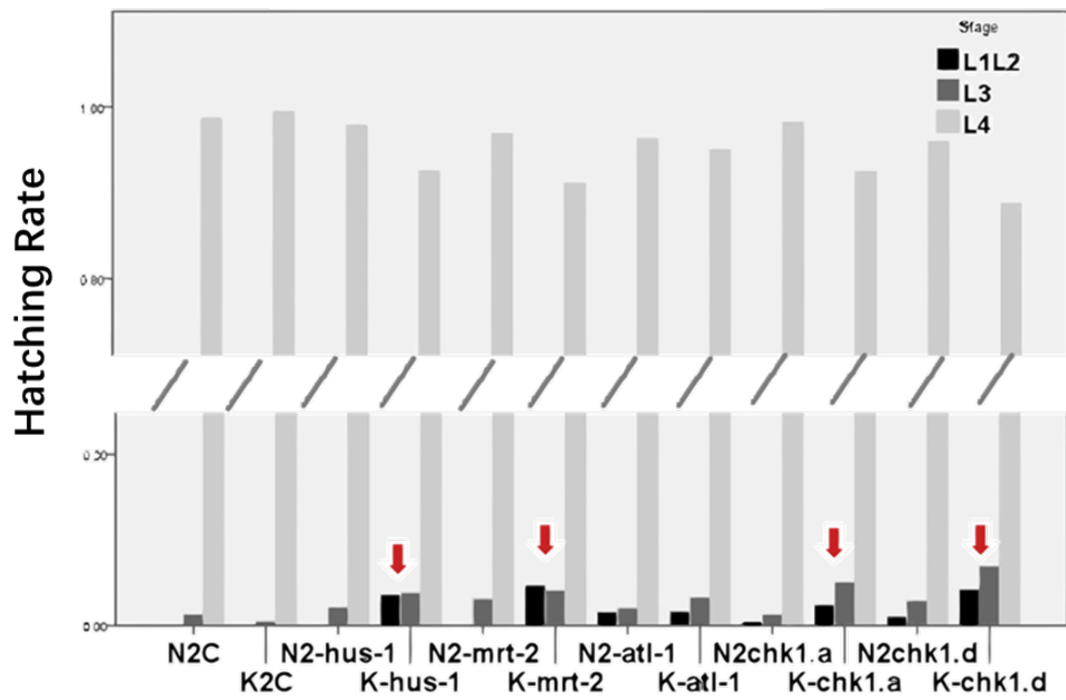
# Figure. 11

a



b

# Figure. 12

## a



## b
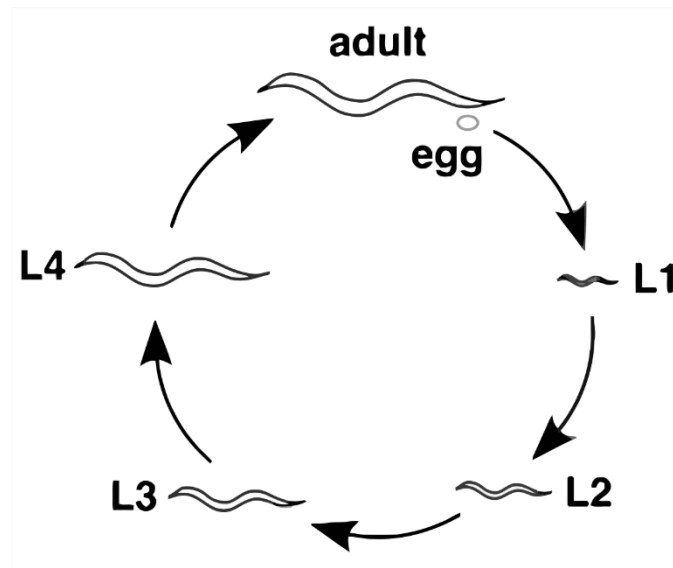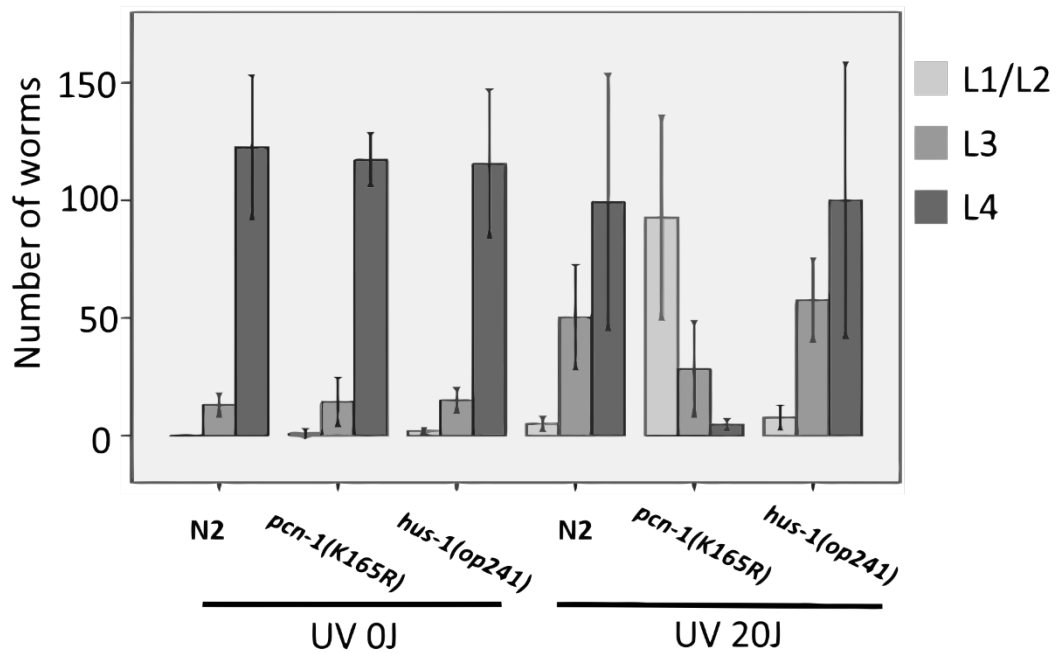
