Cell Biology: Cellular Organization, Division and Processes

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Lecture 13

DNA Replication-Part III

Hello everyone! I am Shikha Laloraya, Professor of Biochemistry at IISc. Welcome to this lecture which is the third in the series of my lectures on DNA replication. This lecture is mainly about eukaryotic DNA replication.

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But first I would like to review what we know about DNA replication in general, and also in the prokaryote, the *E. coli* bacterium. New DNA molecules are produced by copying of template DNA strands; you all know that DNA replication is semi-conservative. The newly synthesizing DNA chains grow in the 5 prime to 3 prime direction by addition of new nucleotides at the 3 prime end.

An RNA primer is formed by a primase enzyme. RNA polymerase can initiate synthesis by joining 2 ribonucleotides but DNA polymerase cannot do this. The enzyme DNA polymerase uses dNTPs and substrates and it adds a complementary nucleotide to the 3 prime hydroxyl of the deoxyribose of a primer paired with the template DNA strand forming a phosphodiester bond and also releases a pyrophosphate in this process.

Replication usually starts at an origin or initiator sequence and progresses bi-directionally. A replication bubble is formed at the site of initiation and the 2 oppositely oriented replication forks progress away from each other. In *E. coli*, the replication origin of *E. coli,* oriC binds an initiator protein DNA a that causes melting of the AT-rich region nearby. A DNA helicase enzyme DnaB unwinds the duplex DNA.

A DNA primase associates with the helicase and an RNA primer is formed by the DNA primase enzyme. The DNA polymerase 3 holoenzyme synthesizes DNA by adding new nucleotides at the 3 prime end of the primer and DNA synthesis occurs in the 5 prime to 3 prime direction.

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At the replication fork, the DNA Pol III holoenzyme synthesizes DNA by adding new nucleotides at the 3 prime end of the primer and due to the anti-parallel nature of the template strands DNA synthesis on one strand is discontinuous. This is referred to as a lagging strand. And there are multiple priming steps and formation of Okazaki fragments on this strand. Replication on the other strand is continuous and it is referred to as the leading strand.

Leading and lagging strands are synthesized simultaneously. Various proteins at the replication fork facilitate DNA replication forming a complex yet coordinated replication machinery termed the replisome. Proteins important for replication include the initiator protein, the helicase, the single stranded DNA binding proteins, primase, clamp loader and the tau protein, sliding clamps and DNA Pol III.

The clamp loader puts the sliding clamp around the primer template duplex at the primer template junction. The sliding clamp also binds to the DNA polymerase core subunit and holds it onto the DNA enhancing its processivity. A loop is formed on the lagging strand between the DNA polymerase extending the primer and the helicase that binds the primase to initiate new primer formation.

The size of this loop increases until the DNA polymerase reaches the end of the last Okazaki fragment and releases the DNA. Then a new loop is initiated and it also increases in size and the cycle is continued till DNA synthesis is completed. And this is referred to as the trombone model of DNA replication on the lagging strand. In the end there is enzymatic removal of the primer and this is followed by DNA synthesis to find the gap and there is also ligation of the remaining nick by a DNA ligase.

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Shown here is a replisome at the replication fork and a somewhat improved version of the trombone model for coordinating replication at the lagging strand. So, the steps are that there's an active helicase on the lagging strand template; the helicase is shown here- it moves in the 5 prime to 3 prime direction. Pol 3 holoenzyme interacts with the helicase via the tau subunit. And this interaction actually stimulates the rate of strand separation by the helicase such that DNA unwinding occurs at the same rate as the rate of replication by the DNA polymerase.

So if this association between the helicase and the DNA pol holo enzyme is not there, the unwinding slows down by about 10 fold. And the polymerase can replicate faster than the helicase can unwind and thus in such a situation the DNA polymerase can catch up with the helicase forming the replisome. The helicase enzyme extrudes a single stranded DNA loop and single-stranded DNA binding proteins bind to the single-stranded DNA to prevent the self-annealing- they are shown here in pink.

Periodically there is a primase which associates with the helicase and this synthesizes a primer on the lagging strand template. This interaction between the primase and the helicase is weak but it actually stimulates the primase function by a thousand-fold. The sliding clamp loader, which is shown here, it recognizes this primer template junction and it assembles a sliding clamp onto the primer which has been formed here.

