Cell Biology: Cellular Organization, Division and Processes

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Lecture - 7
Checkpoints, DNA Damage and DNA Replication Checkpoints

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Hello, everyone, I am Shikha Laloraya from the Indian Institute of Science. Today we will discuss checkpoint controls, which are another type of regulatory mechanism that ensure that cell cycle progression occurs at the right time. We will discuss what are checkpoints and the strategies that were used for the identification of checkpoint proteins. We will also discuss examples of specific checkpoints such as the DNA damage checkpoint, the DNA replication checkpoint, and we will also discuss a mitotic checkpoint, the spindle assembly checkpoint.

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As you know, during the cell cycle, events must be turned on at an appropriate time and requisite time has to be made available for the completion of the process. Events in the cell cycle occur in a particular order and there has to be some kind of mechanism to ensure this. Each major event in the cell cycle such as DNA replication, for example, must be triggered only once per cell cycle. Binary on, off switches that trigger the events in a complete, irreversible fashion should be there, and you can think of as an example phosphorylation brought about by the cyclin CDK complexes. There should also be some backup mechanisms, so, that if one of the mechanisms fails, and things cannot be fixed, and another mechanism can compensate for it and the cell can somehow survive. There should also be a possibility of adaptation to respond to specific environmental conditions that may arise or to perform specific functions.

So, the key regulators of cell cycle progression are the CDK cyclin complexes. These are the complexes that phosphorylate specific targets to trigger cell cycle events. They drive the cell cycle forward when active and therefore, the overall impact of the cyclin dependent kinase on the cell cycle progression is positive that is, it makes the cell cycle move forward. Checkpoint controls on the other hand, prevent entry into the next phase until the events in the preceding phase have been completed. So, these checkpoint pathways inhibit progression of the cell cycle when they are active, and their overall impact on progression of the cell cycle pathway is negative or inhibitory. But they are important to maintain fidelity of cell cycle progression.

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Here are some important concepts postulated by Lee Hartwell. According to him, the cell cycle should be considered as a temporally organized sequence of events linked together in an orderly fashion. Later events in the cell cycle depend on successful completion of earlier events. There could be 2 types of dependencies, direct coupling, or linking via signaling control and this was

referred to as checkpoint controls. In addition, he postulated that certain events in the cell cycle could be rate limiting.

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So Hartwell and Weinert defined the checkpoint concept. They defined checkpoint controls as signaling pathways that act to delay cell cycle progression when perturbations delay the completion of certain cell cycle events. They postulated that checkpoint dependencies can be established in either of 2 ways. One could be the substrate product model, which is a mechanistic linkage, that is, the completion of one event produces a substrate necessary for the execution of the second event. A good example of this would be if we consider that the spindle is composed of microtubules and microtubules are made up of tubulin proteins. So if you were to stop the production of tubulin, you would not get the spindle to be formed. So this is an example of direct coupling or linkage; it is impossible to disconnect the two. So, one of the predictions was that for this model that this would be harder to uncouple. The other model was the checkpoint control model, where the 2 events are mechanistically unlinked. That is there is a regulatory pathway dedicated just for this purpose, which ensures that the later event does not begin until the earlier one is completed. And they thought that perhaps this should be easier to uncouple, than a mechanistic linkage. A prediction of these ideas is that a null allele in checkpoint genes, or inhibitors of checkpoint proteins should result in a loss of dependency. In addition, it was thought that checkpoints are likely to be inhibitory pathways.

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So, what are the functional requirements for a checkpoint response? One most important one would be a monitor or a sensor. So this component of the checkpoint pathway would detect the progress or the completion of one cell cycle event. And then you also need signaling components to relay the signal from the sensor to the effector. The effector is a regulator of the initiation of subsequent events. So it would finally act to stop the cell cycle progression.

