HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2016 August 18.

Published in final edited form as:

Methods Mol Biol. 2014 ; 1170: 29–40. doi:10.1007/978-1-4939-0888-2_2.

Cell Cycle Regulation by Checkpoints

Kevin J. Barnum and **Matthew J. O'Connell**

Abstract

Cell cycle checkpoints are surveillance mechanisms that monitor the order, integrity, and fidelity of the major events of the cell cycle. These include growth to the appropriate cell size, the replication and integrity of the chromosomes, and their accurate segregation at mitosis. Many of these mechanisms are ancient in origin and highly conserved, and hence have been heavily informed by studies in simple organisms such as the yeasts. Others have evolved in higher organisms, and control alternative cell fates with significant impact on tumor suppression. Here, we consider these different checkpoint pathways and the consequences of their dysfunction on cell fate.

Keywords

Checkpoint; DNA damage; Cell cycle; Genome stability; Mitosis

1 Introduction

The cell cycle is the series of events in which cellular components are doubled, and then accurately segregated into daughter cells. In eukaryotes, DNA replication is confined to a discrete Synthesis or S-phase, and chromosome segregation occurs at Mitosis or M-phase. Two Gap phases separate S phase and mitosis, known as G1 and G2. These are not periods of inactivity, but rather periods where cells obtain mass, integrate growth signals, organize a replicated genome, and prepare for chromosome segregation.

The central machines that drive cell cycle progression are the cyclin-dependent kinases (CDKs). These are serine/threonine protein kinases that phosphorylate key substrates to promote DNA synthesis and mitotic progression. The catalytic subunits are in molar excess, but lack activity until bound by their cognate cyclin subunits, which are tightly regulated at both the levels of synthesis and ubiquitin-dependent proteolysis. Cyclin-binding allows inactive CDKs to adopt an active configuration akin to monomeric and active kinases. Layered on top of this regulation, CDK activity can also be negatively regulated by the binding of small inhibitory proteins, the CKIs, or by inhibitory tyrosine phosphorylation which blocks phosphate transfer to substrates.

Checkpoints emerged as a series of cell cycle dependencies. In seminal studies in the fission yeast *Schizosaccharomyces pombe*, Mitchison and colleagues determined that cell size was a determinant of cell division [1–4]. Further, Rao and Johnson used human cell fusion experiments [5–8], and determined a dependency between S phase and mitosis. That is, nuclei undergoing S phase could delay mitotic entry of a G2 nucleus, whereas mitotic cells stimulated nuclei to prematurely enter mitosis. In addition, studies in oocytes had

determined a similar relationship between S phase and mitosis [9, 10]. In addition, Weinert and Hartwell utilized the cell cycle arrest induced by DNA damage in the budding yeast *Saccharomyces cerevisiae* to identify the first DNA damage checkpoint genes [11, 12], which has subsequently been expanded in several systems into a detailed signaling pathway, with significant overlap of signals making mitosis dependent on the completion of DNA replication [13–16]. Similarly, the mitotic arrest caused by microtubule inhibitors was utilized to identify the first spindle checkpoint genes in *Saccharomyces cerevisiae* [17, 18], again leading to a highly conserved checkpoint pathway that governs chromosome segregation [19]. It is these checkpoints acting as feed-forward signalers that give the cell cycle its remarkable fidelity, and ensure normal development and tissue homeostasis.

2 The Checkpoints

There has been enormous progress in the molecular dissection of various cell cycle checkpoint pathways. In many cases, this is very detailed with close dissection of posttranslational modifications, structural biology, enzyme kinetics, and so on. It would take a textbook to adequately detail all these events, which we do not attempt to do here. Rather, we will focus on the key concepts and regulatory events, and refer the reader to excellent articles that describe the molecular details of these pathways [19–25].