The unengaged second lagging strand DNA polymerase recognizes the loaded sliding clamp at the junction and then it goes ahead and synthesizes a new Okazaki fragment. So, now that is shown in this position, the Okazaki fragment has been synthesized, and you can note that the lagging strand DNA is folded to bring this lagging strand polymerase into this complex with the leading strand polymerase molecule.

And also, it is also close to the 3 prime end of the completed Okazaki fragment and also close to the start of the next Okazaki fragment. Since the lagging strand polymerase is in the complex with other replication proteins, in form of the DNA polymerase holoenzyme, it can be reused to make successive Okazaki fragments alternating with the third polymerase core shown here as unengaged.

The size of this loop which is formed between the helicase and the polymerase on the lagging strand changes in size. It initiates and it grows in size and then it is released. And then again a new one is formed that also grows in size. So, hence the name of this model; as you may know a trombone is a trumpet-like instrument having a sliding mechanism that varies the length of the air columns in the instrument to change the pitch, repeatedly.

So, the clamp was put at the primer template junction by the clamp loader and as mentioned the clamp also binds and holds the Pol 3 core enzyme on the lagging strand and it holds it in place so, it does not float away if it falls off. So, it enhances its processivity in this way. The Pol 3 core enzyme extends the chain at the end of the primer till it reaches the end and then it lets go. So, the polymerase is released from the sliding clamp now once it is completed.

But before it lets go, meanwhile another primer had been synthesized and the other Pol 3 core, which was unengaged earlier had started extending it. So, now this one becomes unengaged from the DNA strand and lets go. And then this process continues until the DNA replication is completed.

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In this animation by Drew Berry and Etsuko Uno we can see these steps of DNA replication in quick succession. Especially the repeated expansion of the loop on the lagging strand that resulted in naming of this mechanism as the trombone model. The helicase is shown in bright blue, unwinding the double-stranded DNA at the left side and the leading strand is synthesizing continuously and it can be seen at the bottom.

Whereas the lagging strand is shown at the top giving off the loop every time. The greyish green primase arrives and it binds to the helicase and it forms the primer, which is denoted in yellow colour- there, that is the primase and the primer. The primer template junction is recognized by the clamp loader ,which is shown in lavender shade and it loads a sliding clamp which is the green ring around the primer template junction.

The lagging strand Pol 3 core enzyme, which is shown in purple, it binds and it initiates the synthesis of the Okazaki fragment. Now note that the loop increases in size with the progress of the lagging strand replication. When the polymerase reaches the end of the earlier fragment it lets go and then

it becomes available to re-initiate at another primer template junction. The clamp prevents the DNA polymerase from falling off and floating off while it is synthesizing the DNA and hence it enhances the processivity of the polymerase.

In this model you can see only 2 polymerase molecules shown, not 3, that is there is only one for the lagging strand- not 2 as I explained, probably because this animation was made at a time and it was not known that there are in fact 3 polymerase molecules in the DNA Pol 3 holoenzyme.

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Now in this lecture we will discuss DNA replication in eukaryotes and try to appreciate the similarities as well as the distinct aspects of replication in eukaryotes. This table shows the conservation of the replication machinery in eukaryotes and the enzymatic counterparts of the *E. coli* enzymes in the budding yeast *Saccharomyces cerevisiae* are listed here. So, the helicase in budding yeast is referred to as the CMG complex and it consists of Cdc45, the Mcm complex, and GINS proteins. The primase is referred to as DNA pol alpha and the SSB proteins are referred to as RPA or replication protein A. The sliding clamp loader is referred to as RFC and there are 3 kinds of them as you will see. The sliding clamp is referred to as PCNA which stands for Proliferating Cell Nuclear Antigen because this protein was found to be quite abundant in proliferating cells.

The DNA polymerase: there are in addition to Pol alpha, which synthesizes the primer, there is the Pol delta, which synthesizes the lagging strand and Pol epsilon, which synthesizes mainly the leading strand. Some of the other key differences are that eukaryotic cells of course already mentioned use 3 different DNA polymerases at the replication fork. The eukaryotic clamp loader is not bound to the DNA polymerase. The replication fork progression is much slower, it progresses at the rate of 20 to 60 base pairs per second as opposed to 1000 base pairs per second as we discussed in case of *E. coli*. The Okazaki fragment length also differs it is a 1000 to 2000 nucleotides in prokaryotes, whereas it is 100 to 400 base pairs in eukaryotes.

