✓ Review of Literature
"All things began in an order, so shall they end, so shall they begin again; according to the ordainer of the order and mystical mathematics of the city of heaven" - Sir Thomas Browne, 1568.

The same reasoning holds true for the execution of cell cycle, most eukaryotic cells never re-duplicate before the segregation of the sister chromatids is completed at the previous mitosis, never start the process of mitosis until the process of DNA replication has doubled each and every nucleotide in it’s genome nor it attempts to separate sister chromatids until proper alignment of every pair of chromosomes have taken place on the metaphase plates. Early microscopy based studies recognized the above fact as- cell division precedes mitosis (M-phase). Little information was available of the phase, which preceded mitosis (interphase). It was a momentous discovery that DNA was a carrier of hereditary information. Subsequently a study by Howard and Pelc (1951) established that the actual process of DNA replication and consequent genome duplication occurred in a narrow window of time during interphase. This observation led to the division of interphase into three further intervals: G1- the gap between mitosis and the onset of DNA replication, S- the actual period of DNA replication and G2- the gap between S- and M- phases.

The later work becomes associated with factors controlling the advent of these phases and their proper and orderly execution. The first phase of experiments was based on the unicellular protozoa Physarum polycephalum, which led to the identification of M-phase promoting factor (MPF) due to its ability to accelerate the advent of mitosis when introduced to early G2-phase cells (Johnson and Rao, 1971). Later on, the development of more powerful tissue culture techniques including cell fusion that led to the discovery of S-phase promoting factor (SPF), which could accelerate S-phase in G1- nuclei (Rao and Johnson, 1971). It was the genetic studies in yeast that led to the identification of genes for SPF and MPF (Hartwell, 1974; Nurse, 1980).
Fig 1. Cell fusion experiments: *Human HeLa cells that had been synchronized at different stages of cell cycle were fused and the fate of the marked nuclei was followed.*

The knowledge from yeast genetics, frog biochemistry and mammalian tissue culture suggested that DNA replication and mitosis are induced by the action and prior activation of phase specific cyclin dependent kinases (Cdks). The purified MPF was Cdk1 and soon it became clear that the catalytic subunits are only functional when present with their cognate regulatory subunits i.e. Cyclins, which were found to be unstable in nature and this specific feature governed the fluctuations in the activity of their catalytic partners (Fig.2) and sets the stage for the alteration of phases (Evans et al., 1983).

In animal cells, S-phase is induced through the action of Cdk2 in conjunction with the cyclins E or A and M-phase by the Cdk1 along with Cyclin B, whereas in both budding and fission yeast, S- and M-phases are induced through Cdk1 associated with phase specific cyclins. Both yeast and mammalian cells, however, require the activity of G1 specific Cdk4/6 -Cyclins (D-type), which promote synthesis of proteins required for genome duplication and chromosome segregation. All these activators are crucially feedback controlled through signal transduction pathways, which, provides cues from the external environment and through classes of proteins collectively referred as Cdk inhibitors (CDKI) e.g. p16Ink, p21Waf1, p27Kip1 etc. (Hunter and Pines, 1994).
The fundamental task of the cell cycle is to ensure that DNA is faithfully replicated once during S-phase and that identical chromosomal copies are distributed equally to two daughter cells during M-phase (Heichman and Roberts, 1994). The machinery for DNA replication and chromosome segregation is insulated from disruption by extracellular signals, and its essential and autonomous nature implies that damage to these components would be debilitating if not fatal to cells. Therefore, the genes that carry out such fundamental aspects should not be frequent targets of mutation, deletion or amplification.

The current model of cell cycle control posits that the transitions between different cell cycle states are regulated through checkpoints. These checkpoints are directly regulated by the activity of cyclins and their cognate Cdks (Hunter and Pines, 1994).

2.1 The START Checkpoint: G1/S- Restriction Point Controllers

This is perhaps the most studied and most important of all the checkpoints (also known as restriction point in mammalian cells), it is positioned in the late G1, at which cells commit for a further round of DNA replication and at which both positive and negative external signals are integrated and feed into the cell cycle. Growth factors act throughout the G1-phase by binding to specific cell surface receptors, leading to the transcription of immediate early and delayed early response genes. Most of the known oncogenic processes exert their effect by targeting particular regulators of G1/S-phase progression (Hunter and Pines, 1994). This brings into the play – the G1 cyclins and their regulators.

2.1.1 Cyclin D

In mammalian cells, the cyclin-Cdk complexes most closely linked to the regulation of START are the D-type cyclins and their partner Cdks, primarily Cdk4. There are three types of D cyclin (D1, D2 and D3). Cyclin D1 is encoded by the CCND1 gene on chromosome 11q13 (Inaba et al., 1992; Xiong et al., 1992), which has been identified as the PRAD1 proto-oncogene. Cyclin D1 is
overexpressed in many human cancers as a result of gene amplification or translocations targeting the D1 locus (Sherr, 1996).

**Fig 2. Fluctuation of different Cyclins and p27Kip1 during the cell cycle:** D-type cyclins are expressed throughout the cell cycle in response to mitogenic stimulation. Expression of cyclins E, A and B is periodic. The cyclins assemble with more stably expressed CDKs to temporally regulate their activities. p27 levels are high in quiescent cells, fall in response to mitogenic stimulation, and degraded at G1/S-phase transition, setting the stage for S-phase.

For example, the inversion of chromosome 11 is associated with parathyroid adenomas (Motokura et al., 1991). This study led to the identification of the position of D1 in relation to a recurrent chromosomal amplification unit at 11q13 and to the BCL1 breakpoint in the translocation 11; 14. There is another characteristic translocation of lymphomas of B cell origin in which D1 locus moves under the direct regulation of immunoglobulin heavy chain enhancer leading to onset of carcinogenesis. Amplification of this locus is a frequent event in some common adult cancers, including squamous cell carcinomas of head and neck, esophageal carcinomas, bladder cancer, primary breast cancer and hepatocellular carcinomas (Hunter and Pines, 1994). Cyclin D2 is encoded by the CCND2 gene on chromosome 12p13 (Inaba et al., 1992; Xiong et al., 1992). The cyclin D2 gene was identified as the site of integration of a murine leukemia virus in a mouse T cell leukemia (Hanna et al., 1993) and this results in cyclin D2
overexpression. Although no rearrangement of cyclin D2 has been found in human cancer, but its amplification has been reported in colorectal carcinoma. The CCND3 gene on chromosome 6p21 encodes cyclin D3, which is yet to be identified as a proto-oncogene, although this region is rearranged in many lymphoproliferative disorders.

As cells enter the cycle from quiescence (G0), one or more D-type of cyclins are induced as part of delayed early response to growth factors and both their synthesis and assembly with Cdk4/6 depend on mitogenic stimulation (Sherr, 1994). The catalytic activities of the assembled holoenzymes are detected first time around mid-G1 phase, increase to maximum to G1/S- phase transition and persist throughout as long as mitogenic signals are present. Mitogen withdrawal leads to cessation of cyclin D1 transcription and ubiquitin-proteosome mediated degradation of the D1 protein (half-life ~30 minutes) (Won et al., 1992; Matsushime et al., 1991). Thus, “D” cyclins act as growth factor sensors, whose deregulation would make cell cycle progression less dependent on growth factors and will essentially contribute towards carcinogenesis. Further, deregulation of D-type cyclins synthesis is expected to cause the cell cycle machinery to receive the signals of continuous presence of growth factors, causing cells to proliferate than to differentiate. Cyclin D1 has also been shown to cooperate with ras (Hinds et al., 1994) and myc (Lovec et al., 1994) to transform cells in classic colony formation assays and anchorage independent growth. The “D” cyclins also play an important role in the switch between proliferation and differentiation. Ectopic overexpression of cyclin D2 and D3 prevents myeloid cells from differentiating in the presence of GM-CSF (Kato et al., 1993). While D1 cyclin is known to antagonize the action of MyoD and prevent myocyte differentiation.

Looking at the prominence of Cyclin D1, it is expected that its main kinase partner Cdk4 to play an important role in G1 progression and oncogenesis. Cdk4 expression is also induced when quiescent cells are stimulated with mitogens (Matshuime et al., 1992; Geng and Weinberg, 1993). Surprisingly, coexpression of a dominant negative version of Cdk4 does not block G1 progression as does that of Cdk2 (van den Heuvel and Harlow, 1993). Cdk4 has been proposed to be a target of TGF-β since the later blocks serum dependent
induction of Cdk4 mRNA in human keratinocytes (Geng and Weinberg, 1993) but has no effect on continuously growing cells either at mRNA or protein level (Hanon and Beach, 1994). Elevated Cdk4 is known to counteract the cell cycle inhibitory effect of p15 Cdk inhibitor (Hannon and Beach, 1994). No mutations have been reported in the Cdk4 gene in human tumors, but both Cdk4 and Cdk6 are known to be overexpressed in several tumor cell lines (Tam et al., 1994) and the Cdk4 gene is amplified in certain tumors (Khatib et al., 1993). Cdk4 when coexpressed with H-Ras has been shown to aid in tumor formation in murine model of melanoma (Lazarov et al., 2002).

