Cell Biology: Cellular Organization, Division and Processes

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Lecture 09

S-phase: Regulation of entry into S-phase and DNA replication

Hello everyone, I am Shikha Laloraya, Professor of Biochemistry at IISc. Today's lecture is on the regulation of entry into the cell cycle and the regulation of DNA replication during the cell cycle. We will discuss how the cell cycle control system initiates DNA replication and, also limits it to once per cell cycle.

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During the cell cycle one of the most important events is the duplication of genetic material so that there is only one copy each for each daughter cell that is produced. DNA replication is a process by which the two DNA molecules are produced from one DNA molecule by synthesis of DNA by a complex enzymatic machinery referred to as the replisome. So, shown here is the simple duplication of a prokaryotic cell. First, the DNA is replicated and then it is segregated such that each daughter cell receives one copy upon cell division.

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So, some of the prerequisites for DNA replication are that it must occur very accurately to avoid the possibility of spontaneous mutations that could be then passed on to the next generation, which is not desirable. And all the parts of the chromosome must be duplicated but they must be duplicated only once in one cell cycle to avoid the harmful effects of gene amplification. The chromosome organization must also be reproduced and maintained such that the daughter cells inherit the correct chromosome structure as well.

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So, DNA replication happens in the S-phase of the cell cycle, after G1 phase. The entry into S-phase is highly regulated; there is a regulatory point in G1 termed the restriction point in animal cells or

referred to as start defined by the Cdc28 mutant in budding yeast. This point is crucial for controlling the entry of cells into S-phase. It is the crucial decision point, at which the cell evaluates whether the conditions that it is in are proper to proceed to S-phase, or not.

So, in yeast the external signals such as nutrient availability, the cell size, and the presence or absence of mating pheromones such as alpha factor, can affect the outcome. In animal cells external growth factor signal proliferation. So, in the presence of these extracellular growth factors or mitogens, cells cross the restriction point and they enter S- they become committed for DNA replication. Passage through this restriction point is irreversible. So now that the cell is committed to go through S-phase and the other steps of the cell cycle, even if the external stimulus that had prompted it to enter the cell cycle are withdrawn. So, after this phase it is a point of no return; after this the cell has to initiate and undergo DNA replication and the rest of the cell cycle. If appropriate conditions were not available in the G1 phase then the cell cycle progression stops at the restriction point. It does not progress for further and the cells can arrest and sometimes even enter a quiescent stage of the cell cycle known as G_0.

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Shown here are some of the key regulators of cell cycle transitions. In budding yeast, the passage through start, requires the Cdk-1 in association with the G1 cyclins Cln 1, 2 and 3. Progression through S in budding yeast requires the Cdk-1 with the S cyclins Clb5 and Clb6. Entry into and progression through mitosis requires Cdk-1 in complex with the M-cyclins Clb1 through 4.

In animal cells however, there are multiple Cdks. So, in this case the progression through the restriction point requires the Cdk4 or 6 in a complex with cyclin D. The G1 to the S transition is driven by the Cdk-2 in a complex with cyclin E and progression through S and G2 is driven by the Cdk2-cyclin A complex. And the G2 to M transition is controlled by the Cdk-1 in a complex with cyclin A and progression through mitosis by Cdk-1 complexed with cyclin B.

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Extracellular growth factors control the progression through G1 and the restriction point. Cyclin D synthesis is induced in response to growth factors in part by signalling through this Ras/Raf/MEK/ERK pathway and cyclin D is induced to be synthesized and it continues to be made as long as these growth factors are present and this drives the cell through the restriction point. Cyclin D also undergoes rapid degradation by APC Ubiquitin ligase in G1 and the levels can fall rapidly if these growth factors are removed. So, as long as these factors are present cyclin D levels build up and the Cdk is active and the cell can cross the restriction point.

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Growth factors or mitogens bind to cell surface receptors and this initiates an intracellular signalling cascade. An important pathway involves the activation of the small GTPase, Ras, which activates the Map kinase cascade and this results in the increased expression of many immediate early genes such as Myc. Myc regulates the transcription and increases expression of many delayed response genes. And when Myc is activated cyclin D expression is also induced and hence the G1 Cdk that is the Cdk46-cyclin D is activated and the cell can cross the restriction point in the presence of these growth factors.