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Some of the distinctive features of DNA replication in eukaryotes are that the eukaryotic chromosomes are linear as opposed to the circular bacterial *E. coli* chromosome that we discussed earlier. DNA replication occurs only once per cell cycle in S-phase and the initiation of DNA replication in eukaryotes is highly regulated. So, helicase loading and activation are temporally segregated in different phases of the cell cycle. As was explained in detail in lecture 9, origin, licensing by loading of helicase occurs in the G1 phase while helicase activation to initiate DNA replication occurs in S-phase. We have mentioned that eukaryotic chromosomes have multiple origins of replication. And these multiple origins do not fire simultaneously. Some of them fire early while others fire late in the S-phase. Therefore, different parts of the same chromosome may replicate at different times in S-phase. There is a large multi-subunit complex termed ORC the origin recognition complex that binds to origins at all times in the cell cycle in eukaryotes. Chromosomal

DNA is bound to nucleosomes and along with DNA replication, new nucleosomes have to be assembled behind the replication fork.

The chromosome ends of these linear chromosomes have protective structures; they have repetitive sequences and are associated with the enzyme telomerase that replicates the ends of chromosomes.

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In yeast also, the replication originates at specific sites termed origins or ARSs that are autonomously replicating sequences. These origins in budding yeast were first identified by their ability to confer stable replication to episomes, and for this reason they were referred to as autonomously replicating sequences or ARSs. So, in this type of experiment different DNA fragments from yeast were cloned into a plasmid or an episome, which was having a selectable marker *HIS3*, which is an auxotrophic marker.

So, there was a collection of such plasmids having these DNA fragments from yeast and they were transfected into his3 auxotroph yeast strains and then plated on histidine omission selective plates to select for this marker *HIS3* on the plasmid and they looked for colony formation. It was found that the vector alone that is without any insert or inserts which did not have origin like activity formed very few colonies. Whereas there were certain DNA fragments that when inserted, there was a high frequency of transformants. And then when these colonies were analyzed it was found that in this case where you got rare transformants, they did have the *HIS3* marker of course, but the DNA had been integrated in the chromosome. Whereas in this case, they were autonomously replicating, that is they had replicating plasmid DNA, the DNA which entered these cells was able to replicate on its own. And then the insert sequences were analyzed and they were found to have these sequences, which have the capability of transferring the ability to replicate onto a plasmid. So, in fact later on these sequences were also characterized in the context of the genome and it was found in fact that DNA replication indeed originates at these sequences.

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The yeast ARS element, it has got a conserved 11 base pair ARS consensus sequence or ACS and it is got 3 additional elements B1, B2 and B3. B3 is not mentioned here or shown here, and these are important for origin activity. ORC binds these elements ACS and B1 and then it recruits some additional proteins such as the Mcm helicase to the origin and that binds at B2 and other factors bind at B3.

The ARS elements are AT-rich and of course as you know this makes sense because AT base pairs have only 2 hydrogen bonds and therefore DNA segments which are AT-rich are easier to melt, which is required for initiation of DNA replication, of course. Now this figure has been taken from a review article in Genetics by Steve Bell and Kareem Labib, and also some of the additional figures in this lecture and I would suggest you to look up the article if you are interested.

Some of the additional interesting points about ARSs are that some of these ARS elements were shown to act as the replicators in their chromosomal locations by two-dimensional gel electrophoresis by Brewer and Fangman, who had developed that technique, to actually map a site of replication initiation on a chromosome. And the comparison of these elements identified also an extended ACS, referred to as an E-ACS, which was spanning 19 base pairs. ORC binds this DNA in vitro and also in vivo, it leaves an in vivo footprint on this DNA, which is regulated during the cell cycle. And the genome-wide studies of ORC DNA binding were also done at a high resolution. And another consensus was defined which includes this extended ACS but it spans greater than 30 base pairs and this is referred to as the ORC-ACS (the ORC ARS consensus sequence) and of course this has been analyzed functionally and mutations in this sequence they are not consistent with replication. So mutations in this sequence would inactivate the ability of this sequence to act as a replicator.