2.1.2 Cyclin E

Cyclin E is rate limiting for the G1/S transition: The role of cyclin E in the regulation of G1/S phase transition was directly demonstrated through stable, ectopic overexpression of cyclin E in Rat-1 cells and human fibroblasts where it reduced the G1 interval, decreased the cell size and bypassed the mitogenic requirement for G1/S-phase transition (Ohtsubo and Roberts, 1993). Nevertheless, the overall generation time of cells remained unchanged as cells were delayed in clearing S/G2 and G2/M-phase checkpoints. Given the unchanged doubling time, the decrease in cell size implied its dependence on G1 cyclin levels than on cell cycle length per se. Cyclin E overexpressing cells when cultured in the absence of serum or when progressively lower levels of cyclin E was induced underwent growth arrest, thus these set of experiments established cyclin E as the critical regulator for G1/S-phase transition. Thus, any event that disturbs the normal level of cyclin E or its physiological regulation is expected to reduce the duration of G1/S-phase transition. Recent studies from the laboratory of Stillman suggested that it is due to an impairment of replication initiation and fork movement, due to a defective loading of initiator proteins MCM4, MCM7 and to a lesser extent for MCM2 onto chromatin during telophase and early G1 (Ekholm Reed et al., 2004). Cyclin E without its cognate kinase Cdk2 is known to regulate centrosome duplication although the exact molecular mechanism remains to be elucidated.
Cyclin E is found elevated in many tumors, and often correlates with aggressive disease and poor prognosis (Nielsen et al., 1997; 1998; Schraml et al., 2003). However, it is not simple overexpression of cyclin E, but the loss of its cell cycle regulation might be linked to cancer (Erlandsson et al., 2003; Reed et al., 2004). Expectedly, deregulation of cyclin E promotes chromosome instability in an *in vitro* model (Spruck et al., 1999). Analysis of endometrial carcinomas revealed that mutation of hCDC4, a gene related to cyclin E degradation, correlated with levels of cyclin E, deregulation of cell cycle and chromosomal instability observed in these patients (Spruck et al., 1999). Several hypotheses have been put forward to explain the observed relationship between cyclin E and chromosome instability. First, the interference with pre-replication complex (RC) assembly can lead to a lower number of active replication origins, increasing the average replicon size and resulting in higher frequencies of stalled replication forks and double stranded DNA breaks. Second, the diminished rate of DNA replication can compromise the processes of chromatid cohesion and condensation as they are coupled to DNA synthesis at replication fork. Third, slow DNA replication can lead to incompletely replicated chromosomes at the time when cells would normally enter mitosis, leading to nondisjunction events and ultimately karyotypic anomalies. Finally, deregulation of cyclin E may directly compromise the normal intra-S phase checkpoint mechanisms and consequently allow propagation of DNA (Ekholm-Reed et al., 2004).

2.1.3 Inhibitors of G1/S - Cyclin-Cdks as Potential Tumour Suppressors

Given the roles of pRb and p53 in tumor suppression, it would be expected that other gene products that act epistatically to regulate their expression or functions might also be frequent targets of deregulation in cancer cells. Prominent among these is the INK4 family of Cdk inhibitors, which block the ability of the cyclin D-dependent kinases, Cdk4 and Cdk6 to phosphorylate and thereby inactivate growth suppressive functions of retinoblastoma (Rb). The founding member, p16INK4a was initially identified as a Cdk4-binding protein in SV40 T antigen transformed cells (Serrano et al., 1993). It is found inactivated in cases of familial melanoma (Kamb et al., 1994) and in many other tumor types (Ruas and Peters, 1998). It is presumed to be a competitor of cyclin D1 binding to
Cdk4/6. The level of p16 is elevated in cells lacking functional pRb, such as T-antigen transformed cells, suggesting a feedback regulation (Parry et al., 1994). Intriguingly, the INK4a locus encodes a second, structurally and functionally unrelated protein that is also a potent tumor suppressor. Two alternative transcripts, initiated at separate promoters and incorporating sequences from distinct first exons (designated 1alpha and 1 beta), are each spliced to common downstream exon sequences that are translated in alternative reading frames. Whereas the transcript that contains exon-1alpha sequences specifies p16INK4a, the mRNA incorporating exon-1 beta sequences encodes the alternative reading frame (ARF) protein, designated p14ARF in humans and p19ARF in the mouse (Sherr, 2001). Equally surprising, the ARF protein activates p53 by binding directly to the p53 negative regulator Mdm2 and protecting p53 from Mdm2-mediated degradation (Sharpless and DePinho, 1999; Sherr, 2001). Thus, one locus encodes two proteins, p16INK4a and p14ARF, that functionally interface with pRb and p53, respectively (Figure 3). The simultaneous regulation of both pRb and p53 is an interesting example of economy of genetic organization during evolution. The INK4a-ARF promoters respond to sustained hyperproliferative signals. For example, constitutive activation of oncogenic ras induces both of these proteins leading to cell cycle arrest (Serrano et al., 1997). In contrast loss of INK4a-ARF extends the replicative capacity of cells in culture, contributes to their establishment as continuously proliferating cell lines, and sensitizes them to transformation by oncogenic Ras (Serrano et al., 1996; Kamijo et al., 1997). In short, pRb, p53, p16INK4a, and p14ARF form part of a signaling network that monitors mitogenic signaling and restrains aberrant growth-promoting signals from driving cell cycle progression inappropriately. Inactivation of this signaling network occurs in most, if not all, forms of cancers.

Cyclin E- and A- dependent kinases are negatively regulated by a distinct family of Cdk inhibitors that include at least three proteins: p21Waf1, p27Kip1 and p57Kip2 (Figure 3). Perhaps the single most remarkable feature of p21Waf1 is its inducibility by p53 during growth arrest/DNA damage (El deiry et al., 1993; Wade Harper et al., 1993). Genotoxic agents are known to activate p53 leading to increased expression of p21Waf1, which in turn binds to the S-phase cyclin-Cdk2 complex and block S- phase progression (Sherr, 1996). Cells harboring mutated
p53 have low levels of p21Waf1. Such condition allows DNA replication to proceed in presence of unrepaired genome and contributes to the chromosomal abnormalities and genetic instability in transformed cells (Hartwell, 1992). Related to this is an observation that human papilloma virus (HPV) E6 oncoprotein, which inactivates p53 through its ubiquitin ligase activity, leads to a reduced p21Waf1 expression and consequent association with cyclin-Cdk2 complex (Sarnow et al., 1982). p21Waf1 has also been shown to bind other components of the cell cycle machinery, viz. PCNA (Waga et al., 1994).

Fig 3. Relationship of various Cyclin-CDKs and their inhibitors: In humans, the p16 gene is adjacent to a gene encoding a very similar protein, p15INK (Hannon and Beach, 1994). The sequences of p16 and p15 are 44% identical in the first 50 amino acids and 97% identical in the following 81 amino acids; both the proteins have four ankyrin motifs and both bind and inhibit only CDK4 and CDK6. Unlike p16 however the synthesis of p15 are induced by TGF beta treatment, like that of p27Kip suggesting they work in tandem for observed G1 arrest.

p21Waf1 is 42% identical in its N-terminus to another CDK inhibitor p27Kip1 (Toyoshima et al., 1994; Polyak et al., 1994). Like p21Waf1, p27Kip1
inhibits a wide variety of cyclin-Cdk complexes \textit{in vitro}, including Cdk4 and Cdk2 complexes and overexpression of p27Kip1 blocks progression of cells through G1. The block in G1 progression of murine macrophages induced by cAMP apparently occurs through increased expression of p27Kip1 (Kato et al., 1994). KIP1 is supposed to be the inhibitor most directly involved in restriction point control. In quiescent cells, the level of p27Kip1 is high but falls as cells slowly progress to S-phase (Nurse et al., 1994) (Figure 2). The level of p27Kip1 is largely controlled by the translational (Hengst et al., 1996) and posttranslational mechanisms (Pagano et al., 1995). Cyclin E-Cdk2 mediated phosphorylation of p27Kip1 at T-187 (Clurman et al., 1996) is known to mark it for SCF (Skp/ Cullin/ F-box protein) complex mediated proteasomal degradation (Carrano et al., 1999; Montagnoli et al., 1999). p27Kip1 harbors its cyclin–Cdk inhibition and binding in an N-terminal domain that contacts both the cyclin and the Cdk subunits (Russo et al. 1996). Importantly, to exert its Cdk inhibitory functions, p27Kip1 is required to be localized in the nucleus. p27Kip1 contains a phosphorylation-regulated nuclear localization signal (NLS) harbored in the C terminus (Zeng et al., 1996). Upon mitogen stimulation, the human kinase interacting stathmin (hKIS) phosphorylates p27Kip1 on Ser 10 to signal its nuclear export into the cytoplasm (Ishida et al. 2000; Rodier et al. 2001; Boehm et al. 2002). Furthermore, phosphorylation of p27Kip1 on the NLS Thr-157 by AKT disables its nuclear localization capacity (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002).

Antisense inhibition of p27Kip1 synthesis in cycling cells can prevent them from becoming quiescent (Coats et al., 1996). Mice nullizygous for the gene encoding p27Kip1 grow faster than their littermate controls and exhibit organomegaly, with all tissues containing increased numbers of smaller cells (Nakayama et al., 1996; Fero et al., 1996). This phenotype underscores the importance of p27Kip1 in regulating both cell size and cell number.
Overcoming the G1/S Restriction Point

![Diagram of cell cycle phases and cyclin-dependent kinases]

**Fig 4. G1/S Restriction point control:** Rb phosphorylation triggered by cyclin D-dependent kinases release pRb bound E2F1, which leads to transcriptional activation of its cognate substrates known to play a direct role in DNA replication. This leads to a positive feed back loop promoting pRb phosphorylation by cyclin E-CDK2, contributing to the irreversibility of the restriction point transition and making it mitogen independent.