# Figure. 13

# Figure. 14

# Figure. 15

# Figure. 16

a



Normal     Integration     7-12 Chromosomes     Poorly condense
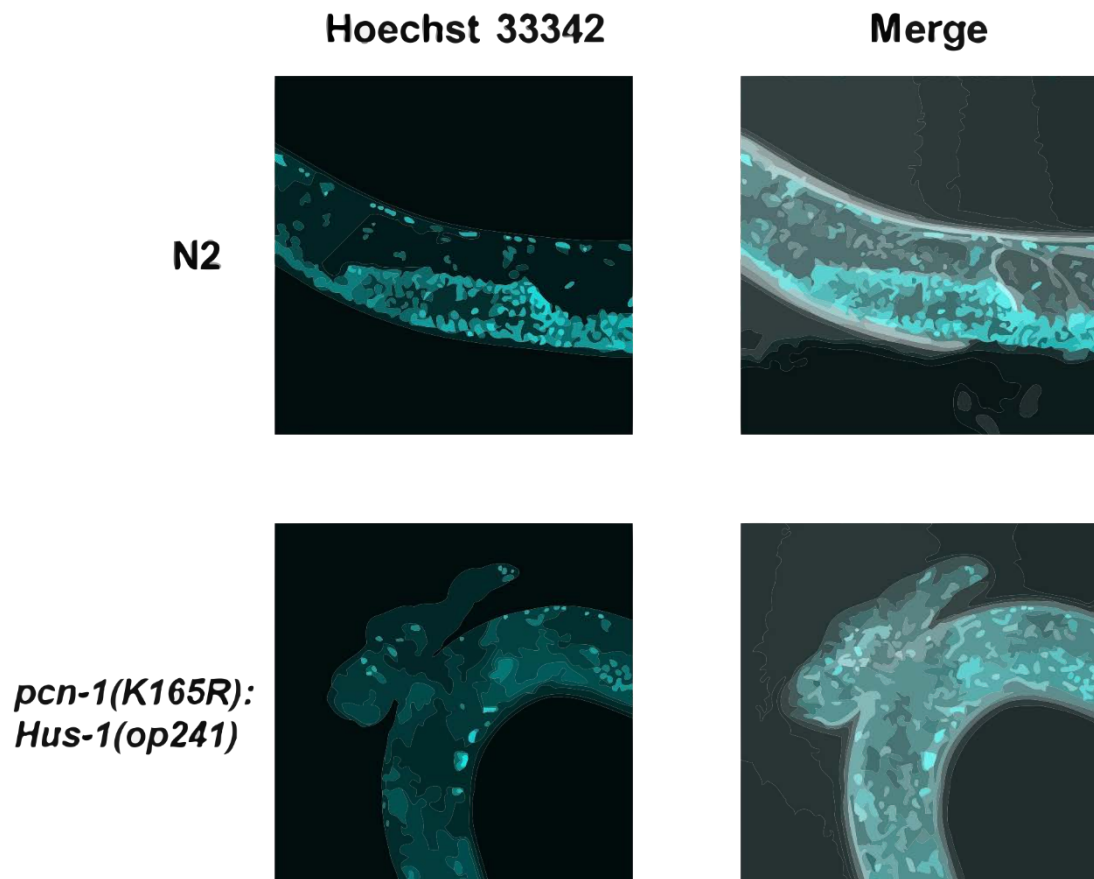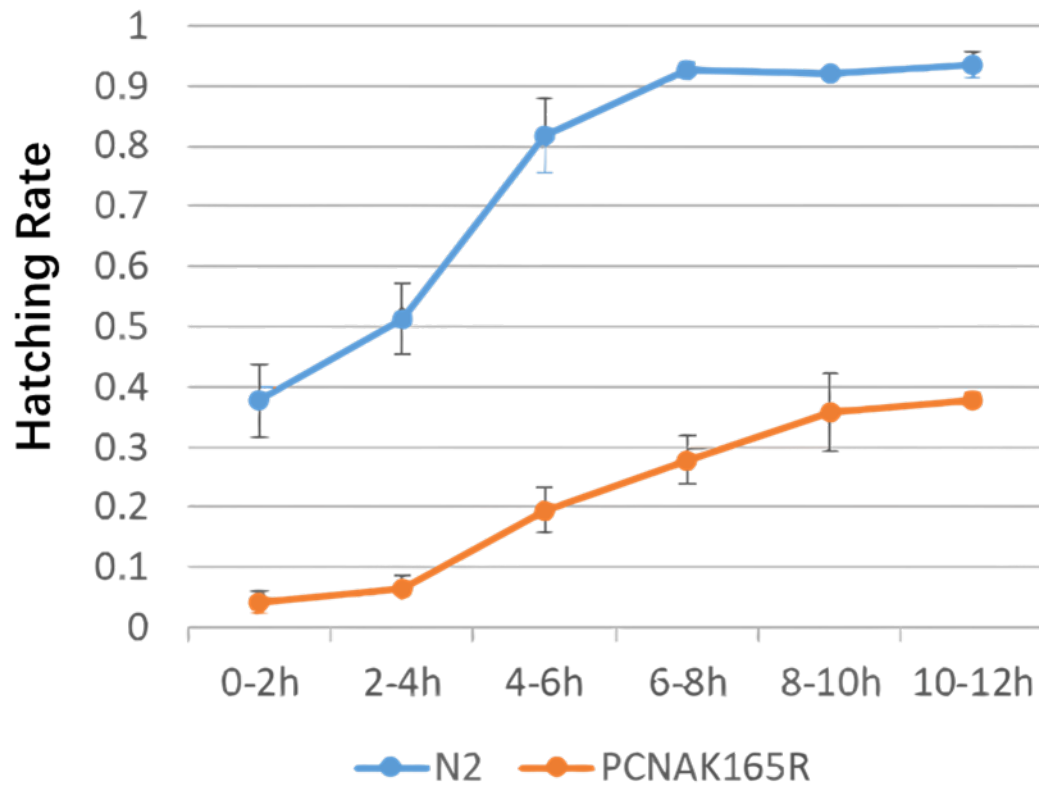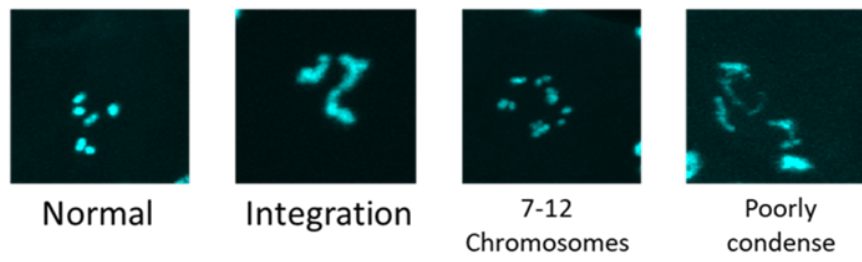
b
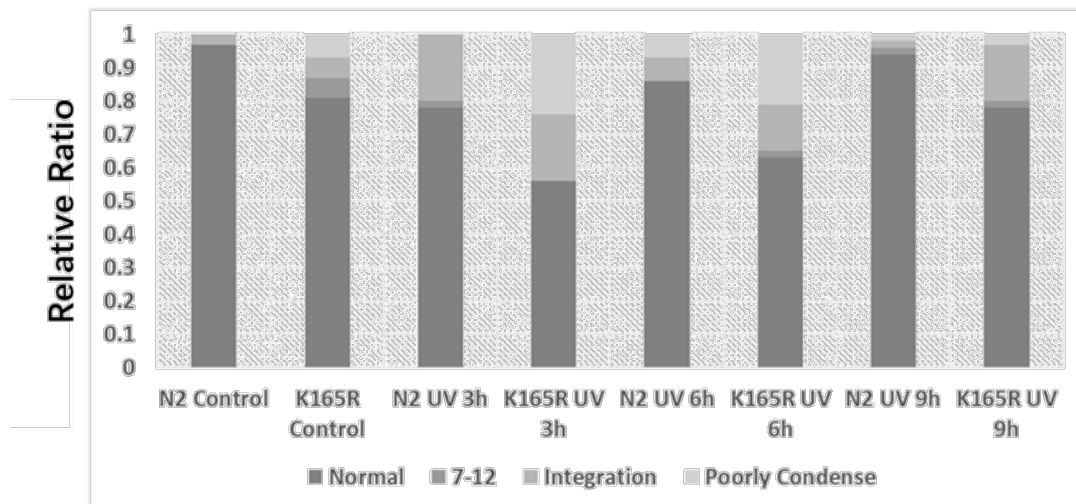
# Table. 1