2.1 Cell Size Control

In order to maintain cell size and ensure that each daughter cell is endowed with the appropriate amount of genetic and biosynthetic material, cells must, on average, exactly double their contents before division. Control of cell size is critical for regulating nutrient distribution for the cell and for regulating organ size and function in multicellular organisms. The existence of cell size checkpoints has been proposed for allowing cells to coordinate cell size with cell cycle progression. Cell size checkpoints have been observed in G1 and G2. Early evidence for these checkpoints came from observations that the size of new daughter cells after mitosis affects cell cycle progression: large daughter cells speed up progression through G1 and/or G2, and small daughter cells delay exit from these growth phases [26, 27]. However, different species and cell types vary widely in the location of these checkpoints within the cell cycle, and thus in how the cell cycle is affected in response to change in cell size.

Not surprisingly, much of what is known about size checkpoints at the molecular level is based on regulation of the proteins involved in G1 and G2/M progression. Control of the G1 cell size checkpoint has been studied most extensively in budding yeast, where the cyclin Cln3, which activates Start, regulates cell size [28, 29]. Control of the G2/M cell size checkpoint has been studied most extensively in fission yeast, where Cdc25 and Wee1 respond to cell size and nutritional status in their control of the Cdc2-cyclin B complex [30, 31].

One proposed mechanism for control of cell size is via the monitoring of protein translation. Ribosomal mass, and thus translational activity, should correlate with the size of the cell, so it is thought that there is some product of translation called a "translational sizer" that increases in abundance with cell size and that exerts control over the cell cycle after a certain

amount has accumulated [32]. Cln3 and Cdc25 are both proposed translational sizers. This hypothesis also offers an explanation for how cell size and the cell cycle respond to nutritional status. In yeast, several signaling pathways, including the PKA and TOR pathways, are proposed to mediate nutrient control of the cell cycle, and the unifying characteristic of these pathways is that they control ribosome biogenesis, such that translational activity serves as a cellular indicator of nutritional status.

Another mechanism by which cells may coordinate cell size with cell cycle progression is via monitoring of cell geometry. The fission yeast *S. pombe* is shaped like a cylinder and grows lengthwise prior to division. A protein called Pom1 localizes to the tips of the cell and halts cell cycle progression via regulation of the Cdr1-Cdr2-Wee1-Cdc2 axis, which is centrally placed in a region called the interphase node. At longer cell lengths, Pom1 can no longer influence this complex, and the cell cycle can progress to M phase [33, 34]. Though this system may depend on the relatively unique cell shape of *S. pombe*, it raises the question of whether similar mechanisms exist in other species.

While a number of explanations for coordination of cell cycle and cell size have been offered, it is possible that any number of them function simultaneously in a cell. How they are all integrated, however, remains unclear.

2.2 DNA Damage Responses

Throughout interphase, DNA damage elicits a cell cycle arrest that allows time for repair pathways to operate prior to commitment to subsequent phases of the cell cycle. The source of DNA damage may be intrinsic, such as intermediates of metabolism, attrition of telomeres, oncogene overexpression, and DNA replication errors. Alternatively, there are many extrinsic sources of DNA damage ranging from sunlight, to carcinogens, ionizing radiation or other anticancer therapeutics. While there are many lesion-specific responses for DNA repair, different lesions in genomic DNA activate common checkpoint pathways whose goal is to maintain CDKs in an inactive state until the lesion is removed. Broadly speaking, DNA damage checkpoints can be separated into those controlled by the tumor suppressor and transcription factor p53, and those ultimately under the control of the checkpoint kinase Chk1, and we will consider the latter first.

The Chk1 pathway is highly conserved from yeast to man. The components of the pathway have come largely from genetic screens in the yeasts among damage-sensitive mutants [11, 14, 35–38], with some additional components identified in mammalian cells [39–42]. Chk1 is activated by all known forms of DNA damage, though this is more efficient in S- and G2-phase than in G1, and restricted to post-replicative lesions [15, 36, 43]. The diversity of activating lesions suggested a common intermediate, which is single- stranded DNA coated by Replication Protein A (RPA), and containing a primer template junction [13, 44]. Complexes of checkpoint proteins assemble on the RPA-coated DNA, including a protein kinase known as ATR (Ataxia Telangiectasia and Rad3-Related) in humans that is targeted by its interacting protein ATRIP, and a PCNA-related clamp called the 9-1-1 complex (Rad9-Rad1-Hus1) that is loaded by a variant Replication Factor C (RFC) complex. Following phosphorylation by ATR, BRCT-domain mediator proteins are recruited to these sites. There are more mediators in mammals than in the yeasts, but they serve the same