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Now the chromosomes of eukaryotes have multiple origins of replication, they are linear. And initiating replication from multiple origins perhaps helps in completing the replication of the large chromosome faster. Of course, that is quite obvious because if you have only one origin it has to traverse a very large distance and these chromosomes are longer than those of prokaryotes in general. The replication fork has to traverse a shorter distance, only the inter-origin distance in this case, when there are multiple origins. So, the cell can manage to still have a short S-phase despite the large size of the genome. In addition, another interesting feature is that there is temporal regulation of origin firing; some origins fire early in S-phase whereas there are others which fire late within the same S-phase.

Some other interesting points are that the origins which fire with a similar timing, they cluster along the chromosomes. And origins near the centromere are early replicating and those near telomeres are late replicating.

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In contrast with prokaryotes in eukaryotes the helicase loading and activation occur in different stages of the cell cycle. So, with this process in mind the cell cycle can be split into 2 phases, okay, with respect to DNA replication. So, helicase loading only occurs in the G1 phase when the cyclin-dependent kinase levels are low and S-Cdk is activated by phosphorylation of Sic1, a CKI inhibitor by the G1-Cdk and then its SCF E3-ligase mediated ubiquitylation and degradation occurs and this results in activation of the S-Cdk that then triggers the S-phase transition.

The increased levels of the S-Cdk in S and also high Cdk levels in G2 and M also, they prevent helicase reloading through multiple mechanisms, which we have discussed in detail. So, the enhanced Cdk levels are required to activate the assembly of the CMG, the helicase complex, and the helicase activation, ensuring that there is no helicase activated during G1. So, this regulation ensures that no origin can initiate more than once per cell cycle.

So, binding happens in G1, activation happens in S-phase; you can try to remember that- that is an important aspect of regulation.

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So, let us try to understand the steps in replication in budding yeast starting with helicase recruitment. The helicase in budding yeast is the Mcm hexamer and this consists of six different subunits having the AAA plus ATPase domain and they are not identical; each subunit is a different protein and they form a ring with a hole in the middle. ORC binds at origins we have already discussed this a few times.

And this initial ORC-Cdc6 complex also is thought to form a ring-like complex of again triple-A plus related subunits and they encircle the origin DNA. This complex may then recruit one Mcm-Cdt1 complex to the adjacent DNA, which is shown here to form what is referred to as the OCCM complex and of course in the end there is a double hexamer of the Mcm hexamer is assembled in a head-to-head orientation on this DNA.

The mechanism for this is still unclear how it happens that 2 of them end up there but indeed they are found to be bound there. So, it is thought that either the Mcm complex itself recruits another Mcm complex or maybe it could happen that another ORC binds and then recruits the second Mcm hexamer. So, the mechanism of this is not exactly clear right now.

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Following recruitment of the helicase and origin licensing in S-phase, the activation of the helicase occurs by the S-Cdk and the DDK. Activation involves a remodelling of this Mcm complex double hexamer and the bound origin DNA. So, initially this double hexamer encircles the double-stranded origin DNA. But the active helicase, which is known as a CMG complex, has only one copy of this complex and it encircles single-stranded DNA.

So, for this transition there have to be a number of steps; there should be dissolution of the interactions between these 2 hexamers and there has to be strand separation at the origin and there has to be opening of each of the Mcm rings and extrusion of the opposite single-stranded DNA from the 2 Mcm complexes and then the ring has to close around the single stranded DNA. So, the details of this process are not very well understood as of now. Because this is a very complex process and there are also several additional factors, which are required for helicase activation for example Sld3, Sld2 and this protein Dpb11 that are required but they are not part of the final replisome. Here is also a model for the mechanism of the initial origin DNA melting by the Mcm complex- the double hexamer. So, these helicases they translocate double-stranded DNA towards itself and they cause the melting of the AT-rich origin DNA.

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So, in eukaryotes interestingly there are 3 multi-subunit DNA polymerases, which are essential for replication and they have been studied in budding yeast in quite some detail these are Pol alpha, Pol delta and Pol epsilon. Each of them has a distinct role at the replication forks. So, you can think of it as a sort of division of labour among these 3 DNA polymerases at the fork.

Only Pol alpha can begin the new DNA chains because its got heterodimeric primase subunits that synthesize 8 to 10 nucleotide RNA primers and it also has a Pol 1 DNA polymerase subunit, which can then extend that same primer to about 10 or 15 nucleotides, and then this enzyme though it has got limited processivity and it also has deficiency in proofreading so it can make frequent errors and therefore it is not suitable for the replication elongation steps.