### 2.1.4 The Rb/E2F Connection in Cell cycle

Rb was identified as the first tumor suppressor. Although the gene was initially cloned as a result of its frequent mutation in the rare pediatric eye tumor, retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987), it is now thought to play a fundamental role in cellular regulation and is the target of tumorigenic mutations in many cell types. The retinoblastoma gene encodes a 928-amino acid phosphoprotein, Rb, which arrests cells in the G1 phase (Weinberg, 1995). Rb is phosphorylated and dephosphorylated during the cell cycle; the hyperphosphorylated (inactive) form predominates in proliferating cells, whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells (Chen *et al.*, 1989). As a demonstration of its tumor
suppressor activity, Rb was reintroduced into Rb-deficient tumor cells and it blocked several features of the malignant phenotype (Huang et al. 1988). Mutations affecting the retinoblastoma gene are frequently encountered, not only in retinoblastoma but also in other cancers such as osteosarcoma, small cell lung cancer, prostate cancer, and breast cancer (Friend et al., 1986; Fung et al., 1987; Harbour et al., 1988; Lee et al., 1988; T'Ang et al., 1988; Bookstein et al., 1990). Indeed, children with hereditary retinoblastoma have 30-fold increased risk of developing a second, nonocular malignancy, especially bone and soft tissue sarcomas in adolescence and cutaneous melanomas in adulthood (Eng et al., 1993; Moll et al., 1997). These second neoplasms occur almost exclusively in patients who have germ-line mutations in the retinoblastoma gene. As a further indication of its fundamental role in tumor suppression, pRb can be functionally inactivated by constitutive hyperphosphorylation in tumors that do not have mutations in the retinoblastoma gene (Sherr, 1996). The activity of pRb depends, at least in part, on interactions with the E2F family of DNA-binding transcription factors (Chellappan et al., 1991; Dyson, 1998; Nevins, 1998). E2F sites are found in the promoters of many genes that are important for cell cycle progression, and pRb appears to repress transcription of these genes through its interaction with E2F (Blake and Azizkhan, 1989; Thalmeier et al., 1989; Dalton, 1992; DeGregori et al., 1995). Recent findings in the pRb/E2F field are clarifying how this pathway regulates the transition from G1 to S phase at the molecular level (Figure 5). pRb in its hypophosphorylated form complexes with E2F family of transcription factors and interferes with the concomitant transcriptional process. Also pRb and other members of the pocket protein family, p107 and p130, are known to associate with HDACs, which through their enzymatic action actively deacetylate histones and maintains a repressive chromatin mark for transcriptional purposes (Figure 5). pRb contains 16 potential phosphorylation sites for Cdk's. Cell cycle progression leads to progressive hyper phosphorylation of pRb through concerted action of stage specific cyclins and their associated kinases and this phosphorylation in itself is considered as a “hallmark” of cell cycle progression.
Fig 5. Mechanism of Rb mediated E2F transcriptional repression

2.2 Cell Cycle deregulation by Viral proteins

The multilayer regulatory systems evolved to regulate the various phases of cell cycle are critical to maintain an orderly progression from one phase to another. Any abnormality in the system is manifested as a potential cause for either initiation or sustaining of carcinogenesis. Oncogenic viruses also have evolved mechanisms to deregulate this orderly transition to maintain a permissive environment for their replication and in the process override various "checkpoints". Over the years, a number of experimental and clinical studies have suggested that many viral oncoproteins play a critical role in different forms of cancer. A selected few examples are discussed below:

2.2.1 The T antigens

SV40 and mouse polyomavirus, the prototype members of the Polyomaviridae family, encode proteins known as large tumor (T) antigens. These are sufficient to induce cell cycle progression, reverts terminal differentiation and induce cell transformation both in vitro and in vivo (Chandrasekaran et al., 1996; Fromm et al., 1994). This protein inhibits the cell cycle inhibitory activities of tumor suppressor proteins like p53, pRb, p107 or activating p300 and thus, promoting progression towards S-phase (Ludlow, 1993).
2.2.2 E6 and E7 proteins

Papilloma viruses encode two non-structural proteins, E6 and E7, which deregulate the expression and activities of cellular regulators. E7 binds to and inactivates pRb, p107, p130 and p21Wafl leading to cell cycle progression into S-phase (Ewen et al., 1991; Jones et al., 1997). E7 also binds to but activates the S-phase cyclin and Cdk s (Dyson and Harlow, 1992). E6, on the other hand, is known to abrogate p53 functioning through its associated ubiquitin ligase activity. E6 induces proteasomal degradation of p53, and thus hamper the transcriptional induction of p21Wafl (Sarnow et al., 1982).

2.2.3 E1A

The adenovirus encoded E1A protein is known to bind and inactivate p53 and hypophosphorylated pRb, p107, p130 (thus alleviating repression of E2F1) thus allowing cells a proliferative advantage and preventing apoptosis (Cobrinik et al., 1993). E1A proteins also contribute to S-phase progression by inducing expression of cyclin A and directly binding to it (Pines and Hunter, 1990; Tsai et al., 1991). E1A proteins can prevent inactivation of Cdk2 activity by differentiation induced cytokines, and thus prevent G1 arrest (Datto et al., 1997).

2.2.4 HHV8

This gene of Kaposi’s sarcoma associated herpes virus (KSHV) encodes a viral homologue cellular cyclins (K-cyclin) (Cesarman et al., 1996 and Chang et al., 1996). It is most closely related to cellular D-type of cyclins, but functionally it can associate with Cdk2 as well as Cdk4/6 and phosphorylate their cognate substrates (Mann et al., 1999). Furthermore, Cdk6 activated by this protein phosphorylates p27Kip1 leading to its proteasomal degradation, and deregulation of G1/S- phase checkpoint (Mann et al., 1999).
2.2.5 HBx

The X protein of hepatitis B virus is known to stimulate cell cycle progression. HBx has transactivating and transforming activities and can induce tumors in transgenic mice (Bouchard et al., 2004; Kim et al., 1991). At the molecular level HBx activates cyclin A expression and cyclinA/Cdk2 activity, thus promoting cell cycle progression (Bouchard et al., 2004). The activation of cyclin A is dependent on a constitutive Src kinase activity. HBx can also inactivate p53 and downregulate expression of p21Waf1, thus promoting cell cycle progression and preventing apoptosis (Wang et al., 1994; Huo et al., 2001).

2.3 Cell Cycle mediated Control of DNA Replication (S-Phase)

The initiation of DNA replication in eukaryotic cells is a highly regulated process, which leads to the duplication of the genetic information for the next cell generation. This requires the ordered assembly of many proteins at the origins of DNA replication to form a competent, pre-replicative chromosomal state. In addition to the competent complex, at least two cell cycle regulated protein kinases are also required to affect a transition to a post-replicative chromosomal state.

2.3.1 Initiation: Cis- Elements and Trans-acting Factors

The starting point required to understand the cell cycle controls on DNA replication is the origin of DNA replication. In eukaryotes, the origin of DNA replication is determined by cis-acting DNA sequences (replicators) and groups of trans-acting proteins that bind to replicator sequences and orchestrate the process of replication (Stillman, 1996). Eukaryotic chromosomes harbor multiple origins, more than the required numbers to replicate each chromosomes. The sequences required for an origin of replication vary significantly between different eukaryotic organisms. The best characterized among the eukaryotic replicators are the one derived from bakers yeast (S. cerevisiae), where 3 to 4 sequences of 10-15 base pairs spread over 100-150 base pairs are sufficient to act as origin (Bell and Stillman, 1992). In its closely related cousin, fission yeast (S.
metazoan origins are still less well defined and are known to extend over thousands of base pairs of DNA. In addition the sites of initiation are not always tightly linked within these regions. Studies on Xenopus embryos revealed that the zone where replication initiates changes during development (Hyrien et al., 1995). In the very early embryonic cell divisions, initiation occurs over a broad region in the ribosomal DNA locus, but during the mid-blastula transition, this zone becomes restricted. A similar phenomenon may occur during every cell cycle in mammalian cells. When very early G1-phase nuclei from human cells were incubated in Xenopus egg extract, initiation near the DHFR gene was dispersed, however when nuclei from cells in late G1 were incubated, initiation was localized to a specific region (Wu and Gilbert, 1996).

The first mammalian origin to be localized resides in the 240 kb amplified dihydrofolate reductase domain in the methotrexate-resistant CHO cell line, CHOC 400 (Milbrandt et al., 1981). Most of the data on this complex origin have converged into a reasonably unified model in which initiation can occur at as many as 40-50 sites within the spacer, with maxima at ori-b and ori-g, spaced about 20 kb apart. At most, 20% of initiations in the spacer occur within the 2 kb encompassing the most active region ori-b; (Dijkwel et al., 2002).

Several origins in other higher eukaryotes are broad zones of inefficient sites, including the hamster rhodopsin origin (Dijkwel et al., 2000), the human rDNA origin (Little et al., 1993), and the Drosophila histone and alpha-polymerase genes (Shinomiya and Ina, 1993; 1994). In contrast to its localized loading on origins in yeasts, MCM2-7 is loaded in a distributive pattern around origins in higher eukaryotes (Edwards et al., 2002). This difference might explain why many metazoan origins correspond to broad zones of potential sites, in contrast to the well-defined initiation sites found in yeast genomes. However, some origins are clearly more circumscribed and come closer to the bacterial paradigm.

These include the human lamin B2 (Paixao et al., 2004) and β-globin (Wang et al., 2004) origins. The human globin origin is actually a mini-zone of initiation, although two genetic elements have been described that behave as
redundant replicators when the β-globin origin is assayed at an ectopic chromosomal site (Wang et al., 2004). In contrast, the lamin B2 origin appears to correspond most closely to a single site (Abraham et al., 2003). Thus, there appears to be a spectrum of origin types ranging from tightly circumscribed to extremely broad initiation zones. Likewise, the efficiency of origin firing can range from nearly 100% [as with human β-globin (Kitsberg et al., 1993) or Physarum polycephalum (Benard et al., 1996)] to less than 20% [as with the origins for DHFR (Dijkwel and Hamlin, 1992; Dijkwel et al., 1994) and rhodopsin (Dijkwel et al., 2000)]. The probability that any given site will efficiently attract an initiation complex will depend upon whether it finds itself in a permissive environment. Thus, in a euchromatic chromatin domain, the overall architecture would be permissive, allowing a non-transcribed (intergenic) region to initiate with more or less efficiency, whereas sites within an active transcription unit, in the same euchromatic domain, would not. Non-permissive heterochromatic regions and/or active transcription units may therefore be replicated passively from active upstream or downstream origins before they fire their own origins.

2.3.2 Components of the Pre-replicative Complex

2.3.2.1 The Origin Recognition Complex

The origin recognition complex (ORC) is a six-sub unit complex that acts as the initiator (the protein that selects the sites for subsequent initiation of replication) at eukaryotic origins of replication. Although identified in S. cerevisiae as binding to the conserved ARS consensus sequence (Bell and Stillman, 1992), subsequent studies have found that this complex is a conserved feature of chromosomal replication in all eukaryotes. Although direct evidence that mammalian ORC functions in the initiation of replication is lacking, studies indicate that replication from oriP of Epstein-Barr virus (EBV) requires ORC function under tissue culture conditions (Dhar et al., 2001). The best understood activity of ORC is its ability to bind DNA. The complex nature of the interaction of ORC with DNA has made identification of a discrete DNA binding domain difficult. ScORC requires the five largest subunits (Orc1p-Orc5p) to recognize
DNA. The DNA sequences identified have a strong propensity to have stretches of poly-A. ORC also binds and hydrolyzes ATP. While ATP binding is a prerequisite for ORC-DNA interaction, but not its hydrolysis. Recent studies in mammalian cells suggest that not all ORC subunits remain tightly associated as part of the complex throughout the cell cycle, e.g. Orc1p is removed from the chromatin during S-phase and rebinds chromatin only during the subsequent G1 phase. This removal of ORC could potentially serve to eliminate pre-RC formation prior to the completion of M-phase (also chromatin condensation could be a leading cause of ORC elimination). ScORC2 and 6 are phosphorylated in a Cdk dependent manner in vivo (Nguyen et al., 2001). The degradation of human ORC has been suggested to require a phosphorylation through Cdk2 (Kreitz et al., 2001).

