Mechanisms that Induce Replication Stress Induced Nucleophagy (ReSIN)

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Abstract:

Cell division, from DNA replication to mitosis, is a highly coordinated process resulting in each cell receiving identical copies of the genome. Genome stability requires coordination between sister chromatid replication and segregation. The final steps in this process occur in anaphase with the resolution of sister chromatids and completion of DNA replication. Failure to properly resolve sister chromatids in a timely manner can give rise to lagging chromosomes that can be inappropriately packaged in nuclear envelope, forming a so-called micronucleus. Micronuclei (MN) are unstable and their disassembly can result in chromosome fragmentation as well as the activation of an inflammatory response triggered by cytosolic DNA. To avoid MN formation, nuclear envelope formation is highly regulated, and it has been proposed that autophagy might surveil and suppress the inadvertent formation of MN. It is unclear how such a surveillance pathway is regulated. We hypothesize that a specific form of autophagy, nucleophagy, is controlled by the Intra-S-Phase kinase cascade that is triggered by changes in chromosome structure that arises from stalled DNA replication. Preliminary findings from our lab demonstrate that the drug hydroxyurea, which depletes nucleotide pools resulting in replication stress, also induces nucleophagy, a pathway we refer to as replication stress induced nucleophagy or, ReSIN. To confirm that ReSIN is triggered by stalled replication forks and not simply depletion of nucleotide pools, we will engineer cells so DNA polymerase type I can be depleted. We predict that loss of Pol1 will result in stalled replication forks that will also induce ReSIN and that this pathway will require the intra-S phase signaling pathway.

Introduction:

Autophagy is a conserved pathway in which the cell targets organelles or large macromolecular complexes for degradation in the vacuole or lysosome under starvation conditions or after other forms of cell stress. There are three different forms in which autophagy can occur: macro-autophagy, micro-autophagy and chaperone mediated autophagy. In macro-autophagy, organelles (i.e., cargo) are surrounded by a double membrane vesicle called an autophagosome, which is then trafficked to the lysosome or vacuole where the membranes and cargo are subject to degradation. (Kilonksy, 2008). The resulting biochemical building blocks are exported back into the cytosol where they can be re-utilized by metabolic pathways necessary to build new organelles and synthesize proteins. Target of rapamycin, or the TOR kinase, is master regulator of nutrient signaling and regulator of autophagy. During starvation or treatment with rapamycin (a drug that indirectly inhibits Tor kinase activity), inhibition of TOR induces a wide range of autophagy pathways. Mis-regulation of autophagy has been implicated in many clinically relevant diseases from cancer to neurodegenerative disease (Uchiyama et al., 2008).

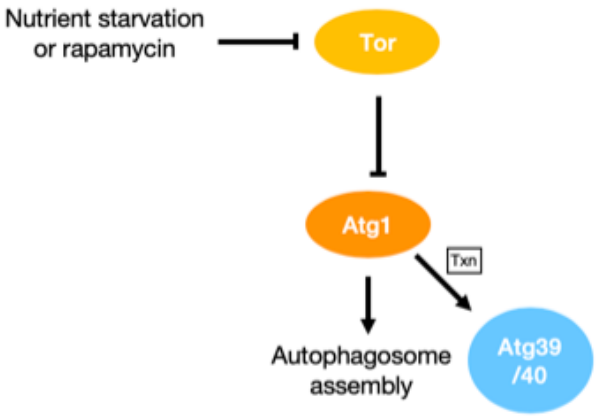

Figure 1: Autophagy Pathway

Figurative diagram of the autophagy pathway. Inhibition of TOR via nutriient starvation or rapamycin allows for activation of Autophage Gene 1,ATG1, and autophagosome assembly. ATG39/40 are receptors that target the cortical endoplasmic reticulum and the nucleus for autophagy.

Genomic fidelity is critical for the normal function of cells. One critical aspect of this process is that genomes are faithfully replicated and segregated. However, under certain growth conditions or in the presence of cancer chemotherapeutics cells experience replication "stress", a state that induces a cell cycle checkpoint called the intra-S phase checkpoint. Signals from checkpoint kinases in this pathway serve to stabilize stalled replication forks, arrest cell cycle progression, induce genes regulating nucleotide biosynthesis and ensure that the genome is repaired before moving forward (Iyer and Rhind, 2017).