Both Pol epsilon and delta are highly processive enzymes and they also have a proofreading exonuclease activity that reduces the rate of errors during the process of replication. Pol epsilon is mainly responsible for extending the leading strand at the replication fork whereas Pol delta completes the synthesis of each Okazaki fragment on the lagging strand that had been started by Pol alpha.

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Also in budding yeast there are multiple sliding clamp loaders, which are referred to as RFCs as I mentioned. So, after Pol alpha detaches from the template following the synthesis of an RNA-DNA primer, the clamp loader, which has RFC 1, RFC1-RFC can effectively compete for access to the 3 prime end and that is shown over here and so, the 3 prime end of the primer now can be bound by this RFC which also brings along the clamp.

And the clamp in this case is referred to as PCNA. So, ultimately there is loading of PCNA around the double-stranded DNA. Now this loading of the clamp leads to recruitment of Pol delta. So, now Pol delta comes and it is attached to the clamp which then extends the new Okazaki fragment. So this is going on, on the lagging strand. Another RFC the CTF 18 containing RFC, associates with Pol epsilon and this is thought to contribute to the loading of PCNA onto the leading strand side of the fork.

And finally, another RFC which has Elg-1, I should refer to it as Elg1-RFC, is also recruited to PCNA. Now this is a bit different; this recruitment is helped by sumoylation. And after ligation of the Okazaki fragments, this results actually in the removal of PCNA from the replicated DNA.

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Shown here is the structure of the budding yeast replicative Pol delta which is complexed with the primer template and the PCNA clamp and the structure was deduced by electron microscopy. This is a 3.0 Angstrom cryo-EM structure deduced by Zheng et al, of the budding yeast complex. So, Pol delta is in a complex with primed DNA; the template DNA primer is shown here in green colour.

And the PCNA clamp, because it is multi subunit, is shown as this multi colour ring or disc like structure. Now this model reported in this study showed some very interesting features. For example, they found that Pol delta binds only one of the subunits of the PCNA trimer. And this

interaction though, it is only with one subunit but it is extensive and it holds the DNA such that the 2 nanometer wide DNA it threads through the center of the 3 nanometer channel of the clamp without making direct contact with the protein. So, thus there is a water mediated clamp DNA interface which enables the PCNA clamp to water skate, as per the author's terminology, along the duplex with a minimum drag.

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So, to summarize chromosomes of eukaryotes have multiple origins and they are linear. There are 3 essential DNA polymerases showing division of labour. The DNA Pol alpha, which is important for primer synthesis and DNA Pol delta and epsilon, which are important for lagging strand and leading strand DNA synthesis. And also as the process continues, in the end the primers have to be removed and there are enzymes Fen1 and Dna2; they are important for cleavage of the displaced RNA primer flap. So, this is formed because Pol delta displaces the primer as it synthesizes the DNA and forms this flap-like structure and this is then cleaved by the flap specific endonucleases. The gap which is formed by this cleavage is then later sealed by the DNA ligase.

In the next part of this lecture we will deal with some of these additional steps and also other aspects of replication in eukaryotes and we will also talk about the completion of DNA replication.

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This slide shows the generic DNA elongation process during replication as has already been discussed. Note the additional enzyme topoisomerase, which is shown in front of the fork. These enzymes, they help in relieving torsional stress which is created by unwinding of the DNA double helix by the helicase enzyme at a progressing replication fork, especially when free rotation around the ends is not possible. For example in case of long chromosomes, it becomes difficult to achieve this type of rotation, and the role of these enzymes becomes very important.

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When helicase unwinds the DNA then torsional stress builds up and this can actually be relieved by the rotation of the DNA ahead of the fork. However, when this cannot happen, for example in case of the long chromosomes or here it is shown that the DNA itself is tethered to a support and therefore it cannot undergo that rotation. Then, when you have unwinding of the DNA during DNA replication, in the absence of such free rotation possible ahead of the fork to relieve the torsional stress, this results in the DNA in front of the fork becoming overwound and then it can become supercoiled as is shown here, these are the supercoiled regions. And after a certain point the fork progression itself would be hindered when there is a build-up of torsional stress beyond a certain level then it cannot really progress forward.