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Now certain events are linked in dependent pathways, for example, DNA replication anaphase, cytokinesis, and they happen in a particular order. So if you think about whether the dependencies between them are due to a mechanistic link, or a checkpoint control mechanism, Hartwell and Weinert suggested an empirical criterion that is, can we find specific mutations or drugs that would uncouple the dependent events and would permit the second event to occur even when the first one was incomplete or blocked. Dependencies resulting from the mechanistic links should be harder to uncouple, than the dependencies that are resulting from checkpoint controls. Therefore, the existence of uncoupling mutations or drugs would support the checkpoint model of regulation.

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To understand these concepts further, let us look at this simple experiment by Weinert and Hartwell. They were looking for proof for their hypothesis. And so they searched for mutations that might inactivate a checkpoint that detects DNA damage, or incomplete replication. So, in this experiment, they have taken the budding yeast *cdc9* mutant; *cdc9* mutant is defective in completion of DNA replication, and it is also a temperature sensitive mutant. *CDC9* encodes a DNA ligase that joins Okazaki fragments during DNA replication. And hence, it is required for the completion of

replication, particularly on the lagging strand. So, here is shown the phenotype of this mutant, it is temperature sensitive and here at the permissive temperature, it is fine, but at the non-permissive temperature, it arrests with this morphology that is mother cell attached to medium sized daughter bud, which is typical of an S phase arrest.

So, in order to test the hypothesis, they wanted to find mutants that would be defective in the checkpoint that brings about this arrest. So it would be predicted that in such a mutant, if the checkpoint was inactivated, then rather than arresting, this mutant should keep on dividing, it should go forward. And to do this screen they combined this mutant *cdc9*, with other mutants, that is they created additional mutations in this mutant. And one of the mutants that they came across was *rad9*. So, rad mutants were obtained in screens for radiation sensitive mutants. And when they introduced a *rad9* mutation in the *cdc9* mutant background, then they saw this interesting result that when the mutant was shifted to the restrictive or the non-permissive temperature of 36 degrees, then instead of arresting as you would expect the *cdc9* mutant to do, this mutant kept on dividing that is, beyond this arrest phenotype seen in the *cdc9* mutant it would actually go and give rise to additional daughter buds. So this indicated that whatever mechanism was restricting *cdc9* to the stage in the cell cycle and causing this arrest, is not operational in the double mutant *cdc9 rad9*, indicating that *rad9* mutant might be defective in some aspect or some player of the checkpoint that monitors a defect in the *cdc9* mutant which recall is, it is defective in the completion of DNA replication and it is not able to ligate Okazaki fragments that are found on the lagging strand. Furthermore, it is not as if the double mutant was dividing more than the single mutant and was happy about it; they actually tested these mutants for viability after incubating at the non-permissive temperature. And of course, the *cdc9* mutant because it has some defect in replication certainly showed a decrease in viability as time increased at the non-permissive temperature. But the double mutant showed a precipitous decline in the viability, much more compared to the *cdc9* single mutant alone, which indicated that this double mutant although it is undergoing divisions and giving rise to more cells, these cells are not viable, they have some problems. So now we know that *rad9* encodes a mediator of this checkpoint.

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They concluded that Rad9 is involved in an important checkpoint that perhaps ensures completion of DNA replication or at least it checks for unligated DNA, that is DNA gaps, or breaks in the DNA. So this DNA damage checkpoint, it operates in interphase, and Rad9 of course, is an important mediator of this checkpoint. The DNA damage checkpoints operate in G_1, S and G_2 phases. They sense DNA damage and prevent progression till the damage has been repaired. The G_1 checkpoint allows time for repair to happen prior to entry into the S phase. And the S phase checkpoint similarly monitors damage during DNA replication. The G_2 DNA damage checkpoint senses damaged DNA or incomplete replication in G_2, and it prevents the initiation of M phase until replication is complete and until any damage has been repaired.