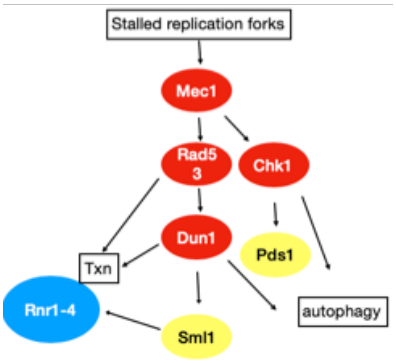

Figure 2: Intra-S-Phase Checkpoint

Figurative diagram of the Intra-S-Phase checkpoint. This pathway is activated when replication fidelity is compromised which is denoted by stalled replication forks. Pds1 halts cell cycle progression once phosphorylated by Chk1. Rn1-4 encodes for ribonucleotide reductase, an enzyme that produces nucleotides.

Like any other organelle, the nucleus must be maintained in order to ensure proper function. This is especially important for the nucleus because it houses the genome of the organism. The loss of nuclear integrity is associated with multiple diseases such as cancers (Lever and Sheer, 2010). It is well characterized in the literature that lagging chromosomes, or chromosome bridges, in mammalian anaphase cells contribute to MN formation. For example, MN form when topologically linked sister chromatids are prematurely packed and separated into nuclear envelopes. A high frequency of MN is a pathology associated with high chromosome instability and fast evolving tumor cells (Luzha and Kovalchuk, 2013). It has been proposed that formation of MN are suppressed by autophagy pathways that target partially formed nuclei, also known as nucleophagy (Mijalicija and Devenish, 2013). In Saccharomyces cerevisiae, the Kaplan lab has observed what appears to be an orthologous nucleophagy pathway that targets nucleolar cargo to the vacuole under replication stress conditions induced by the drug hydroxyurea (Van El Gort et al.).

The nucleophagy pathway has been shown to be under control of the Tor pathway; when Tor is repressed by rapamycin two nucleophagy receptors, Atg39 and Atg40 are up-regulated and also target nucleolar cargo to the vacuole (Mochida et al., 2015) However, in the context of replication stress it is unclear if the same Tor dependent pathway is involved, or whether nucleophagy is induced by the intra-s-phase kinases known to be activated under these conditions. In this study, we set out to test whether stalled replication forks per se, as opposed to

Tor dependent nutrient signaling, activates ReSIN and whether the activation signals depend on the intra S-phase checkpoint pathway.

Materials and Methods:

Cultivation of Yeast

Yeast were cultivated on yeast peptone dextrose (YPD) agar plates for two days in a 30ºC growth chamber. If the yeast contained an autonomously replicating plasmid with a selectable marker, then they were plated on a drop out (SD) plate to ensure plasmid integrity. For yeast cell lines that were integrations, they were plated as normal. To prepare for genomic extractions, microscopy experiments, or western blots, a sterilized wooden stick was used to pick up a colony of yeast and inoculate in 5mL of liquid media. However, for microscopy experiments, YPD was not used. A dropout media (SD) replenished with complete amino acids were used instead to avoid auto-fluorescence. Target concentrations of cells/mL of liquid culture varied on the type of experiment.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to in order to amplify the desired target locus. Forward and reverse primers were designed on serial cloner based on the genomic sequence from SGD. For a two-reaction genomic PCR, 134uL of sterile H_2O, 40uL 5X HF buffer, 16uL 2.5mM dNTPs, 2uL each of forward and reverse primers, 4uL of genomic DNA and 2uL of Phusion enzyme. For a two-reaction plasmid PCR, 118uL of sterile H_2O, 40uL 5X HF buffer, 16uL 2.5mM dNTPs, 2uL each of forward and reverse primers, 20uL of plasmid diluted 1:1000 in Tris buffer, and 2uL of Phusion enzyme. Everything was prepared on ice to prevent non-specific amplification. The master mix was then pipetted into 100uL aliquots into reactions tubes before placed into the heated machine.