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So, after activation of G1 Cdk, the G1Cdk phosphorylates its target proteins, one of which is the retinoblastoma tumor suppressor protein. So, G1 Cdk phosphorylates RB as cells pass through the restriction point in G1. Normally the unphosphorylated Rb binds to the E2F transcription factor. E2F normally binds to its target sequences whether or not Rb is present. However, when Rb is bound to it, Rb actually acts as a repressor of gene expression. So, this E2F Rb complex represses the transcription of these E2F regulated genes and many of these are S-phase genes. So, their transcription is off as long as Rb is present. Phosphorylation of Rb releases it from this complex and hence the repression is lifted and now E2F can activate the transcription of its target genes, which is shown on this slide. One of these target genes is cyclin E of the G1 Cdk cyclin complex. And when cyclin E is transcribed and its protein levels build up then the G1 S-Cdk is active and this triggers the entry into the S-phase. Growth factor signalling also reduces the transcription and translation of p27 a CKI, it is a Cdk-2 inhibitor. So, the levels of p27 are lowered, and the Cdk is also activated. The activated Cdk-2 also phosphorylates p27 and targets it further for degradation by the Ubiquitin mediated proteasomal degradation.

Thus, the G1S Cdk is active and this can drive the entry into the S-phase. In addition, cyclin A is also transcribed and hence the S-Cdk is also activated. So, now S-phase can also proceed further.

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So, here is shown additional regulation of Cdks by the CKIs, the cyclin dependent kinase inhibitors in mammalian cells. There are two main families of these inhibitors, one is the Ink4 family, which has p15, p16, p18 and 19, and they act to inhibit progression through the restriction point in G1. Then there is the Clp or Klp family which includes p21, p27 and p57; these bind to the Cdk-2 cyclin E or the Cdk-2 cyclin A complex and they block the entry into S and progression through S and G2.

So, as mentioned earlier the growth factor signalling reduces the transcription and translation of p27, the Cdk inhibitor, lowering its level. Hence, S-Cdk is relatively active. The activated Cdk-2 also phosphates p27 and targets it for degradation by the ubiquitin-mediated proteosomal degradation, driving S-phase progression.

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From the previous descriptions we can see that the protein cyclin D is a critical target of growth factor signalling. So, defects in the cyclin D regulation might result in the loss of growth regulation as is seen in cancer. And indeed, this is found to be the case. For example, there are mutations, which cause the continued unregulated expression of cyclin D and such mutations indeed contribute to lymphomas and breast cancers.

Mutations that inactivate Ink4 CKIs, which are the CKIs that bind and inhibit Cdk 4 and Cdk6, the G1 cyclin complex, are also quite common in the human cancers. And finally the Cdk46 cyclin D target retinoblastoma is frequently mutated in the disease retinoblastoma and also in a variety of human cancers. Retinoblastoma is a tumour-suppressive protein and it binds E2F and represses E2F mediated transcription of S-phase genes. So, its inactivation may actually allow for S-phase entry and progression similar to when it is phosphorylated by G1Cdk and it dissociates from E2F.

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Entry into S-phase can occur in this way via the activation of S-Cdk or the Cdk-2 cyclin E complex. Cdk-2 cyclin E complex initiates S-phase by activating DNA synthesis at replication origins that were previously licensed in the G1 phase of the cell cycle. But how really does chromosome duplication occur? Chromosomes of eukaryotes have a very complex organization. First of all they are linear and they have many DNA associated proteins and they have a complicated yet dynamic three-dimensional organization.

The entire set of chromosomes has to be replicated accurately and also only once per cell cycle. The chromatin organization also has to be maintained after the replication. So, linear chromosomes have multiple replication origins. Replication origins were actually first identified in budding yeast and these origins are known to be sites for initiation of DNA synthesis. This is a highly controlled process so that DNA replication is initiated at the origin only once during the entire cell cycle. In S-phase the replication is initiated at origins when a DNA helicase enzyme unwinds the DNA forming a replication bubble. So, this results in the separation of the 2 DNA strands of the DNA double helix, and they can be used as templates now for DNA synthesis. The DNA replication enzymes are loaded onto the two single stranded DNA templates forming a complex known as a replisome. DNA replication proceeds bi-directionally from each replication origin at the replication fork.