| Oligo | Oligo sequence | Oligo function |
|-------|----------------|----------------|
| ce-pcn1-K165-tg-F-2 | tcttgAACACGATGCCAGC**CTT**GG | K165R gRNA |
| ce-pcn1-K165-tg-R-2 | aaacCC**AAG**GCTGGCATCGTGTTc | K165R gRNA |
| ce-pcn1-K165R-100bp-2 | TGCAAGGATCTGTCGACGTTCTCCGACTCGTTGAACATCAC CGCCACC**CGT**GCTGGCATCGTGTTCACCGGAAAGGGAGA**TA** TCGGATCTTCTGTCGTCA | K165R ssODN |
| ce-pcn1-K108R-ssODN | aaaaattccaattttaagcccaaaaattaccaattttcatt gcagTATGAGGAAAACGAAGGCGACTCGATCATCTTCACAT TCGCCGATCCAcgtCGTGACAAGACCCAAGACGTGACAGTC AAAA | K108R ssODN |
| ce-pcn1-K201R-ssODN | AGAGAGAGACGAACTCTGTCGGAAAGTGCCGTCGCCTTGGT GAACTGATTCATGTACTTGATCGAGAAGTTCACATTAACCG GATCacgGACCTCCAGTGTTACAGCCTCAGTCTCATCGTCA GTGT | K201R gRNA |
| ce-pcn1-K108-tg-F | tcttgGTCTTGTCACGCTTTGGAT | K108 gRNA |
| ce-pcn1-K108-tg-R | aaacATCCAAAGCGTGACAAGACc | K108 gRNA |
| ce-pcn1-K201-tg-F | tcttgGCTGTAACACTGGAGGTCA | K201 gRNA |
| ce-pcn1-K201-tg-R | aaacTGACCTCCAGTGTTACAGCc | K201 gRNA |

# VII. Figure Legends

## Fig. 1 Generate the mutation at the ubiquitination site of PCNA in C. elegans via CRISPR/Cas9 system.

(a) Alignment of PCNA amino acid sequences between seven species. The ubiquitin binding site K164 (K165 in *C. elegans*) was marked in red. * represents unmatched amino acids.

(b) Procedures to generate mutation via CRISPR/Cas9 in this study. All listed compounds were injected to the gonads of healthy young adult worms. F1 worms with dumpy phenotype were selected individually in each single plate. F2 worms (at least 8/plate ) were picked into individual plate and do worm PCR after F3 came out. Selected homozygous mutant then backcross with wild-type worms for at least two times.

## Fig. 2 Hatching rate was significantly reduced in the PCNA modified worm strain.

(a) & (b) Quantification of egg hatch ability among F1 progeny of wild type N2 and pcn-1(K165R) mutants expose to UV or X-ray. Young adult worms were exposed to UV or X-ray, the hatch ability of eggs laid during first 2h was counted. Data are represented as log [mean] ± SEM; At least 100-200 eggs of each strain were counted.

**Fig. 3 Ubiquitination-like bands appeared in both N2 and *pcn-1* (K165R) worms.**

Young adult worms were collected (at least 500 worms) and extracted total protein. Anti-*C. elegans*- PCNA antibody was used as 1$^{st}$ antibody to show the modification of PCNA. Times indicate the minutes that worms were collected at indicated time after UV exposure.