Cycles	1		25		1	
Temp (ºC)	Denaturing 98	Denaturing 98	Annealing 58	Extension 72	Extension 72	Cooldown 4
Time (mins)	5:00	0:30	0:30	1:45	10:00	hold

Table 1: PCR protocol for *GAL1p-POL1*. Annealing temperature will vary based on the primers and extension time will be dependent on size of the targeted locus.

Confirmation of PCR Product

In order to confirm desired amplification of the target locus, a 1% agar, 1:20000 gel green and 200mL solution of 1X TBE was melted and poured into a lane cast. The gel was allowed to solidify before being subjected to gel electrophoresis of 50V for one hour. The buffer for the electrophoresis treatment was 1X TBE.

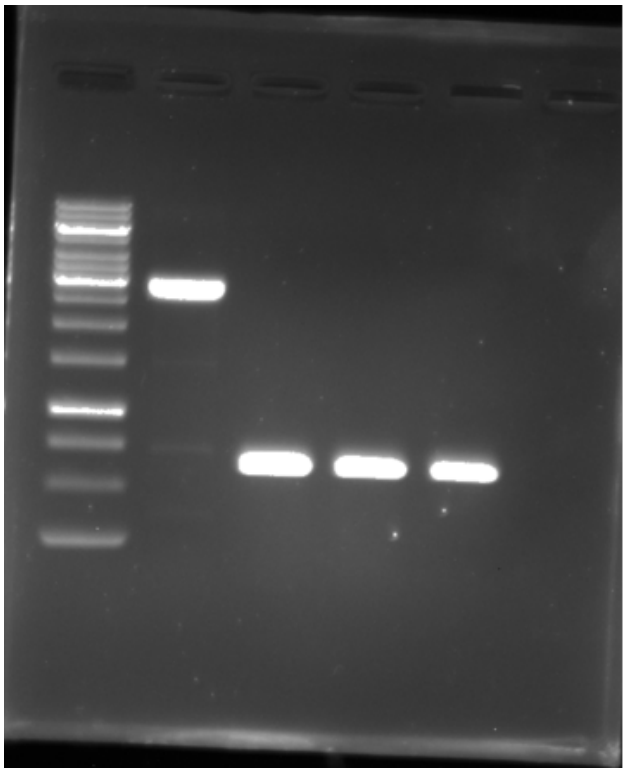

Figure 3: PCR screening of ATG39-GFP Transformation

The lane furthest to the left is the ladder, following that is the positive control and the subsequent cell lines being tested for presence of locus integration which was not successful.

Yeast Transformation

Yeast were inoculated in 5mL of YPD liquid media at 30ºC, the day before allowing growth. They were then diluted to 0.5 x 10^7 cells/mL and allowed to grow for three hours at 30ºC, 2 doublings, to achieve the final concentration of 2.0 x 10^7 cells/mL and to assure that the yeast were in logarithmic phase. They were then transferred into a disposable 15mL conical and centrifuged at 3k RPM for 10 mins. The supernatant was disposed and the pellet resuspended int 5mL of sterile water before being centrifuged once more. The water was then removed, and the pellet resuspended in 100mM lithium acetate (LiAc) before being transferred to a 1.5mL Eppendorf tube. The cells were spun and 14kRPM for 15 seconds and resuspended in 4x cell volume of 100mM LiAc. The cells were then vortexed and pelleted before adding 50uL of LiAc and transferred into transformation tubes. The LiAc was removed and yeast were subjected to 360uL the transformation mix (TRAFO Mix) which consisted of: 240uL 50% Polyethylene glycol, 1M LiAc, 20uL PCR/Plasmid Product with a concentration of .25ug/uL, 50uL salmon sperm DNA and 14uL of sterile H_2O. The pellet was vortexed thoroughly to ensure proper mixing. The mixture was then subjected to a 30ºC water bath for 30mins then transferred to a 42ºC water for 30mins. The mixture was spun at 5k RPM for 15s and the supernatant removed. The pellet was resuspended in 200uL of sterile water and gently mixed to prevent cell lysis. The mixture was plated onto appropriate drop out plates and incubated for two days at 30ºC.