2.3.2.2 The Cdc6 protein

This protein is a member of the AAA+ATPases and is highly related to Orc1p. Cdc6 plays a crucial role in the assembly of the pre-RC at a step after ORC and before the MCM2-7 proteins (Liu et al., 2000). Regulation of Cdc6 activity plays a critical role in regulating the formation of the pre-RC during the cell cycle. Studies of human Cdc6 support the idea that the function of this replication factor is dependent on its ability to bind and hydrolyze ATP (Herbig et al., 1999) since mutations affecting either the ATP binding or hydrolysis affects the S-phase entry or its completion respectively. In mammalian cells, with S-phase, phosphorylation of Cdc6 by Cdns leads to a nuclear export whereas in mitosis Cdc6 is degraded through APC mediated recognition of its KEN box. The phosphorylation of Cdc6 by Cdk activity is a well-documented event (Dutta and Bell, 1997). These modifications appear to control either the degradation (yeast) or nuclear export (human). The fact that Cdc6 is selectively removed from the chromatin at G1/S-phase transition with the help of the cyclin-Cdk action, whereas ORC and MCM are retained is consistent with the latter two molecules having a direct role in replication initiation (Figure 6).
2.3.2.3 The Cdt1 protein

Originally identified in *S. pombe* as a gene whose mutation affected S-phase progression and a defective DNA replication. This protein has been shown to associate with the carboxy terminal of Cdc6 to cooperatively promote association of MCM proteins with chromatin (Nishitani *et al.*, 2000). Consistent with its function, the Cdt1 protein level peaks at G1 and decreases as cells progress through S-phase. Experiments conducted with *Xenopus* extracts demonstrate a requirement of ORC but not of Cdc6 in the chromatin association of Cdt1. Perhaps the best understood role of Cdt1 is in context of geminin which can stoichiometrically inhibit the function of Cdc6 (Wholschelgel *et al.*, 2000).

2.3.2.4 The Mini Chromosome Maintenance 2-7 Protein Complex

The genes that encode the MCM2-7p complex were originally identified in genetic screens for proteins involved in plasmid maintenance, cell cycle progression, and chromosome missegregation (Chong *et al.*, 1996). Each MCM is highly related to all others, but unique sequences distinguishing each of the subunit types are conserved across eukaryotes. Both biochemical and genetic studies indicate that these proteins function together as a complex in the cell. The assembly of MCM proteins onto chromatin requires the coordinated function of ORC, Cdc6 and Cdt1. Interestingly, once MCM proteins are loaded onto chromatin, ORC and Cdc6 can be removed from chromatin without preventing subsequent DNA replication (Hua *et al.*, 1998). ChIP assays suggest that several of the MCM proteins associate with the origin and origin proximal DNA sequences in a temporal manner (Zou and Stillman, 2000). Although it is clear that the MCM proteins act at both the replication fork and origin, their biochemical function at these sites remain to be determined. Biochemical data supports the hypothesis that MCM proteins act as a DNA helicase. A complex containing the MCM 4,6,7 retains 3'-5' DNA helicase activities (Ishimi *et al.*, 1997).

The MCM hexamer also demonstrate a robust ATPase activity and this activity helps the helicase activity, as mutation in the ATP hydrolysis site interferes with the functioning under *in vitro* conditions (Blow and Dutta, 2005).
Cdk modification also controls the function of MCM proteins. Cdk mediated phosphorylation of both MCM2 and MCM4 subunits have been documented both in vitro and in vivo (Perverzeva et al., 2000) and may have role in regulation of its helicase activity.

2.3.2.5 Chromatin Loading of MCMs

DNA replication in all eukaryotes starts with the process of loading a replicative helicase onto chromatin in late M phase of the cell cycle. This helicase is a hexamer composed of the proteins MCM2 to MCM7. MCM2 to MCM7 are closely related proteins that harbor AAA+ ATPase domains, which are required for both elongation and initiation in the *Xenopus* in vitro replication system (Pacek and Walter, 2004). Loading of the MCM2-7 complex is achieved by the concerted action of ORC, Cdc6, and Cdt1 (Figure 6), although metazoans tend to have MCM proteins distributed over the chromosomes instead of being concentrated near origins as in yeast. Although ORC, Cdc6, and Cdt1 are required to load MCM2-7 helicases onto chromatin, the exact mechanism of action of these proteins is poorly understood. The ring-shaped structure of MCM2-7 encircles the DNA, suggesting that ORC and Cdc6 might act as an ATP-dependent clamp-loader analogous to the replication factor C clamp-loader for the proliferating cell nuclear antigen (PCNA) (Mendez and Stillman, 2003). Among the pre-RC components in metazoans, Orc1, Orc4, and Cdc6 have both Walker A and Walker B motifs, whereas Orc5 has only a Walker A motif. The Walker A motif is critical for ATP binding and the Walker B motif is required for ATP hydrolysis. Mutation of the Walker A motif of Orc1, but not of Orc4 and Orc5, reduces the ATPase activity of metazoan ORC (Chesnokov et al., 2001), suggesting that the ATPase domain of Orc1 is responsible for the ATPase activity of ORC. Mutagenesis studies indicate that ATP binding by ORC (or Cdc6), but not hydrolysis of ATP, is required for DNA binding by ORC or Cdc6 (Chesnokov et al., 2001; Frolova et al., 2002; Herbig et al., 1999). Given that a non-hydrolysable ATP analog inhibits the loading of MCM2-7, but not loading of ORC and Cdc6 (Harvey and Newport, 2003), it seems likely that ATP hydrolysis is used to load MCM2-7 on chromatin. The interaction between Cdt1 and MCM2-7 is greatly enhanced by the presence of Cdc6 (Cook et al., 2004).
Fig 6. Cell cycle dependent assembly of replication complex
2.3.3 Origin Firing: The Transition to Replication

DNA is detectably not unwound until S-phase, suggesting that the MCM2-7 loaded onto origins during G1-phase remain inactive. MCM2-7 does not show a strong helicase activity \textit{in vitro}, supporting the possibility that it requires activation to initiate replication. Loading of Cdc45 seems to be a crucial step for the activation of the MCM2-7 helicase (Figure 7). Immunoneutralization
of Cdc45 prevents both origin firing and elongation in the *Xenopus in vitro* replication system (Pacek and Walter, 2004), indicating that Cdc45 is required for both activation of MCM2-7 on origins and chromosome unwinding at the replication forks. Consistent with the idea that Cdc45 is a helicase cofactor, helicase activity was associated with Cdc45 immunoprecipitates from *Xenopus* egg extracts (Masuda et al., 2003). MCM7 also interacts with pRb in a yeast two-hybrid assay (Sterner et al., 1998). This interaction has a negative effect on DNA replication in *Xenopus* egg extracts. Furthermore, Cyclin D-Cdk4 prevents binding of pRb to MCM7 (Gladden and Diehl, 2003). Thus, pRb might suppress MCM2-7 helicase activity until activation of Cdk in a manner similar to how it represses E2F-mediated transcription until S-phase. The MCM3-associated protein (MCM3AP) may also be involved in repression of MCM2-7 through acetylation of MCM3 (Takei et al., 2001) and suppression of the MCM2-7 helicase activation until S-phase (Takei et al., 2002). The MCM2-7 activation and derepression models are not mutually exclusive, and both mechanisms may be required for its tight regulation and coordination of the replication cycle.

Two kinases, Cdc7 and Cdk2, are involved in the loading of Cdc45 on origins (Figure 7). Cdc7 phosphorylates the subunits of MCM2-7 causing a conformational change to facilitate the loading of subsequent factors such as MCM10 and Cdc45. Although the role of metazoan Cdk2 in replication initiation is not known, phosphorylation of an initiation factor, Sld2/Drc1, by Cdk is essential for initiation of DNA replication in yeast (Masumoto et al., 2002; Noguchi et al., 2002). The amino terminus of human RecQL4, a helicase, shares homology with a corresponding region in Sld2/Drc1, suggesting that RecQL4 is a metazoan ortholog of yeast Sld2/Drc1. However, the Cdk phosphorylation sites on Sld2/Drc1 are not conserved in RecQL4, raising a possibility that RecQL4 is not a bona fide Cdk2 target for replication initiation in metazoans. Consistent with this, RecQL4 is required for the loading of RPA, the single-stranded DNA (ssDNA) binding protein that is recruited after Cdc45 loading, whereas RecQL4 is not required for the Cdk2-dependent Cdc45 loading step (Sangrithi et al., 2005) (Figure 7).
2.3.4 Replication Timing