**Fig. 4 Modification at K108R and K201R did not change the PCNA modification detected after UV exposure.**

(a) The 3D structure of human PCNA homo-trimer.

(b) The position of K108, K165 and K201. Arrows indicate the location of these three lysine.

(c) Western blotting of N2, K165R, K108R and K201R worms 30min after UV exposure. Anti-PCNA was used as 1$^{st}$ antibody.

**Fig. 5 Procedure of generating His-tagged PCNA in *C. elegans*.**

His-tagged PCNA sequence was infused into miniMos-plasmid then injected into worms.

**Fig. 6 His-tagged PCNA strains failed to express His-PCNA.**

The western blotting result of His-tag PCNA strain using anti-PCNA (up) and anti-His (down).

**Fig. 7 The UV sensitivity on PCNA-ub defect worms depend on DNA replication bypass.**

(a) & (b) Effects of UV irradiation on egg hatching of strain N2 and pcn-1(K165R) mutants under ubc-1 or polh-1 knockdown background. Synchronized young adults were exposed to UV, eggs laid at first 8h were counted. All data in this experiment are presented as mean values.

**Fig. 8 The hatchability was not changed in *pcn-1(K165R):hus-1(op241)* double mutant.**

Quantification of egg hatch ability among F1 progeny of wild type N2, *pcn-1(K165R)*, *hus-1(op241)* and double mutant expose to UV irradiation (35J/m2). Young adult worms were exposed to UV. The hatch ability of eggs laid during first 8h was counted. Data are presented as mean values ± standard errors of SEM.

**Fig. 9 Early mitosis arrest appeared in K165R embryogenesis with UV exposure.**

The 2-cell stage eggs were selected and taken photo before/20 min after

UV exposure. Each strain was examined at least 25 samples in this experiment.

**Fig. 10 UV damage induces cell mitosis arrest in the absent of PCNA ubiquitination.**

Synchronized young adults were exposed to mild UV (35J/m2), the worms were dissected and eggs on 2-cells stage were located. After 2h, we checked cell stages of these located eggs. The n value means the number of 2-cells stage eggs that were counted in each line.

**Fig. 11 Development retardation appears in pcn-1(K165R) mutant after low dose of UV exposure.**

(a) Schematic of the life cycle of C. elegans. C. elegans has four larval stages (L1 to L4) and finally reach adulthood.

(b) Effects of UV irradiation on larval development of strain N2 and *pcn-1(K165R)* mutants. Synchronized L1 larvae were exposed to UV (20 J/m2), and after 3d,  animals in L1/L2, L3, and L4 developmental stages were counted. Data are presented as mean values ± standard errors of SEM.

**Fig. 12 In the DNA damage checkpoint-deficient background, PCNA ubiquitination is crucial for larval development.**

(a) Larva development of strain N2, pcn-1(K165R), hus-1(op241) and pcn-1(K165R)::hus-1(op241) double mutant without DNA damages. Synchronized L1 larvae were transferred to new plates and after 3d, animals in L1/L2, L3, and L4 developmental stages were counted.

(b) The abnormal protruding vulvas rate of N2, pcn-1(K165R), hus-1(op241) and double mutant. Synchronized L1 larvae were transferred to new plates and after 4d, young adult worms with normal or protruding vulvas were counted.

Data are presented as mean values ± standard errors of SEM.

## Fig. 13 Embryogenesis defect appeared in K165R strain after knockdown different components of checkpoint system.

Quantification of egg hatch ability among F1 progeny of wild type N2, *pcn-1(K165R)* in different checkpoint components knockdown background. Red arrows indicate considerably differences. Data are presented as mean values ± standard errors of SEM.

## Fig. 14 Germline formation defect on *pcn-1(K165R):hus-1(op241)* double mutant.

Young adult worms were stained with Hoechst 33342 without UV exposure.

In photos of double mutation, protruding vulva and unformatted germline were showed.

**Fig. 15 Time course of hatchability after UV exposure.**

Young adults worm were irradiated with 35J of UV. Hatching rate were counted each 2 hours after UV dose. Data are represented as mean value $\pm$ SEM.

**Fig. 16 UV exposure triggered abnormal segregated chromosomes appears in late pachytene during meiosis.**

Worms were fixation 3h, 6h, 9h after UV exposure (35J/m2) then stained by Hoechst 33342.

(a) The chromosomes of -1 and -2 oocyte on diakinesis were checked and divided into 4 patterns.

(b) Quantification of oocytes in different patterns of individual strain. At least 80 nucleuses were counted in each series.

**Table.1 Oligoes used in this study.**

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# Reference

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