Rapamycin

Yeast were grown to a concentration of 1.00 x 10^7 cells/mL in 5mL of liquid media and the was bud index measured to assure that the culture was in log phase which was denoted by a bud index of around 50%. The culture was then treated with rapamycin to achieve a final concentration of 200uM. The cultured was then incubated at 30ºC for three hours before analysis.

Hydroxyurea

Yeast were inoculated overnight in 5mL of appropriate liquid media at 30ºC. They were then counted, diluted back to 0.25 x 10^7 cells/mL, and counted after three hours to achieve the target concentration of 1.0 x 10^7 cells/mL. They were counted again, and the bud index measured to assure at least 50% of cells were budded to indicate log phase. The cultures were split into two and one was treated with 1M hydroxyurea such that the final concentration of hydroxyurea was 0.1M. They were then allowed to grow for three hours at 30ºC and counted to assure >80% budded to indicate successful replication stress. The cells were pelleted, washed three times with sterile water and resuspended in media. Nocodazole (15mg/mL) was added to both the treated and untreated to achieve the final concentration of 15ug/uL. The cells were allowed to grow for one hour before being washed three times with sterile water. Four percent low melting agarose pads were prepared and 2uL of culture was added and sealed with vLAP before imaging via microscopy.

Galactose Inducible Polymerase

Yeast with the *Galp-POL1* construct were cultured in 5mL SD 2% Raffinose/ Galactose media. These were grown up overnight, counted to make sure they were in log phase, diluted to a concentration of 0.25 x 10^7 cells/mL and switched into 5mL SD 2% Dextrose media. The media was changed every two hours to prevent starvation.

Statistical Significance

In order to determine whether the numbers collected were significant, a two-tailed student T-test was applied in excel using the mean of taken fields. The means of each cell line at each timepoint were then tested against one another. A value of $P<0.05$ was determined to be statistically significant.

Results:

GAL1p-POL1 induces cell cycle arrest consistent with stalled DNA replication

It is well established that depletion of nucleotides via the drug hydroxyurea arrests cells in early S phase, an arrest that causes budding yeast to accumulate in a large budded state. To test the efficacy of *Galp-POL1* in engaging the intra-S phase checkpoint, we monitored the bud index of cells grown under repressive conditions (i.e., dextrose in the media) to deplete the Pol1 polymerase. Because the Pol1 protein is quite stable it requires “dilution” during cell division to be depleted and thus cause cell cycle arrest (Falconi et al., 1993). Consistently, we observed an increase of large-budded cells after 5 to 7 generations or 10-14hrs after switching cells into dextrose containing medium. This is consistent with the induction of replication stress and stalled replication forks after depletion of Pol1.