Although S phase-promoting kinases (Cdk2 and Cdc7) are highly active after S phase entry, activation of those kinases does not trigger all origins immediately. Origins instead fire at different times during S phase. Yeast released from a hydroxyurea block can complete the replication of the entire genome under conditions where the late-firing origins do not fire. In contrast, the larger size of metazoan genomes, makes it very unlikely that all late-firing origins will be completely dispensable for the complete replication of metazoan chromosomes. Recent large scale profiling of replication in Drosophila and human cells provides insight into how replication timing is determined in vivo. Replication timing correlates with a range of genomic features. Chromosomal regions that are gene-rich, and rich in GC or alu repeat sequences replicate earlier (Jeon et al., 2005; Woodfine et al., 2004). In contrast, gene-poor regions, heterochromatin and regions with high concentrations of LINE repeats tend to replicate late (Jeon et al., 2005; Schubeler et al., 2002; Woodfine et al., 2004). Although heterochromatin replicates in late S phase, it is not yet clear whether this is because replication timing is influenced by heterochromatin or whether heterochromatin is a consequence of late-replication. Mutation of the histone deacetylase (HDAC) Rpd3 in Drosophila induces genome-wide hyperacetylation and stimulates origin activity (Aggarwal and Calvi, 2004), supporting the notion that heterochromatin can suppress replication. The second model, in which late-replication plays a role in heterochromatin formation, was proposed based on the observation that HDAC is recruited to replication foci only during late S-phase (Rountree et al., 2000). In this model, late replicating chromosomal regions form heterochromatin simply because this is the only type of chromatin that is assembled in late S-phase. Consistent with this idea, DNA injected in early S phase is packaged into acetylated histones and transcriptionally active, whereas DNA injected in late S-phase harbors deacetylated histones and is transcriptionally inactive (Zhang et al., 2002). There is a positive correlation between replication timing and the probability of gene expression in Drosophila and humans (MacAlpine et al., 2004; Schubeler et al., 2002; Woodfine et al., 2004). In yeast, however, there is no correlation between replication timing and the likelihood of gene expression (Raghuraman et al., 2001). Superimposition of
the genome-wide distribution of origins and ORC binding sites in *Drosophila* reveal significant colocalization of ORC and RNA polymerase II (MacAlpine *et al.*, 2004). However, many of the origins mapped so far in humans are located in intergenic regions. Together, these results suggest that transcription can dictate both origin choice and initiation timing. Nevertheless, it is still unclear whether transcription factors help recruit initiation factors to chromatin or whether open chromatin at active genes increases the chance of loading initiation factors. In any case, the positive effect of transcription on replication timing is seen over large domains (>100 kb) rather than with individual genes (MacAlpine *et al.*, 2004), suggesting that the effect is mediated through large-scale chromatin structure changes. Recent studies suggest that the ATM/ATR-mediated checkpoint regulates origin firing even in unperturbed metazoan cells (Shechter *et al.*, 2004). Abrogation of the checkpoint pathway stimulates Cdc45 loading and origin firing in *Xenopus* egg extract and human cells (Marheineke and Hyrien, 2004; Shechter *et al.*, 2004; Syljuasen *et al.*, 2005). Thus, ATR, which is probably activated by ssDNA at normal replication forks, suppresses other unfired origins through the intra-S-phase checkpoint pathway. Chances are that factors such as chromatin structure determine which origins fire early and efficiently, and then checkpoint pathways suppress inefficient origins once efficient ones fire. Increasing the number of nuclei in *Xenopus* extracts prolongs S phase, which can be partially reversed by inhibition of the checkpoint, suggesting that the number of firing origins is restricted by checkpoint pathways (Marheineke and Hyrien, 2004; Shechter *et al.*, 2004). Checkpoint pathways could restrict the number of origins that fire in a given time-interval so that fork density is maintained at an optimal level.

2.4 The DNA Replication Fork in Eukaryotic Cells

Replication of the two template strands in eukaryotic cell DNA replication forks is a highly coordinated process that ensures accurate and efficient genome duplication. Biochemical studies, principally of plasmid DNAs containing the Simian Virus 40 (SV40) origin of DNA replication, and yeast
genetic studies have uncovered the fundamental mechanisms of replication fork progression (Waga and Stillman, 1998).

**Cellular Replication Fork associated Proteins:**

2.4.1 DNA Polymerase Alpha/Primase Complex

This is the only enzyme capable of initiating DNA synthesis de novo by first synthesizing an RNA primer and then extending the primer by polymerization to produce a short DNA extension (RNA-DNA primer) (DePamphilis, 1993). The short RNA-DNA hybrid then serves as a primer for extension by other polymerases for leading and lagging strand synthesis, because this protein in itself is not capable of processive DNA synthesis and dissociates from the template DNA following primer synthesis (Murakami and Hurwitz, 1993). The human primase consists of four subunits. Of the four subunits, the 180 kDa and 70 kDa subunits are phosphorylated during G2/M-phase transition and considered to play a role in resetting the replication apparatus for the next S-phase (Nasheuer et al., 1991).

2.4.2 Replication Protein A (RPA)

This is a single-stranded DNA (ssDNA)-binding protein that exists as a heterotrimeric complex consisting of subunits with apparent masses of approximately 70, 34 and 14 kDa. The trimeric protein was initially identified as an essential factor for both cellular and SV40-dependent *in vitro* DNA replication (Fairman and Stillman, 1988), but subsequently its role has been established for DNA recombination and repair (Wold, 1997). RPA promotes extensive unwinding of duplex DNA and also promotes primase activity under certain conditions and is required for RFC-PCNA dependent DNA synthesis by DNA polymerase delta (Tsurimoto and Stillman, 1991). Although the human p70 subunit alone can bind single-stranded DNA, it cannot support DNA replication *in vitro*, pointing towards a role of the trimeric RPA complex for this function (Gomes and Wold, 1995). Mutational analysis provides evidence for a N-terminal DNA binding and C-terminal subunit association function for the p70 subunit
(Lin et al. 1996). Both the p70 and p34 subunits of human and yeast RPA are phosphorylated in a cell cycle dependent manner (Din et al., 1990). Increased levels of similar phosphorylated forms of RPA are also seen in response to DNA damage (Carty et al., 1994). The DNA damage dependent phosphorylation of p34 subunit is delayed to abrogate in cells from patients having mutated ATM/ATR kinases suggesting a close functional link between the checkpoint kinases and RPA phosphorylation (Brush et al., 1996).

2.4.3 Replication Factor C (RFC)

This is the key protein complex involved in loading the replicative polymerases to create the replication fork. It is composed of five subunits (p140, p40, p38, p37 and p36) that is conserved in all eukaryotes. RFC was first identified as an essential factor for in vitro SV40 DNA replication (Tsurimoto and Stillman, 1990). RFC preferentially binds to a primer-template junction created by the annealing of an oligonucleotide to single stranded DNA, or by synthesis of a DNA primer on a single stranded DNA template. This binding requires ATP and the DNA bound RFC functions as a DNA dependent ATPase, an activity stimulated by associated PCNA (Fien and Stillman, 1992). The main role of RFC is to load PCNA on the primer-template junction, which is a prerequisite for the assembly of polymerase delta onto template DNA to form a processive holoenzyme (Podust et al., 1995) that functions during synthesis of both leading and lagging strand. Of the five subunits of RFC only p140 subunit is known to interact with PCNA (Fotedar et al., 1996). p140 is also a target of caspase mediated cleavage during apoptosis (Rheaume et al., 1997).

2.4.4 Proliferating Cell Nuclear Antigen (PCNA)

Also known as DNA polymerase clamp, PCNA is perhaps the most intensely studied proteins amongst the one comprising the replication fork. Not only does this protein plays a central role in DNA metabolism, but also has become a significant clinical diagnostic marker for proliferating cells. A protein with an apparent mass of 36 kDa forms a homotrimeric complex and functions as a DNA polymerase accessory factor (Kelman, 1997). The primary amino acid
sequence of PCNA is not highly conserved among species, but yeast and human PCNA nevertheless have an identical three-dimensional structure. The trimeric PCNA forms a closed ring with a hole in the centre to accommodate the duplex DNA. PCNA itself does not have a DNA binding activity, but it can be loaded onto DNA by RFC in an ATP-dependent manner (Podust et al., 1995). PCNA associates with polymerase delta at the primer-template junction. Mutational analyses of PCNA have shown that distinct regions of trimeric ring are required for the stimulation of polymerase delta and the ATPase activity of RFC (Fukuda et al., 1995). PCNA is also capable of binding to several other proteins, including FEN1, DNA ligase1, p21Waf1, cyclin D1, Caf1 etc. (Waga and Stillman, 1998). The identification of proteins that interact specifically with PCNA has led to the emergence of PCNA as a key player in replication and other events at the replication fork, such as DNA methylation and DNA repair.

2.4.5 Geminin

Ever since its discovery as an anaphase-promoting complex (APC) substrate with an inhibitory activity on pre-RC formation, geminin has emerged as a key regulator of metazoan replication. Geminin is conserved from the worm Caenorhabditis elegans to humans, but homologs have not been reported in yeast. Geminin is destabilized during G1-phase and accumulates during S-, G2-, and M-phases of the cell cycle. At the metaphase to anaphase transition, geminin is ubiquitinated by APC and degraded by the proteasomes to allow pre-RC formation in G1-phase. Geminin binds to Cdt1 and inhibits loading of MCM2-7 onto chromatin. In Drosophila and human cells, geminin seems essential for preventing re-replication, because geminin depletion by RNAi in cultured cells causes rereplication, as does overexpression of geminin’s target, Cdt1 (Blow and Dutta, 2005). In contrast, geminin depletion is not sufficient to induce robust rereplication in vitro in Xenopus egg extracts (Arias and Walter, 2005; Li and Blow, 2005). However, Cdk-mediated suppression is still important in preventing rereplication in metazoans, particularly in G2/M cells (Blow and Dutta, 2005) where Cdk inhibition, but not geminin depletion, is sufficient to induce pre-RC formation. Thus, different mechanisms suppress re-replication in S-phase and G2/M-phase in metazoans. Similar to the role of geminin in stabilizing Cdt1 for
pre-RC formation, recent work has uncovered a positive role for Cdk activity in pre-RC formation in humans (Mailand and Diffley, 2005). Phosphorylation of Cdc6 by Cdk2 protects it from polyubiquitination by APC, leading to the stabilization of Cdc6. This stabilization is critical when cells enter the cell cycle from a quiescent state. Thus, for both the major inhibitors of rereplication, geminin and Cdk, the exact effect on pre-RC formation depends on the stage of the cell cycle.