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How the DNA damage checkpoint leads to cell cycle arrest is depicted here. So, when you have single stranded DNA or unreplicated DNA, or, when you have a double strand break in the DNA this would be sensed by the checkpoint proteins. Two important proteins involved in this process are ATM and ATR and these are activated in complexes that recognize and they bind to these damaged

DNA sites. ATR is activated by single stranded DNA breaks or unreplicated DNA, and the ATM kinase is activated mainly by double strand breaks. So, these are kinases and then they phosphorylate and activate, Chk1 and Chk2 protein kinases respectively, and activated Chk1 and Chk2 phosphorylate Cdc25. Now, recall that Cdc25 is a phosphatase and it is important to activate Cdks because it removes the inhibitory phosphorylation that inhibits the cyclin dependent kinase activity on the Cdks. So, Cdc25 which normally would be activating Cdks, is not able to do so, and hence, the Cdks remain in an inactive phosphorylated form resulting in cell cycle arrest in the presence of DNA damage and due to the activity of these checkpoints in G 1, S or G 2 phases.

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In mammalian cells the DNA damage checkpoint dependent arrest is mediated by the activity of additional proteins, p53 is an important one; it is a tumor suppressor protein whose gene is often found to be mutated in human cancers. So, p53 is a target of ATM and Chk2; it is phosphorylated by them, when DNA damage is detected and this checkpoint is active. Now, normally p53 is complexed with another protein Mdm2 and this binding of Mdm2 targets p 53 for ubiquitylation and ubiquitin dependent proteasome mediated degradation. Upon phosphorylation though, p53 no longer binds Mdm2 and so, p53 level is stabilized and it builds up. And whenever there is DNA damage, the p53 levels rise, p53 is a transcription factor. So, it is high levels induce the expression and accumulation of a protein known as p 21, which is a CKI, a cyclin dependent kinase inhibitor; p21 protein inhibits the cyclin dependent kinase cyclin complex, the G 1, S and the S Cdk complexes, thus preventing the cell cycle progression.

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Coming to the DNA replication checkpoint, sometimes also referred to as the replication stress checkpoint. This checkpoint monitors the completion of S phase, so it operates in S phase and it checks whether the DNA replication is completed or not. So, here is a small experiment using HU or hydroxyurea: this is a chemical that causes depletion of the deoxy nucleotide triphosphates which are the precursors as you know for DNA synthesis. HU is an inhibitor of ribonucleotide reductase which is important for the formation of dNTPs. So, when you add HU, enough substrates are not available and this causes slowdown of fork progression, or fork stalling and sometimes even fork collapse, if it goes on for a long time.

So, here is a test of uncoupling of checkpoint control using a checkpoint inhibitor. Here are normal cells which are untreated they go through the cell cycle normally as you would expect, and 1 cell gives rise to 2 cells, which are viable. In the next experiment, these cells have been treated with caffeine and also they do undergo division and give rise to 2 cells although they may be perhaps small problems in those cells, which are not immediately obvious. In presence of hydroxyurea, because of the shortage of the dNTPs and the cell arrests in S phase and its progression is slowed down dramatically. And so, you do not get cell division as long as HU is present. However, when HU is combined with caffeine treatment, it was observed that the cells could actually go through mitosis, which is inappropriate, it is referred to as a suicidal mitosis, because, although 2 cells are produced, these cells are usually not viable, because they have tried to segregate the chromosomes which have not yet completed replication. So, they do not have a full complement of the chromosomes, and hence the cells are inviable.

So, now, how does this happen? Caffeine is allowing the progression of the cell cycle, even when DNA replication has been stalled. And this is because it is an inhibitor of one of the signaling kinases in this checkpoint pathway. In fact, my lab has shown that in budding yeast caffeine can inhibit the checkpoint kinase Mec 1 mediated phosphorylation of Rad53, which is another checkpoint protein in this pathway. So, this indicates the existence of a checkpoint that prevents progression in the presence of HU.