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So topoisomerases are enzymes that have got a reversible nuclease activity and they help in relieving such torsional stress. The topoisomerases they can attach covalently to a phosphate in the DNA chain backbone and they can bring about strand break; they can break a phosphodiester bond. And then the bond is reformed as this protein or the enzyme is released. For example, here is shown the action of topoisomerase. So, topoisomerase one can create a transient single stranded DNA break. And then the DNA on either side of the nick can actually rotate freely around this phosphodiester bond on the opposite strand of the point opposite the break. The tension in the helix will drive this rotation in the direction that relieves the tension. And then after this rotation then the gap is rapidly resealed using the energy that was released by its earlier cleavage of this bond and was stored in the Top1-DNA phosphate bond. So, additional energy is not really required for this process.

Another enzyme topoisomerase 2 can bind and form covalent linkage to 2 strands of the DNA helix that cross each other simultaneously and it creates a double strand break. ATP hydrolysis is used to break one DNA strand and it creates a DNA gate. And now the second helix which is unbroken can pass through this gate. After this the double strand break is sealed and the topoisomerase is released and the 2 strands can be separated. So, here is the example shown for 2 interlocked circles but this can also occur in the supercoiled regions produced in front of the replication fork. So, topoisomerase I can relieve the tension in front of the replication fork during DNA replication. At crossovers and supercoils, the passage of these strands, again it occurs in the direction that reduces supercoiling. Topoisomerase 2 is also important for untangling chromosomes during DNA replication to help in their proper separation later on.

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Here is shown a model for the removal of the RNA primer and the completion of synthesis of Okazaki fragments. When polymerase delta reaches the 5 prime end of the preceding Okazaki fragment, then it displaces a small flap. And this flap is then cut by an enzyme Fen 1. So, Fen 1 it is a nuclease, and it can cut this flap but if there is a longer flap formed it can be cut by another enzyme Dna2.

Now after this again the strand displacement it goes on till the polymerase delta reaches a midpoint of a nucleosome on the preceding fragment. So, now when it reaches this nucleosome, Pol delta detaches from this template and then ligation and completion of DNA synthesis can take place.

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In eukaryotes, during DNA replication the chromatin organization also has to be maintained. DNA unwinding by the CMG helicase displaces the parental histones, but there is a tetramer of the histone H3-H4, which is actually retained and this is possibly because some of the replisome components such as Mcm2 and FACT, they can actually bind histones. So, this helps in the local re-deposition of the parental H3-H4 tetramers onto the newly synthesized DNA.

So, the deposition of the newly synthesized histone H3-H4 also occurs and this is helped by chaperones such as CAF1. The histones H2A and H2B, though they are released from the DNA during replication, but these histones H2A and H2B are restored by another histone chaperone NAP 1 using

both the old as well as the new histones. So, in this way the nucleosomes also can be regenerated on the daughter strands along with the process of DNA replication.

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Now the termination of replication occurs at converging replication forks. Replication forks which were initiated at a single origin, they move away from each other in opposite directions. The other one on this side is not shown here obviously, and then the progression of these forks would stop when one replication fork collides head on with another replication fork which is moving in the opposite direction and in fact this is coming from an adjacent replicon. It will also stop when the replication fork reaches a chromosome end in case of linear chromosomes. The process of termination, it involves the disassembly of the CMG helicase. So, the E3 ligase SCF, we have discussed this earlier, the SCF-Dia mediated ubiquitylation of the Mcm 7 subunit of the CMG helicase occurs and this prepares it for disassembly by another protein known as Cdc48, it is referred to as a segregase. And actually this process is not very well understood but the CMG helicase is removed and disassembled and then this process of termination is completed.

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Now, as replication is going on there could be replication defects. And when these defects occur then they tend to accumulate or expose longer length single stranded DNA at the replication forks. And there is accumulation of the single strand binding protein RPA ,and this structure recruits the Mec 1 and Ddc2; Mec 1 as you know is a checkpoint kinase, to initiate the S-phase checkpoint pathway.