purpose: the recruitment of Chk1, which undergoes activating phosphorylation by ATR, and is then released to maintain the mitotic CDK Cdc2 in its Y15 phosphorylated and inactive state. Chk1 phosphorylates both the kinase (Wee1) and phosphatase (Cdc25) that regulate Y15 phosphorylation. This leads to increased Wee1 stability and decreased Cdc25 activity and/or protein levels. Subsequently, Chk1 is subject to dephosphorylation by type 1 phosphatases [45–47], and the cells resume cycling into mitosis.

In *S. cerevisiae*, the upstream signaling events are identical to those described above, but the effector kinase is different. Although Chk1 is conserved, the major effector is an unrelated kinase known as Rad53 [48, 49]. Moreover, the point of cell cycle arrest is not the G2–M transition, but the metaphase to anaphase transition. This is brought about by Rad53 controlling the activity of the cohesin protease, separase, through phosphorylation of its regulator securin [50]. This damage-induced mitotic arrest is not seen in other species including fission yeast, and notably human mitotic cells are unable to mount a delay to mitotic progression [51]. Further, another kinase known as Dun1 is activated in budding yeast [52], which controls transcriptional responses to DNA damage including activation of ribonucleotide reductase, the enzyme required for dNTP synthesis.

In higher organisms, the transcription factor p53 is a critical component of DNA damage checkpoints [25], particularly in G1 phase. p53 is regulated by a plethora of posttranslational modifications, including N-terminal phosphorylation on serine-15, which is catalyzed by ATR and its cousins ATM (Ataxia Telangiectasia Mutated) and DNA-PKcs (DNA-dependent protein kinase, catalytic subunit). Similar to ATR, these kinases are targeted to double-strand DNA breaks by interacting proteins: the MRN (Mre11-Rad50-Nbs1) complex for ATM, and the Ku70–Ku80 complex for DNA-PKcs. Activated p53 is stabilized through protection from its E3 ubiquitin ligase Mdm2, and as a tetramer transactivates the expression of a large number of genes, including the cyclin-dependent kinase inhibitor (CKI) p21. Through this mechanism, G1 CDKs are inhibited, and DNA damage is repaired prior to DNA replication. However, p53 can also repress the expression of genes, and is required for prolonged G2 arrest in the face of persistent DNA damage [53, 54]. Moreover, p53 can direct the alternative cell fates of apoptosis or senescence [55]. Indeed, the cell cycle arrest function of p53 seems to be a later adopted function, as *Drosophila* p53 regulates apoptosis, but not cell cycle progression [56].

2.3 Monitoring DNA Replication

S phase marks a particularly vulnerable time for cells to cope with DNA damage. Not only must lesions be repaired as in G1 and G2 cells, but they also act as a physical impediment to the replicative polymerases. DNA replication is initiated at specific sites, the replication origins. These are epigenetically defined by a number of proteins that ensure they fire (start replicating) once and only once per cell cycle. Replication origin firing is controlled by the phosphorylation of two proteins, Cdt1 and Cdc6, which is catalyzed by both CDKs and the Dbf4-dependent protein kinase (DDK) Cdc7. Such phosphorylation not only initiates replication but also leads to degradation of these proteins, and hence the origin cannot refire [57].

When the polymerase and its associated proteins (the replisome) encounter a blockade to progression, it is imperative that the replisome remains stably associated with the replicating chromatid so that replication can resume once the blockade is removed. Such blockades can be modified dNTPs, abasic sites, protein–DNA complexes, or result from the depletion of dNTPs. This replisome stabilization is the function of the intra-S-phase checkpoint.