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To understand more about the DNA replication checkpoint, Osborn and Elledge, tried to identify a mediator of this checkpoint, using the strategy where they tested for the progression of the cell cycle in the presence of HU induced replication stress in budding yeast mutants. They did this screen in which they had these mutants, which were HU sensitive, and then they examined them for those which were elongating their spindles, even in the presence of hydroxyurea. So normally in the presence of hydroxyurea, there is a delay in S phase and the cells, the normal wild type cells are arrested with a short spindle. So if you look for mutants which do not arrest in this way, but they keep on elongating the spindle like a normal cell, even though the DNA replication is incomplete, you might be able to find mutants which are defective in this pathway. And so these mutants are likely to be defective in the mechanisms that arrest in response to replication stress. And one such mutant, which came from their lab was the *mrc1* mutant; this mutant is sensitive to hydroxyurea, it elongate spindles, even in the presence of hydroxyurea, and it fails to phosphorylate Rad53, another protein which is a checkpoint protein, in response to hydroxyurea or MMS treatment, unlike wild type cells. So normally, when wild type cells are treated with hydroxyurea, one can detect phosphorylation of Rad53, which is not observed in the *mrc1* mutant, indicating that it is required in some way for this modification. Mrc1 itself is phosphorylated and its phosphorylation depends on the ATR homologue Mec1 in budding yeast, and Mec1 is a kinase and it phosphorylates proteins at these SQ-TQ cluster domains. And there is a mutant *mrc1AQ* which is a phosphorylation defective mutant isolated in the same lab. This mutant showed compromised viability in the presence of replication stress. And this mutant which was defective in Mec1 mediated phosphorylation also failed to activate Rad53 in response to the replication stress, indicating that phosphorylation is somehow important for this signaling.

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More interesting points about Mrc1 also came out from the same study. So these mutants, the phosphorylation defective mrc1 mutants are replication checkpoint effective and they fail to activate Rad53 in response to replication stress, but the checkpoint and the replication phenotypes of Mrc1 are separable, and the replication initiation is required for Mrc1 protein to bind chromatin near the replication origins. Also, as replication proceeds Mrc1 is seen to move along with the replication forks and the checkpoint activity of Mrc1 itself is not required for its localization to the forks. Mec1 is recruited to sites of the DNA replication interface. So, Mec1 is an enzyme which phosphorylates Mrc1, and indeed it is present at the site of action. Mrc1 phosphorylation in response to DNA replication stress is required also for Mec1 accumulation at the stalled fork.

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So, similar approaches are also used to search for mitotic checkpoint defective mutants. There are chemicals known such as benomyl or nocodazole, or colchicine, which inhibit various aspects of spindle assembly or dynamics and these chemicals can cause an arrest in M phase prior to metaphase. So, Andrew Murray's lab looked for mitotic arrest deficient mutants in budding yeast in the presence of benomyl and they referred to them as *mad* mutants, mitotic arrest deficient mutants. So, these are mutants which are sensitive to benomyl and in which the completion of mitosis is not delayed in the presence of benomyl. These mutants die due to premature exit in the presence of benomyl. And if you look at these mutants under the microscope, you will see that they divide initially more rapidly than wild type cells to form small micro colonies of 20 to 50 cells and of course, then they cease to divide. And the cells in these micro colonies show increased frequency of chromosome loss. From this screen, several such mutants are discovered, which were named as *mad1*, *mad2*, *mad3* and so on.

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At the same time, Andy Hoyt's lab also performed a similar screen to look for such mutants. They described *bub* mutants, budding uninhibited by benzimidazole. And, of course, they again used benomyl which causes the disruption of the microtubule structure and spindle assembly. And they knew that the arrest in presence of this drug is as a large-budded cell with a single nucleus and in this case, cytokinesis and the G_1 of the next cycle does not proceed. And so they looked for mutants where it could actually progress. So they looked for mutants where there was new bud emergence, even in the presence of benomyl, hence the name. They found a few mutants which fell in this category. These mutants also showed low survival in nocodazole containing media as would be expected. So shown here is the wild type and when it is treated with benomyl, it undergoes this kind of arrest, where you have the mother cell attached to a medium sized daughter bud and its remains arrested in that stage. However, the *bub* mutants, the particular mutant shown here is *bub2-2*, so this mutant, when benomyl is added, it actually does not stay arrested as seen for wild type cells, but it keeps on dividing that is giving off another daughter bud beyond the second bud. So, this collection of mutants, the *bub* mutants, also were likely to be defective in a checkpoint that monitors the spindle assembly.