So, Mec 1 then goes ahead and it phosphorylates its numerous targets which includes the replisome component Mrc1, which was discussed as the mediator of the replication checkpoint. And it recruits the downstream checkpoint kinase Rad 53 and recruitment of Rad53 promotes its own auto phosphorylation and activation. Now, these 2 activated enzymes Mec1 and Rad53 bring about a number of responses in the presence of these stalled replication forks. And these include a block to mitosis in the presence of replication defects or stimulation of the ribonucleotide reductase activity, maintenance of transcription factors, which are expressed during S-phase. So, these factors they are expressed during S-phase and they continue to be expressed when this pathway is activated. And inhibition of the replication initiation factor such as Sld3 and Dbf4 at replication origins. So, that new forks are not created until the source of this original problem in replication has been removed or rectified. And also, they bring about the phosphorylation of histone H2A so, that they can recruit chromatin remodelling enzymes near the replication fork.

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Post-translational modifications of PCNA also help in preserving genomic integrity. The sliding clamp, PCNA, is sumoylated on lysine 164 by the SUMO E3 ligase Siz1 along with the Ubc9 SUMO conjugating E2 enzyme. Sumoylated PCNA recruits Srs2 translocase, this is a factor that displaces

recombination factors and hence it reduces the illicit recombination events that might interfere with the progression of DNA replication forks.

Now accumulation of the RPA coated single-stranded DNA at replication forks also has another effect, it can recruit the E3 ubiquitin ligase Rad18. Rad18 helps in the monoubiquitylation of lysine 164 of PCNA by Rad6 and this leads to the recruitment of translesion DNA polymerases. Unlike the polymerase epsilon or delta, these trans lesion polymerases are able to incorporate dNTPs opposite damaged bases also. And this allows the replication machinery to bypass the damaged base, which can potentially be repaired post-replicatively. Alternatively the monoubiquitylated PCNA can be modified by Rad5 in association with the E2 complex Mms2-Ubc13 producing a K63-linked Ubiquitin chain at the lysine 164 of PCNA and this activates an error-free DNA repair pathway.

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So, recall that the chromosomes of eukaryotes they have got multiple origins and they are linear and we also discuss that there is a temporal order of origin firing. What this means is that some origins fire early, whereas others fire late, within the same S-phase. And this can be useful because if a replication fork such as the one shown here, gets blocked due to damage and then it could not be repaired in a timely fashion and the replication ceases. So, there is an incomplete replication here. It could later be rescued by a fork which is coming from a late firing origin. And this unreplicated region could be replicated later in the same S-phase and this possibility does not arise if all the origins had fired at the same time and the block was there and it could not get rectified and was left unreplicated. So, this is a useful feature to ensure the complete replication of the chromosomes even though some problems may arise at the replication forks.

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Now coming to the end, the linear chromosomes they have an end replication problem due to incomplete replication on the lagging strand. During lagging strand replication, as you know, a back stitching mechanism is used to form short Okazaki fragments. So, this results in a problem when the fork reaches the end of the linear chromosome then there is no place to produce the RNA primer needed to initiate the last fragment at the end of the linear DNA molecule.

After the removal of the last primer then this region which corresponds to the final RNA primer synthesized on the lagging strand cannot be copied into DNA because there is no 3 prime OH end available for the extension.

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If this problem is left unresolved, this end replication problem can result in loss of DNA, that is genetic information, from the chromosome ends each time the cell divides. There are repetitive sequences term telomeres which are present at the ends of chromosomes in eukaryotes. In the absence of a mechanism to address this end replication problem, with successive divisions ,the

shortening of telomeres can occur as is shown here that is the number of telomeres, telomeric repeats, will keep on shortening. And at some point even the unique sequences may get exposed.

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The repetitive sequences present at the ends of the chromosomes protect the ends. These repetitive sequences are tandem repeats of a short sequence that is similar. So, in humans this sequence is GGGTTA, it is repeated thousands of times at each telomere. So, the sequence is actually recognized by specific DNA binding proteins that recruit the enzyme telomerase. Telomerase binds the tip of the telomere repeat sequence as shown here and it extends the 3 prime end of the parental strand using an RNA template that is part of this telomerase enzyme itself. In fact, the enzymatic part of the telomerase enzyme is similar to reverse transcriptases, that is the telomerase RNA also contributes functional groups to make the catalysis process efficient. So, now the replication of this lagging strand can be completed as a normal process using the extension as a template to synthesize the complementary strand.

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Here is the reference to a useful review article on the topic of chromosome duplication in budding yeast that is quite an interesting read. I hope this lecture has provided all of you with useful information about the process of DNA replication in eukaryotes. Thank you.