2.5 Restricting DNA Replication to “Once” per Cell Cycle

The key mechanism employed to ensure that chromosomes are replicated once and only once per cell cycle is separation of pre-RC formation and replication initiation into mutually exclusive phases in the cell cycle (G1 and S, respectively). MCM2-7 remains inactive in G1-phase, and once DNA replication is initiated following MCM2-7 activation, no additional MCM2-7 complexes are loaded onto origins. The lack of activity of MCM2-7 in G1-phase can be explained by the fact that activation requires Cdk activity, which is low in G1-phase. At least three mechanisms might contribute to inhibition of pre-RC assembly after the onset of S-phase: (1) prevention of pre-RC formation by Cdk, (2) prevention of pre-RC reformation by geminin and (3) replication dependent inactivation of origin (Figure 7). Cdk-dependent suppression of pre-RC formation is a major (and perhaps only) mechanism for preventing rereplication in yeast. Even in metazoans, phosphorylation of components of pre-RCs by CdkS has a negative effect on pre-RC formation (Figure 8). For example, *Xenopus* MCM4 loses its chromatin loading ability after phosphorylation by Cdk. Cdk also facilitates nuclear export of excess Cdc6 protein in human cells and regulates cell cycle progression by phosphorylating destabilizing Cdk inhibitor, p27Kipi via E3 ubiquitin ligase Skp2. Cdk-mediated phosphorylation of Cdt1, a component of pre-RC involved in loading MCM2-7, also triggers its ubiquitination by SCF (Skp2) and degradation by the proteosome. Human Orc1, the ATPase subunit of ORC, is also ubiquitinated by SCF (Skp2) and degraded in S-phase. Therefore, at least two pre-RC components are destabilized by the combined action of Cdk2 and SCF (Skp2).
2.6 Cell Cycle Checkpoints

The genomes of eukaryotic cells are under continuous assault by environmental agents (e.g., UV light and reactive chemicals) as well as the byproducts of normal intracellular metabolism (e.g., reactive oxygen intermediates and inaccurately replicated DNA).

![Diagram showing three possible mechanisms for the inhibition of the second-round pre-RC formation.](image)

**Fig. 8. Three possible mechanisms for the inhibition of the second-round pre-RC formation:** Rereplication is prevented by inhibition of pre-RC formation by three mechanisms: (A) An inhibitory complex between geminin and Cdt1; (B) Cdt1 degradation in S phase by Skp2 dependent and independent pathways; (C) High activity of cyclin dependent kinase (CDK) in G2 and M phases inactivates (or destabilizes) pre-RC components Cdc6, Cdt1, MCM2-7, and/or ORC.

Whatever may be the trigger, genetic damage threatens cell survival, and, in metazoans, leads to organ failure, immunodeficiency, cancer, and other pathologic sequelae. To ensure that cells pass accurate copies of their genomes on
to the next generation, evolution has overlaid the core cell-cycle machinery with a series of surveillance pathways termed cell-cycle checkpoints. The overall function of these checkpoints is to detect damaged or abnormally structured DNA, and to coordinate cell-cycle progression with DNA repair. Typically, cell-cycle checkpoint activation slows or arrests cell-cycle progression, thereby allowing time for appropriate repair mechanisms to correct genetic lesions before they are passed on to the next generation of daughter cells (Figure 9). In certain cell types, such as thymocytes, checkpoint proteins link DNA strand breaks to apoptotic cell death via induction of p53. Hence, loss of either of two biochemically connected checkpoint kinases, ATM or Chk2, paradoxically increases the resistance of immature (CD4+ CD8+) T cells to ionizing radiation (IR)-induced apoptosis (Xu and Baltimore, 1996). In a broader context, cell-cycle checkpoints can be envisioned as signal transduction pathways that link the pace of key cell-cycle phase transitions to the timely and accurate completion of prior, contingent events. It is important to recognize that checkpoint surveillance functions are not confined solely to the happenings within the nucleus–extranuclear parameters, but the availability of growth factor and cell mass accumulation, also govern the pace of the cell cycle (Stocker and Hafen, 2000). These checkpoints, as their most proximal signaling elements, sensor proteins that scan chromatin for partially replicated DNA, DNA strand breaks, or other abnormalities, and translate these DNA-derived stimuli into biochemical signals that modulate the functions of specific downstream target proteins. Despite the recent explosion of information regarding the molecular components of cell-cycle checkpoints in eukaryotic cells, the identities of the DNA damage sensors and the mechanism of their initiation and termination is poorly understood. Members of the Rad group of checkpoint proteins, which include Rad17, Rad1, Rad9, Rad26, and Hus1 (Nomenclature based on the *Schizosaccharomyces pombe* gene products) are the prime suspects in the lineup of candidate DNA damage sensors (Green *et al.*, 2000). Three of these Rad proteins, Rad1, Rad9, and Hus1, exhibit structural similarity to PCNA and accumulating evidence supports the idea that this similarity may extend to function as well (Thelen *et al.*, 1999; Burtelow *et al.*, 2000). During DNA replication, Rad1, Rad9, and Hus1 are also found as a heterotrimeric complex in intact cells similar to PCNA that encircles DNA at or near sites of damage by
forming a checkpoint sliding clamp (CSC) (Green et al., 2000). The analogy between PCNA and the Rad1–Rad9–Hus1 complex extends even further. The loading of the PCNA clamp onto DNA is controlled by the RFC. Interestingly, yet another member of the Rad family, Rad17, bears homology to the RFC subunits and, in fact, associates with RFC subunits to form a putative checkpoint clamp loading complex (CLC) that governs the interaction of the Rad1–Rad9–Hus1 CSC with damaged DNA (Green et al., 2000). Although this model is fascinating, rigorous biochemical evaluations of the interplay between the CLC and CSC complexes, and their interactions with damaged chromatin, are needed before the model can be universally acceptable.

2.7 Phosphoinositide Kinase-Related

Cell-cycle checkpoint kinases belong largely, if not entirely, to the serine–threonine kinase family, and the proteins they target for modification range from more downstream members of the checkpoint pathway itself (e.g., additional protein kinases or noncatalytic scaffolding proteins) to distal elements that mediate cell-cycle arrest and DNA repair responses (e.g., the Cdc25C phosphatase or type 2A histones) (Durocher and Jackson, 2001). During the very early stages of checkpoint activation, DNA damage sensors relay information, via a still elusive mechanism, to members of a recently defined family of phosphoinositide 3-kinase related kinases (PIKKs) (Tibbetts et al., 2000). In mammalian cells, two PIKK family members, ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad 3-related), play critical roles in early signal transmission through cell cycle checkpoints (Figure 9). Homologs of ATM and ATR are present in all eukaryotic cell types examined to date, including budding and fission yeast. This review focuses on the biochemistry and function of the mammalian checkpoint kinases, ATM and ATR, with only brief references to the precedent literature from yeast and other model systems.
In eukaryotes, PIKKs initiate cellular stress responses when genome integrity, mRNA translation, or nutrient availability is compromised. Central to the DNA damage response are the ATM and ATR kinases that modulate cell cycle progression, DNA repair, and even, apoptosis. Cells from ataxia-telangiectasia (AT) patients typically lack detectable ATM protein, contain abnormalities in telomere morphology and have abnormal responses to ionizing irradiation (IR), including increased cell death, increased chromosomal breakage and cell-cycle checkpoint defects. However, AT cells have normal responses to ultraviolet radiation (UV), alkylating agents, or inhibitors of DNA replication. Doublestrand breaks (DSBs) arise during spontaneous or local metabolic processes, following exposure to ionizing radiation (IR) or radiomimetic chemicals such as neocarzinostatin (NCS), through the interaction of a
replication fork with a single-strand break in the template, and in a programmed manner during meiosis and immunoglobulin gene rearrangement. Thus, the AT phenotype is consistent with defects in DSB responses and ATM-dependent phosphorylation events are seen following IR in all phases of the cell cycle including G0 (Bakkenist and Kastan, 2003).

2.7.1 Ataxia Telangiectasia Mutated and Rad3 Related protein (ATR)

ATR is also essential for cell and organism survival implying that PIKKs are not only surveyors of occasional damage, but are firmly integrated components of cellular physiology. ATR is required for the appropriate responses to numerous chemical and physical DNA-damaging agents. For example, the expression of a dominant inhibitory kinase-inactive form of ATR sensitizes mammalian cells to IR, UV, hydroxyurea (HU), and the base-damaging agent, methylmethanesulfone (MMS) (Cliby et al., 1998). Since many DNA damaging agents can induce an ATR-dependent response, a central question in checkpoint signaling is whether there is a sensor for each type of damage or whether all damage is converted to a common intermediate that is detected by a single sensor. Given that the DNA replication checkpoint is ATR-dependent and the initiation of DNA replication is required for ATR activation in a Xenopus cell free system, it has been proposed that ATR activity is initiated following the disruption of DNA replication. A founder mutation that affects ATR splicing resulting in reduced but residual levels of normal ATR transcript and ATR protein has been identified in Seckel syndrome (O’Driscoll et al., 2003). Seckel syndrome is characterized by intrauterine growth retardation, dwarfism, microcephaly, and mental retardation. Cells from these patients show enhanced sensitivity to UV and reduced or absent phosphorylation of ATR substrates following DNA damage, not only in S-phase, but also in G0- and G1-phase cells. This suggested that ATR activity is not always dependent on DNA replication, rather it requires DNA synthesis into a region of DNA damage to form an abnormal structure generally, but not exclusively, associated with DNA replication.
2.7.2 hSMG-1:

A recently identified PIKK, hSMG-1, has roles in both the elimination of RNA species containing premature termination codons through nonsense-mediated mRNA decay (NMD) and in the maintenance of genome integrity (Yamashita et al., 2001; Brumbaugh et al., 2004). NMD is essential to eliminate mRNA species that could produce truncated proteins, which may threaten survival by acting as dominant interfering over wild-type proteins. The hSMG-1-deficient cells are also sensitive to IR and exhibit a defective G_1-checkpoint response following both IR and UV (Brumbaugh et al., 2004).