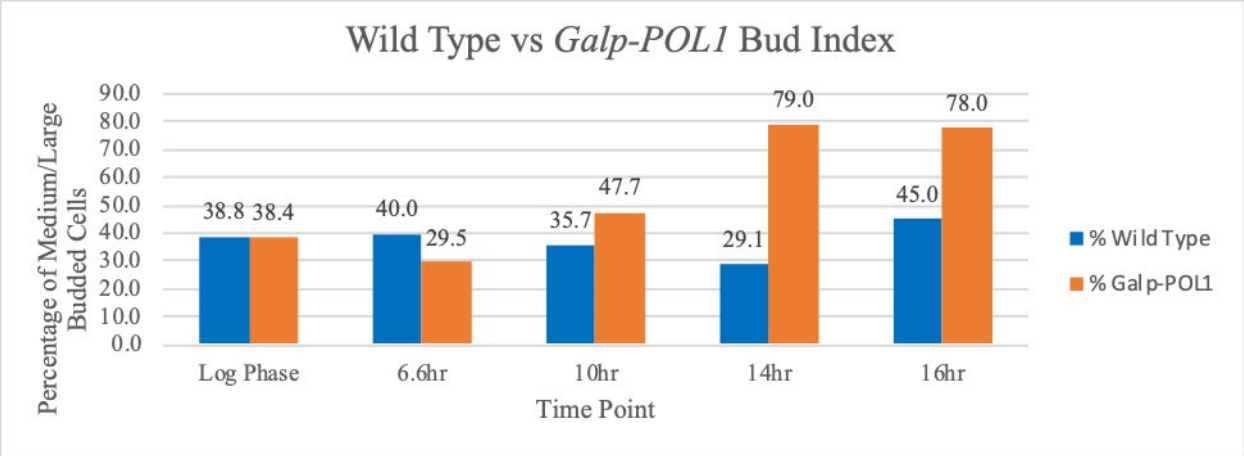

Figure 4: Wild Type and GalPol1 cultures cultured in dextrose. During log phase, cultures were grown in SD uracil dropout with 2% raffinose and galactose. For the following timepoints, the cultures were grown in SD uracil 2% dextrose

GAL1p-POL1 are normal for rapamycin induced macroautophagy

To monitor nucleophagy induced by replication stress, GFP-Atg8 was used to mark the formation of the so-called phagore-assembly site, or PAS, where autophagosomes are assembled. We first showed that the *GAL1p-POL1* cells are able to induce PAS normally by treating them with rapamycin to inhibit Tor and induce bulk autophagy. When cultures were treated with rapamycin, we indeed saw an increase in foci compared to normal, vegetative growing cells, indicating that these cells have fully functional autophagy pathways.

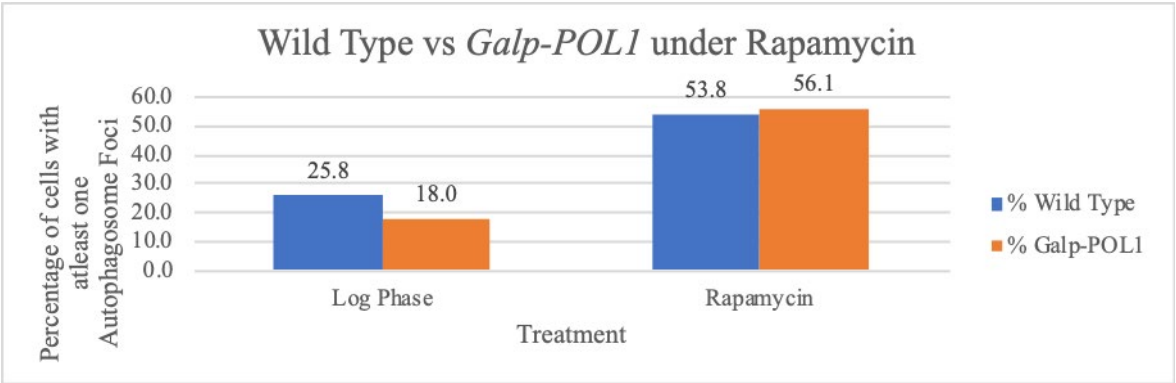

Figure 5: Wild Type and GalPol1 treated with Rapamycin. During Log phase, cultures were grown in SD uracil dropout media with 2% raffinose and galactose. For Rapamycin treatment, the cultures were grown in log phase in SD uracil dropout with 2% raffinose and galactose then treated to achieve a final concentration of 200uM and incubated for three hours.

GAL1p-POL1 inhibition increase GFP-Atg8 marked PAS

Using the conditions that we established to cause activation of the intra-S phase checkpoint and cell cycle arrest, we monitored the kinetics of PAS formation. We cultured cells where *POL1* is expressed under its native promoter simultaneously with the *GAL1p-POL1* repressible cell line under repressive conditions. We expected to see a PAS formation starting 14hrs of transcriptional repression based on our cell cycle experiments. Consistent with this expectation,

we observed a statistically significant (p=0.04813) increase in PAS that peaked after 16hours. We speculate that the increase in PAS observed at the six-hour time point is due to poor culture conditions for that single *Galp1-POL1* sample. In conclusion, this data supports our hypothesis that ReSIN is induced by stalled replication forks and not by depletion of nucleotide pools caused by the cancer chemotherapeutic, hydroxyurea.