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These findings led to the discovery of components of the spindle assembly checkpoint. The spindle assembly checkpoint monitors whether assembly of the spindle is complete or not. That is have all the sister chromatid pairs attached to the microtubules of the spindle or not? So, unattached kinetochores lead to the assembly of the mitotic checkpoint complex, shown here. So at the top you can see a spindle where the assembly is incomplete. One pair of sister chromatids has one attached kinetochore; it has not yet attached on both ends, whereas the other chromosomes are attached. So this is an example of an incomplete spindle assembly whereas the bottom one is in metaphase, where all the chromosomes are at the equatorial plane and each of these pairs have attached to the spindle microtubules coming from opposite poles, which is when the cell cycle would progress. So unattached kinetochores lead to the assembly of this complex, so as long as there is even 1 unattached kinetochore, you have this mitotic checkpoint complex active, and its role is that it inhibits APC, the anaphase promoting complex which is a ubiquitin E3 ligase complex that I mentioned earlier. And one of the subunits of this complex is Cdc20. So, when all the chromosomes

are attached and aligned, this inhibitory complex is no longer formed. And so this wait-anaphase signal is extinguished. And Cdc20, which was part of this complex is a subunit of APC. So now it can go and it binds APC, and APC now is active and it can ubiquitylate its targets. Two important targets of APC are cyclin B and securing, also known as Pds1 in budding yeast. So, these proteins undergo ubiquitylation and ubiquitin-mediated degradation by the proteasome. And upon cyclin B destruction, the Cdk1 which was active-the mitotic Cdk, is inactivated. Upon securin destruction, securin itself was in a complex with a protease known as separase, so when securin is destroyed separase is set free, and it is a protease whose target is the cohesin complex. So, it goes and cleaves one of the subunits of the cohesin complex. The cohesin complex hold sister chromatids together; it brings about sister chromatid cohesion. So, when cohesin is degraded, then these 2 sister chromatids they can come apart and the metaphase to anaphase transition occurs.

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To summarize the checkpoints that I discussed today, the DNA damage checkpoints operate in interphase; they operate in G 1 phase, in S phase as well as G 2 phase. These checkpoints slow down progression of the cell cycle to allow for repair of the DNA damage. So as long as DNA damage is there, the cell cycle cycles is stopped. And when the damage has been repaired, then the checkpoint is inactivated and then the cell cycle can progress forward. Mitotic checkpoints also monitor complex events in mitosis. So there are multiple mitotic checkpoints, but today we have discussed a little bit about the spindle assembly checkpoint. The spindle assembly checkpoint checks for the proper attachment of chromosomes to the spindle, and it provides time for all the chromosomes to attach to the spindle microtubules. If the attachment is incomplete, this checkpoint is active and it prevents cell cycle progression. It prevents the metaphase to anaphase transition, and when the spindle assembly is complete, the checkpoint is inactivated and the cell cycle can progress forward.

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To summarize, checkpoints ensure that the cell cycle does not proceed under potentially dangerous conditions. Checkpoints require a mechanism to detect errors or problems in a cellular process via a sensor. They require a transducer of the signal, and an effector that generates a reversible signal that inhibits cell cycle progression. Checkpoints generally slow down or arrest cell cycle progression to enable cells to fix the damage before proceeding further. Checkpoint mechanisms may be dispensable for a given cell division or for viability, but they are critical for maintaining the fidelity. Mutations in checkpoint genes have been reported to be associated with cancer predisposition and with progression of cancer. And sometimes, if a cell is unable to fix the damage, it may undergo apoptosis, that is a kind of a cell death that removes that defective cell from the milieu. External stimuli such as nutrient availability or certain factors such as mitogens can affect the entry into the cell cycle by simulating the activities of specific Cdks. So, these also provide a point in the cell cycle on which control can be exerted. Thank you.