Thus, ATM, ATR, and hSMG-1 all mediate DNA damage response pathways that operate in parallel but that are presumably initiated by different signals. However, these pathways are also interdependent at some level since constitutive hChk2 and histone H2AX phosphorylation are seen when hSMG-1 is suppressed even in the absence of extrinsic genotoxic agents suggesting that an ATM or ATR-dependent DNA damage response can be initiated in the absence of hSMG-1. Other PIKKs include the DNA-dependent protein kinase (DNA-PK), which is required for the repair of DSBs by non-homologous end-joining repair (NHEJ), and the target of rapamycin (mTOR), a kinase inhibited by the antifungal and immunosuppressive agent rapamycin. mTOR plays no identifiable role in genome surveillance, but coordinates G_1 phase progression with the supply of nutrients and growth factors. Stringent regulation of the kinase activities in PIKKs controls the initiation of multifaceted responses to diverse stresses. In addition to the sequence homology in the catalytic domains, the PIKK family members exhibit a similar overall structural organization (Figure 10). When compared with other kinases (protein or lipid), the PIKKs stand out immediately as very large polypeptides, with molecular masses ranging from ~300 kDa to more than 500 kDa. The catalytic domains (~300 amino acids) of the PIKK family members are located near their carboxyl termini, and are flanked by two loosely conserved domains termed FAT (FRAP/ATM/TRRAP) and FATC (the “C” indicates carboxy-terminal) (Bosotti et al., 2000). Although the FAT/FATC domains contain no identifiable catalytic sequences, the fact that these domains are always expressed in pairs has raised the still untested hypothesis that they interact in an intramolecular fashion, and thereby regulate
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the conformation of the interposed kinase domain (Bosotti et al., 2000). Despite the sequence similarity to phosphoinositide kinases, the catalytic domains of the PIKKs appear to transfer phosphate exclusively to protein rather than lipid substrates. The current members of the PIKK family can be grouped into six subfamilies on the basis of both sequence homology and function (Durocher and Jackson, 2001) (Table 1). The mammalian members of five of the six subfamilies are known to phosphorylate protein substrates on serine or threonine residues. In mammalian cells, ATM and ATR are thought to share responsibilities as the apical protein kinases in all of known cell cycle checkpoints, with the possible exception of the mitotic spindle checkpoint, which is activated by treatment with nocodazole, an inhibitor of microtubule polymerization.

Table 1. Organization of PIKK subfamilies in human and yeast cells

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Homo sapiens</th>
<th>S. cerevisiae</th>
<th>S. pombe</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>ATM</td>
<td>TEL1</td>
<td>tel1</td>
</tr>
<tr>
<td>ATR</td>
<td>ATR</td>
<td>MEC1</td>
<td>rad3+</td>
</tr>
<tr>
<td>TOR</td>
<td>mTOR/FRAP</td>
<td>TOR1</td>
<td>tor1+</td>
</tr>
<tr>
<td>SMG-1</td>
<td>hSMG1/ATX</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DNA–PK</td>
<td>DNA–PKcs</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>TRRAP</td>
<td>TRRAP</td>
<td>TRA1</td>
<td>tra1+</td>
</tr>
</tbody>
</table>

The organization shown is based on overall deduced amino acid sequence homology. Dashed lines indicate the absence of homologs in yeast. Sequence comparisons among the different subfamilies suggest that the members of the ATM, ATR, SMG-1, and TOR subfamilies may have diverged from a common ancestor.
Fig 10. Schematic representation of ATM and ATR structures: Known structural domains are shown for each protein, with numbers above ATR diagram indicating percent identity/similarity of primary amino acid sequence of ATR compared with that of ATM. Numbers on right of each diagram provide total number of amino acids for each polypeptide based on the predicted open reading frame. FAT and FATC © domains are aligned for ATM and ATR (see text for details on FAT domains). No significant homology was detected between the predicted FATC domains of these two proteins. The PI 3-kinase related catalytic domain (PI3Kc) is also shown for ATM (residues 2715–3011) and ATR (residues 2324–2627). The amino-terminal crosshatched box indicates a functionally undefined region of homology between ATM (residues 1493–1773) and ATR (residues 1191–1463).

2.8 Biochemistry of the PIKK Proteins

The PIKKs are large molecules with homology in the catalytic domain to phosphatidylinositol 3-kinases (PI3K), but which phosphorylate proteins rather than lipids. The PI3Ks are dual specificity enzymes with a lipid kinase activity that phosphorylates phosphoinositides at the 3-hydroxyl and protein kinase activity. The class I PI3Ks consist of either a p110α, p110β or p110γ catalytic subunit associated with a p85 adaptor protein that is essential for interactions with receptor tyrosine kinases and Ras.
The class1B PI3K consists of a PI3Kalpha catalytic subunit that is activated by heterotrimeric G protein alpha subunits and Ras, and associates with a p101 adaptor protein that is essential for full responsiveness to G protein alpha subunits. The structure of porcine PI3Kα revealed a modular organization around a helical domain backbone with the Ras binding domain adjacent to the catalytic domain where it could drive allosteric activation of the enzyme affecting substrate or cofactor binding (Abraham, 2001). The kinase domain of PI3Kα is made up of a smaller N-terminal lobe and larger C-terminal lobe with a fold similar to that of the protein kinases. The C-terminal lobe contains a segment analogous to the activation loop in the protein kinases that is essential for substrate specificity and could be controlled by allosteric activation. The helical domain has a fold similar to the HEAT sequence motif that may be involved in protein-protein interactions. HEAT motif has also been described in the TOR kinases.

The first PIKK family member to be characterized as a protein kinase was DNA-PK (Gottlieb and Jackson, 1993). Like most protein kinases, DNA-PK displayed Mg2+ dependent serine-threonine kinase activity in the presence of ATP and substrate (e.g., the transcription factor, SP1) (Gottlieb and Jackson, 1993; Hartley et al., 1995). Consistent with its role in signaling during DNA DSB repair, the in vitro kinase activity of DNA-PK is optimal when both enzyme and substrate are bound to the same DNA fragment (Gottlieb and Jackson, 1993). In addition, the preferred target sequence for phosphorylation by DNA-PK was serine or threonine followed by glutamine at the +1 position. Hence, DNA-PK is commonly identified as an “S/T-Q-directed kinase. The protein kinase activities of ATM and ATR, as measured in immune complex kinase assays, also display unusually strong dependencies on the presence of millimolar concentrations of Mn2+ in the kinase assay buffer. Under test-tube conditions, it appears that the presence of the Mn2+/ATP complex is virtually essential for the phosphorylation of various substrates by ATM and ATR (Kim et al., 1999).

Immune complex kinase assays quickly revealed that, like DNA-PK, ATM and ATR displayed S/T-Q-directed kinase activities under in vitro conditions, and that a physiologically relevant substrate for both protein kinases was p53 (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). The
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major \textit{in vitro} phosphorylation site for ATM and ATR was Ser 15, which resides in the sequence context, LSQE (phosphorylation site is underlined). Kastan and coworkers generated a panel of glutathione S-transferase (GST)–p53 fusion proteins in which the residues surrounding the ATM/ATR target site, Ser 15, were systematically varied (Kim \textit{et al.}, 1999). The general conclusion from these experiments was that hydrophobic or acidic residues surrounding the targeted Ser–Gln motif favored phosphorylation of the Ser residue by immunopurified ATM, whereas positively charged amino acids were inhibitory. Furthermore, ATM exhibited a strong preference for Ser over Thr as the phosho-acceptor site, at least in the context of the p53 amino-terminal sequence. A second group of investigators used an interactive peptide library screening approach to define an optimal sequence context for phosphorylation by ATM (O’Neill \textit{et al.}, 2000). These efforts identified a consensus phosphorylation site [(M/F)-(Q/P)-L-S-Q-(E/Q)] that was in reasonable agreement with that defined by Kastan and coworkers (Kim \textit{et al.}, 1999). Remarkably, the relatively unbiased peptide selection strategy zeroed in on a core L-S-Q-E target sequence, which was identical to that surrounding the Ser 15 site in p53. The available evidence suggests that the consensus sequence for phosphorylation by ATR overlaps extensively with that defined for ATM (Kim \textit{et al.}, 1999). The identification of favorable sequence contexts for phosphorylation by ATM/ATR prompted \textit{in silico} searches for candidate physiological substrates for these protein kinases (Kim \textit{et al.}, 1999). These database mining efforts yielded a rich harvest of checkpoint/DNA repair proteins, including p53, hRad17, hChk1, the Nijmegen breakage syndrome protein (NBS1, also termed p95 or nibrin), BRCA1 and BRCA2. Many of these early candidates have now been established as substrates for either ATM only or both ATM and ATR.

2.8.1 Activation of the ATM Protein Kinase

Exposure of cells to IR rapidly triggers ATM kinase activity \textit{in vivo} and ATM is required for the initial phosphorylation of several key substrates required for cell cycle arrest (Kastan and Lim, 2000). These include p53, Mdm2, and Chk2 in the G1 checkpoint; Nbs1, BRCA1, FancD2, and SMC1 in the transient IR-induced S phase arrest; and BRCA1 and hRad17 in the G2/M checkpoint.
Many of these phosphorylation events are detectable in irradiated AT cells, but they are delayed and decreased, indicating that additional PIKK kinases respond to IR. Nevertheless, the phenotype of AT cells shows that the ATM protein, and presumably the activation of its kinase activity, are required for optimal cellular responses to DSBs. Mutations in the genes encoding the ATM substrates p53, Chk2, Nbs1, BRCA1, and FancD2 are also found in families predisposed to cancer. An IR-induced ATM autophosphorylation at serine-1981 has been identified, but this modification has no direct effect on ATM kinase activity in vitro (Bakkenist and Kastan, 2003). However, antisera against ATM phosphoserine 1981 allowed a detailed characterization of the process of ATM activation in cells and led to the description of a novel mechanism for ATM activation. In this model, ATM is held inactive in undamaged cells as a dimer or higher order multimer, with the kinase domain bound to a region surrounding serine1981 that is contained within the FAT domain (Figure 11). Cellular irradiation induces rapid intermolecular autophosphorylation of serine-1981 and this phosphorylation event results in dimer dissociation and initiation of cellular ATM kinase activity. In this model, the access of substrates to the catalytic domain of ATM is impeded by the intermolecular association. This regulatory model is similar to pseudosubstrate inhibition, with the main distinctions being that the pseudosubstrate is a domain of this protein itself (albeit in trans) and that the partner is not a mimic, but actually becomes a substrate in order to release the inhibition. In support of this model, hydrodynamic analysis reveals that in undamaged cells ATM exists in equilibrium among various oligomerization states, with the dimer being the predominant species (Unsal-Kacmaz and Sancar, 2004). ATM activation, as indicated by autophosphorylation of serine-1981, is detectable as quickly as cells can be collected following an insult, and is already maximal in cells within minutes after exposure to an IR dose as low as 0.5 Gy (Bakkenist and Kastan, 2003). Over 50% of ATM protein in primary cells becomes phosphorylated within 5 min after exposure to 0.5 Gy IR, a dose that would be expected to induce only about 18 DSBs in cellular DNA. While DSBs initiate a signaling pathway that results in ATM activation, the kinetics and quantitation of ATM autophosphorylation on serine-1981 in vivo do not appear to be consistent with a mechanism in which all of the ATM molecules that are phosphorylated have made direct contact with a structure at the DSB. Thus,
initial ATM activation appears to occur at a distance from the DSB. This could result if ATM autophosphorylation is initiated by changes in higher order chromatin structures associated with relaxation of DNA winding caused by breaks or if protein sensors bridge the nucleoplasm between inactive ATM and DSBs. Once activated, phosphorylated ATM appears to move to foci, presumed sites of DNA breakage, where it can phosphorylate certain substrates (Bakkenist and Kastan, 2003; Kitagawa et al., 2004). The phosphorylation of Chk2 and Nbs1 by ATM has been examined in live human cells in which DSBs and single-strand DNA (ssDNA) breaks were introduced into small, restricted subnuclear areas (Lukas et al., 2003). Nbs1 is rapidly recruited to the chromatin damage where it is phosphorylated by ATM. Phosphorylated Nbs1 subsequently undergoes a dynamic exchange but remains in the close vicinity of the damage. In contrast, Chk2 that has been phosphorylated by ATM continues to move rapidly throughout the entire nucleus, irrespective of the region of DNA damage and including DSB free areas thereby disseminating the response.