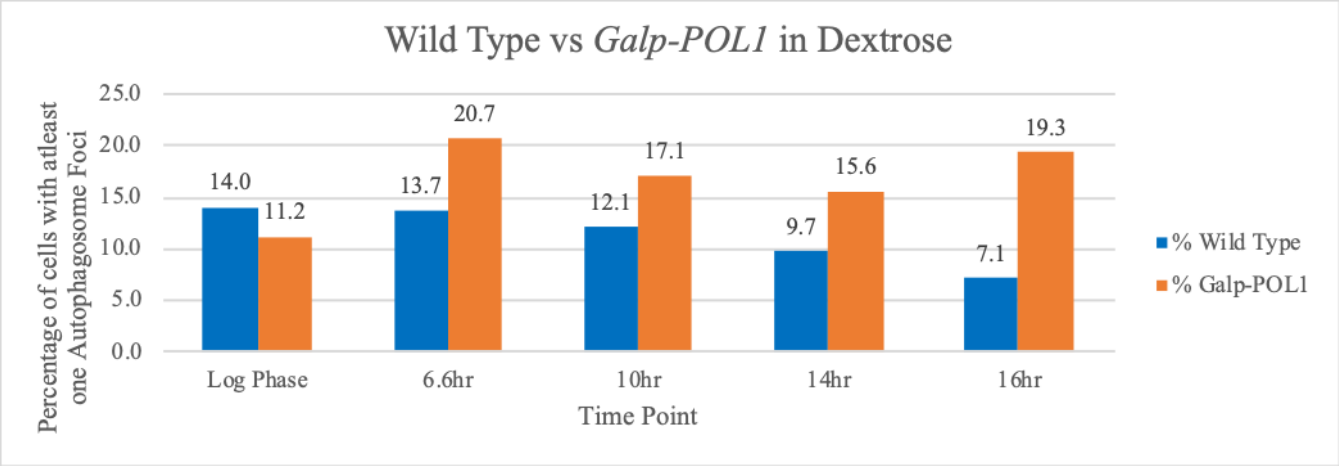

Figure 6: Wild Type and GalPol1 cultures cultured in dextrose. During Log phase, cultures were grown in SD uracil dropout with 2% raffinose and galactose. For the following timepoints, the cultures were growing in SD uracil 2% dextrose. However, at 6.6hrs, the *Galp-POL1* culture was left in hypoxic conditions possibly explaining the increase in PAS.

Discussion:

In this study, we showed that replication stress can be induced by inhibiting transcription of the *POL1* gene and the resulting depletion of Pol1 protein from cells. Significantly we found that cell cycle arrest due to Pol1 depletion was accompanied by an increase in ReSIN as measured by the increase in PAS and autophagosome formation. Though the kinetics of PAS formation due to ReSIN are delayed compared to what we observed with the drug hydroxyurea, this is expected given the slow kinetics of polymerase depletion compared to nucleotide depletion. These initial results are consistent with the prediction that replication stress, whether induced by a reduction in polymerase or nucleotide pools, can activate an autophagy pathway. This finding further supports the hypothesis that intra S-phase signaling is involved in replication stress induced nucleophagy. In combination with previous observations from our lab that the intra-S-phase checkpoint kinases are necessary for autophagic flux after nucleotide depletion, we conclude that that there is a novel branch of this checkpoint that induces a replication stress induced form of nucleophagy, or ReSIN.

This conclusion raises a number of very interesting questions. How do the intra S-phase kinases mediate the induction of nucleophagy? One possibility is that these checkpoints induce a transcriptional response that helps to activate nucleophagy specifically. This idea is consistent with observations in rapamycin treated cells where the ER-nucleophagy receptors Atg39 and Atg40 levels are increased (Mochida et al, 2015). What is the relevant cargo that is being targeted by ReSIN and how does it contribute to genome instability? Our observations that nucleolar proteins appear to be cargo during ReSIN raises the possibility that stalled replication places a stress on the nucleolus, a sub-compartment of the nucleus that houses the rDNA array. The rDNA array is highly repetitive and thus difficult to replicate and is a hot spot of

transcription, creating an additional challenge for the replication machinery to copy this region in a timely manner. Finally, the fact that rDNA containing chromosomes are some of the last to resolve in anaphase raises the intriguing possibility that ReSIN is tied to its timely resolution and the suppression of these unresolved loci contributing to genome instability.

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