The effector kinase of the intra-S-phase checkpoint is known as Cds1 in fission yeast or Chk2 in humans. Despite its related name, Chk2 is not biochemically or functionally related to Chk1. Cds1/Chk2 has an N-terminal phospho-S/T-binding Forkhead-Associated (FHA) domain followed by a kinase domain. Upon replication stalling, the replisome component Mrc1 (Mediator of the replication checkpoint) is phosphorylated by ATR. This creates a binding site for Cds1/Chk2, which is then phosphorylated by ATR, and then fully activated by autophosphorylation [58]. Activated Cds1/Chk2 then stabilizes the stalled replisome by phosphorylation of several subunits, notably the MCM helicase [59, 60]. In budding yeast, the Rad53 kinase serves the function of Cds1/Chk2. Like Cds1/Chk2, Rad53 has an N-terminal FHA domain followed by a kinase domain. However, Rad53 has an additional C-terminal FHA domain not seen in Cds1 that is important in its activation by DNA damage [61].

2.4 S–M Dependency

Upon stabilization, the replisome may stay in position until the blockade is removed or dNTPs restored. Alternatively, the cell can employ post-replication repair pathways to bypass the lesion, either by recruiting mutagenic bypass polymerases, or switching templates by recombination and then replicate using the other nascent strand as a template [62]. In either case, checkpoints must be employed to ensure mitosis is not attempted until replication is complete, or else cells run the risk of reduced ploidy. It is clear that mitotic entry is blocked via Y15 phosphorylation of Cdc2, and that the checkpoint components that act upstream of mediator recruitment are required for this [36, 37, 63]. How this leads to cell cycle delay remains less than clear.

If the source of the blockade is DNA damage, then the Chk1 pathway is activated as described above. However, if the blockade is due to dNTP depletion only, for example by hydroxyurea treatment, Chk1 is not activated and yet the cells will not enter mitosis [64]. Some studies have concluded that Cds1 also regulates cell cycle progression [65, 66]. However, if cells lack Cds1, then the stalled replisome disassociates from its template, a process known as fork collapse, and this is seen as DNA damage that activates Chk1. Consequently, it is difficult to experimentally separate the phenomena of replication fork stability and function for the effector kinases.

A more extreme uncoupling of the dependency of mitosis on prior replication can be seen when fission yeast cells lack both Y15 kinases (Wee1 and Mik1), or when origin firing proteins are deleted [67]. In these cases, cells enter lethal mitoses from G1, a process originally termed mitotic catastrophe, a moniker that has subsequently been used to describe mitotic death in mammalian cells.

In order to maintain ploidy, there is an equally important dependency relationship to ensure one round of replication per cell cycle. This can be uncoupled when degradation of Cdt1 and Cdc6 is defective [68], and replication origin firing becomes constitutive. Complete rounds of S phase without mitosis can also be observed in fission yeast when the CKI Rum1 is overproduced [69]. Similarly, cells devoid of mitotic cyclins bypass mitosis [70], suggesting these confer knowledge of a G2 state [71]. In each of these situations, mitosis is completely bypassed, and ploidy continues to increase.

2.5 The Mitotic Spindle Checkpoint

The segregation of sister chromatids at anaphase is under the mechanical control of the mitotic spindle. The spindle is comprised of microtubules and several motor proteins at both the centrosomal and kinetochore ends, plus additional motors that provide force between overlapping microtubules that do not attach to kinetochores [72]. It is essential that spindle attachment occurs in a bi-oriented fashion such that sister chromatids are under tension at metaphase, and attached to both poles of the spindle. Once all kinetochores are attached and aligned at the metaphase plate, anaphase can proceed as is promoted by the activity of a large E3 ubiquitin ligase known as the Anaphase-Promoting Complex or Cyclosome (APC/C). This ligase targets a number of proteins, but most essential are the mitotic cyclins, which abolishes CDK activity, and securin, the degradation of which allows separase to be released and cleave cohesin complexes at the kinetochores. APC activity is controlled by two accessory proteins: Cdc20, which functions up to the metaphase–anaphase, and Cdh1, which continues to facilitate APC-mediated ubiquitination once cyclin and separase degradation has begun [73]. Once sister chromatid cohesion is released, spindle tension and the associated motor proteins enable sister chromatids to move apart and form identical daughter nuclei.