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So, the preparation for S-phase actually occurs in G1 phase and this is known as licensing. The initiation of replication is divided into two steps, which occur at two different phases of the cell cycle. In the late M and early G1 there is a pair of replicative helicases referred to as MCMs, Mini Chromosome Maintenance protein complex that bind at origins and they form the pre-replicative

complex or the preRC. This is termed as licensing of replication origins; initiation in S-phase can occur at only those replication origins that have a preRC. In S-phase DNA helicases are activated and this results in the unwinding of the DNA double strand and initiation of DNA synthesis. The helicase moves out of the origin and along with the replication forks, as replication proceeds. So, now the origin site lacks the bound helicase and it cannot reinitiate replication until after it binds it again in the next G1. Hence, this is one way that the origins can be activated only once in the cell cycle.

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Now the step one of replication initiation occurs early in G1. And, as I mentioned this is referred to as licensing of the origin. A large multi-protein complex the origin recognition complex or ORC is bound to the replication origins throughout the cell cycle. And it serves as a place for the recruitment of the additional proteins that regulate the initiation of replication. Another protein, Cdc6 is normally present at low levels during most of the cell cycle but its levels increase transiently in early G1 and it binds to ORC at the origins in early G1. Cdc6 is required for the binding of the complex composed of a group of closely related proteins, the MCM proteins, that constitute the DNA helicase. Another protein Cdt1 also associates with the helicase and it helps in loading it at the origin. So, ORC, Cdt1 and Cdc6 collectively help in loading two copies of the inactive helicase near the DNA, which is next to the origin. And there is a large complex therefore, which is formed at the origin and this is known as the pre-replicative complex or the preRC. This origin, once it has established this configuration and has this complex bound here, is now licensed for replication, which will happen in the next S-phase.

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The step two of initiation of DNA replication occurs in S-phase. In S-phase the S-Cdk stimulates the assembly of several initiator proteins on this helicase to help in its activation. Another protein DDK phosphorylates the helicase subunits and it activates the helicase. Now this activated helicase can unwind the DNA and other factors, obviously the DNA polymerase, and other replication proteins, all of which are not shown here, are recruited to the origins and DNA replication starts.

The ORC is transiently displaced when DNA replication goes through it but it rebinds and it is also inactivated.

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So, how does the cell ensure that DNA replication is initiated only once in a cell cycle? Along with the initiation of DNA replication the S-Cdk inactivates the preRC components ORC and Cdc6 and it prevents the reformation of a new preRC until the end of mitosis. The S-Cdk causes a dissociation of Cdc6 from the origins and its degradation by the SCF E3 ligase that recognizes the phosphorylated Cdc6 and ubiquitylates it and any unbound Cdc6 is thus degraded by proteasomes.

S-Cdk also phosphorylates some of the Mcm proteins, which triggers their export from the nucleus, making sure they are out of the site of action. And it further ensures that the Mcm protein complex now cannot rebind to the origins. Hence, Cdc6 and Mcm proteins cannot return and rebind to the origin to reset the ORC containing origin for another round of DNA replication until the end of M when M-Cdk has been inactivated, much later on in the cell cycle.

So, in addition the E3 ligase APC is turned off in late G1 its target is geminin which is a Cdt1 inhibitor and it accumulates and inhibits free Cdt1. So the Cdt1 which is unbound to DNA. And in addition Cdt1 at active forks associates with a protein that stimulates its destruction. Hence Cdt1 is not available to help in reloading the helicase after the origin firing. So, this is how the pre-replicative complex formation is prevented until mitosis, ensuring that the origin is fired only once in the cell cycle in early S-phase.

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To summarize, there is a very complex mechanism that ensures that DNA replication occurs only once in the cell cycle. And this involves the licensing of replication origins and removal of some of the factors which are required for initiation after the origin has fired. For example, the S-Cdk it initiates origin firing but it also brings about phosphorylation of some of the important proteins required for this assembly, Cdc6 and Mcm. And as I already mentioned these proteins are either degraded or in case of Mcm they are exported from the nucleus. So, this prevents the reassembly of the preRC at the origin and prevents the reinitiation of replication at the same origin. So most importantly the S-Cdk maintains a high activity not only in S-phase but also during G2 and early M. And M-Cdk also phosphorylates the Cdc6 and Mcm. So, it further ensures that these proteins cannot rebind to the origins in M phase also and this helps in preventing re-replication.

However, at the end of mitosis the Cdk activity in the cell reduces and therefore the Cdc6 and Mcm are no longer being phosphorylated; their dephosphorylated forms accumulate. And the preRC assembly at origin can happen again, which readies the DNA for another round of DNA replication. In this way, DNA replication and origin firing is limited to once per cell cycle. Thank you.