The spindle checkpoint functions to prevent activation of APC ^{Cdc20} under conditions where kinetochores are not occupied by spindle microtubules, or are attached but not under tension (for example, when attached to the same pole, known as a merotelic attachment). Under these conditions, the spindle checkpoint protein Mad2 (Mitotic Arrest Deficient) inhibits Cdc20 activity both in the context of Cdc20 at unattached kinetochores, where it forms a mitotic checkpoint complex, and at APC-bound molecules. Cdc20 is also regulated by the mitotic checkpoint kinase Bub1 in yeast (Budding uninhibited by benomyl) and its cousin Bub1R in mammals. As Cdc2 is inactive, so is APC, and hence, cells cannot enter anaphase.

The spindle checkpoint includes a number of other proteins, with the list growing with evolutionary complexity. In addition, the formation of the spindle and the detection and correction of spindle defects are under the control of the Polo, Aurora, and NIMA-related (Nek) kinases [74, 75]. In this regard, the spindle checkpoint shares the same basic premise as those controlling DNA integrity discussed above—prevent a cell cycle transition while other effectors correct a genome-altering defect. However, the mitotic checkpoint is unique in that it functions to maintain CDK activity, whereas those functioning in interphase aim to maintain CDK inactivity.

3 The Fate of Checkpoint Dysfunction in Human Disease

Depending on the severity of the cell cycle defect, checkpoint dysfunction can result in outcomes ranging from cell death to cell cycle reprogramming, which can lead to cancer. In the case of p53 loss, arguably the most common genetic defect in cancer, several cell fate decisions are affected. Among these are a lack of CDK control by p21, and hence a loss of the G1 checkpoint. However, p53 can also direct cells into apoptosis and/or senescence, and so the physiological consequence of p53 in cancer is both at the level of cancer etiology and the ability for therapies to kill cells [25]. Interestingly, p53 loss presents a heightened requirement for the Chk1 pathway, which is often up-regulated in cancer cells, and required for the viability of many cancer cells [15, 16, 76]. Hence, there has been a lot of interest in targeting Chk1 [77] and its substrate Wee1 [78] as a therapeutic regimen in the clinic.

Loss of the Chk1 pathway in a fission yeast only manifests a significant phenotype with extrinsic DNA damage, or when combined with DNA repair defects [79]. Entry into mitosis with fragmented or incompletely repaired chromosomes does not trigger the spindle checkpoint, which only measures kinetochore attachment. Therefore, such mitoses are either immediately lethal or result in a significant loss of chromosomal fragments. In mouse and in *Drosophila*, the Chk1 pathway is essential for passage through early embryogenesis [80, 81]. However, this is a bottleneck of rapid cell cycling, and the S–M dependency is critical for genome integrity. Similarly, conditional Chk1 loss is lethal in some tissues and cell lines [82–84], though not others [76, 82, 85, 86], where again proliferation rates may be critical. Nevertheless, mutations in Chk1 pathway genes in human cancers are extremely rare (if at all existent). While cancers exhibit genome instability, they cannot survive in the complete absence of genome integrity checkpoints.

A characteristic of most solid tumors is highly aneuploid karyotypes. Chromosome loss and rearrangement is a rapid means to tumor suppressor loss and oncogene activation. However, while mutations in the spindle checkpoint genes have been reported, they are comparatively rare [87]. Still, with the complexity of the mitotic apparatus and extreme consequence of whole chromosome loss or gain, modest dysfunction can have profound consequences. As with the DNA integrity checkpoints, where high level DNA damage tends to induce cell death, altering the dynamic instability of spindle microtubules can also be lethal, with the advantage that spindles are only present in cycling cells.

4 Conclusions

We have described here the basic principles behind the common cell cycle checkpoints. They share the feature of detecting a defect in the division program, and then sending signals forward to alter the oscillations of CDK activity and therefore cell cycle events. Some aspects of checkpoint signaling remain to be clarified or determined (known unknowns), either as a simple principle, or in the context of human development and disease. Doubtless, we will uncover unforeseen aspects of checkpoint signaling (unknown unknowns), and the ever-growing arsenal of highly sophisticated experimental tools and technologies will enable a more complete picture of the remarkable fidelity of the cell cycle.

Acknowledgments

We thank our colleagues and collaborators for a stimulating discussion. This work was supported by NIH grants RO1-GM087326 (M.J.O.) and T32-CA078207 (K.J.B.).

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