Fig 11. Model of ATM activation and substrate phosphorylation
Since a chromatin immobilized form of Chk2 that was fused to histone (H2B) was phosphorylated exclusively at the sites of damage, ATM appears to only phosphorylate Chk2 at the sites of damage.

2.8.2 Activation of the ATR Protein Kinase

In contrast to ATM and hSMG-1, ATR isolated from damaged cells does not have increased specific activity in kinase assays suggesting that the protein is not stably modified following DNA damage. In human cells, ATR exists in a stable complex with a potential regulatory partner ATRIP (Cortez et al., 2001). Hydrodynamic analysis suggests that cellular ATR exists as an ATR-ATRIP heterodimer in undamaged cells and that this interaction is not changed following UV irradiation (Unsal-Kacmaz and Sancar, 2004). Mec1 and Rad3, the homologous PIKKs in *S. cerevisiae* and *S. pombe* respectively, form similar complexes with Ddc2 (also called Lcd1) and Rad26. Loss of ATRIP, DDC2 or Rad26 abolishes the DNA damage response to the same extent as loss of ATR, Mec1 or Rad3 (Osborn et al., 2002). The phosphorylation of RPA by ATR-ATRIP *in vitro* is strongly stimulated by ssDNA, perhaps due to conformational changes induced in RPA upon ssDNA binding, but an increase in the specific activity of isolated ATR-ATRIP has not been detected (Unsal-Kacmaz and Sancar, 2004). ATR-ATRIP associates with chromatin after the initiation of DNA replication and this association is increased in the presence of aphidicolin, an inhibitor of DNA polymerase delta (Hekmat-Nejad et al., 2000). The association of ATR-ATRIP and chromatin is inhibited by treatment with actinomycin D, an inhibitor of RNA primase. Chromatin is only able to initiate a DNA replication checkpoint after DNA polymerase alpha has been loaded onto unwound DNA and an RNA primer has been synthesized by primase (Michael et al., 2000). ATR-ATRIP accumulates at the sites arrested replication forks and DNA damage through an interaction between ATRIP and RPA-coated ssDNA (Zou and Elledge, 2003). Hence, the ATR-ATRIP heterodimer may have a principal function in the recognition of a region of DNA containing a damage site, perhaps as the sensor. However, the localization of ATR-ATRIP to sites of DNA damage is not sufficient to activate the checkpoint response. The Rad9-Rad1-Hus1 (RHR) complex, Rad17-Rfc2-Rfc3-Rfc4-Rfc5 (RSR) complex, and Claspin are also
required (Osborn et al., 2002; Ellison and Stillman, 2003) (Figure 12). The RHR complex is related in structure to PCNA. PCNA is loaded onto chromatin by RFC (Rfc1-Rfc2-Rfc3-Rfc4-Rfc5 sub-complex) and circles DNA creating a sliding clamp for DNA polymerase delta during DNA replication. Similarly RSR catalyzes the ATP-, RPA- and DNA-dependent loading of the RHR complex onto DNA where it functions as a sliding clamp (Ellison and Stillman, 2003). The loading of RHR onto DNA by RSR requires a 5’ recessed primer-DNA template and RPA. Thus, the checkpoint clamp and clamp loader complexes do not appear to function in checkpoints as initial sensors of damage. Rather these complexes bind DNA after lesions have been detected and processed into structures suitable for clamp loading, perhaps by stabilizing stalled replication forks and reactivating DNA replication. Claspin binds chromatin independently of ATR-ATRIP and the RSR and RHR complexes. Claspin is phosphorylated in an ATR-dependent manner following replication stress or IR- or UV-irradiation, and phosphorylated Claspin interacts with BRCA1 facilitating the ATR-dependent phosphorylation of BRCA1. The Claspin-BRCA1 complex is required for the ATR-ATRIP-dependent phosphorylation of Chkl- and ATR-dependent checkpoint activation in S-phase and at the G2/M transition. Thus, in the case of ATR, there is no evidence that a conformational change in the ATR kinase domain affects either its catalytic activity or the access of substrates to the ATR active site. However, ATR-dependent checkpoint activation requires the independent loading of DNA polymerase, ATR-ATRIP, the RHR complex, and Claspin onto unwound DNA. Perhaps recruitment of ATR-ATRIP and its substrates to the site of damage within this multiprotein replication associated complex is the principal mechanism of control of cellular ATR kinase activity.
2.9 Cell-cycle checkpoint functions of ATM and ATR

Investigations of the checkpoint signaling functions of ATM and ATR began with the identification of substrates and proceeded through analyses of the functional consequences of substrate phosphorylation. Before the cloning of the \textit{ATM} gene, the dedicated efforts of many laboratories documented that ATM-deficient cells displayed significant defects in the G1-, S- and G2- checkpoints. These observations prompted intensive searches for ATM substrates among the numerous proteins that function in each of these checkpoints. Most of these ATM targets have also been tested, in an empirical fashion, as substrates for ATR. Although the empirical approach has been rewarding, the inevitable outcome has been that the list of documented ATR substrates overlaps largely, if not entirely, with that linked to ATM. Despite the seeming overlap, the emerging picture suggests that the checkpoint signaling functions of ATM and ATR are far from redundant—a conclusion that becomes apparent as apparent from many independent investigations on G1, S, and G2 checkpoints.

2.10 The G1- checkpoint

At the heart of the G1-checkpoint lies the series of events leading to the accumulation of the tumor suppressor protein, p53. Although p53 exerts a pervasive influence on checkpoint functions during the mammalian cell cycle, the
G1-checkpoint represents the only case in which loss of p53 leads to total checkpoint abrogation (Ko and Prives, 1996; Giaccia and Kastan, 1998; North and Hainaut, 2000). DNA damage induced by most, if not all, forms of genotoxic stress induces a rapid increase in the level of p53, a response that is mediated primarily through an increase in protein stability. In addition to triggering the accumulation of p53, genotoxic stress induces posttranslational modifications that regulate the transcriptional activating functions of this protein. With respect to the G1-checkpoint, a key target for transcriptional activation by p53 is the cyclin-dependent kinase inhibitor, p21Wafl. The p53-dependent increase in p21Wafl expression suppresses cyclin E and cyclin A-associated Cdk2 activities, and thereby prevents G1-to S-phase progression. In addition to p21Wafl, the activated form of p53 stimulates the expression of a large panel of genes, which, depending on the cellular context and type of initiating insult, may modulate intracellular redox status, or induce the host cell to undergo apoptosis. An intricate web of protein kinases and phosphatases, as well as histone acetylases and ubiquitin-conjugating enzymes, regulates the accumulation and transcriptional-activating functions of p53 (Ko and Prives, 1996; Giaccia and Kastan, 1998). A link between ATM and p53 was predicted on the basis of earlier studies, which demonstrated that A-T cells exhibited a delayed and reduced level of p53 protein induction following exposure to IR (Kastan, et al. 1992). The subsequent cloning of ATM allowed several groups to test the straight-forward hypothesis that ATM was a direct effector of p53 phosphorylation in IR-damaged cells (Banin et al., 1998; Canman et al., 1998). These studies pinpointed a single serine residue (Ser 15) in the amino-terminal region of p53 as a phosphorylation site for the ATM kinase in vitro. Moreover, phosphorylation of Ser 15 was rapidly induced in IR-treated cells, and this response was ATM dependent, as IR-induced Ser 15 phosphorylation was significantly, but not completely, suppressed in A-T cells (Siliciano et al., 1997; Banin et al., 1998; Canman et al., 1998). The residual phosphorylation at Ser 15 in A-T cells hinted that ATM was not the only IR-regulated Ser 15 kinase, and this suspicion was confirmed with the observation that UV-induced Ser 15 phosphorylation was virtually unimpaired in A-T cells. Subsequent studies showed that ATR was also capable of phosphorylating p53 at Ser 15 in immune complex kinase assays (Hall-Jackson et al., 1999; Lakin et al., 1999; Tibbetts et al., 1999). In cells rendered functionally
deficient for ATR by overexpression of a kinase-inactive ATRKI mutant (Cliby et al., 1998), the early phase (0–2 h) of IR-induced p53 phosphorylation was not impaired, which is consistent with the idea that ATM serves as the major Ser 15 kinase during the acute response to IR (Tibbetts et al., 1999). However, the ATRKI overexpressing cells did show a significant defect in their ability to maintain phosphorylation of Ser 15 at later times and suggested that the maintenance phase of p53 phosphorylation was more highly dependent on ATR. On the other hand, treatment of human fibroblasts with UV light triggered a Ser 15 phosphorylation response that was largely independent of ATM expression, and was strongly reduced by overexpression of ATRKI (Tibbetts et al., 1999).

This pattern of dual regulation of substrate phosphorylation by ATM and ATR in cells exposed to different forms of genotoxic stress has become a recurrent theme in the checkpoint-signaling field. Of the PIKK family members, ATM represents the primary responder to IR or radiomimetic agent-induced DNA damage. In the absence of ATM, or in normal cells that incur a high level of IR-induced DNA damage, ATR serves mainly as a backup kinase for ATM. On the other hand, ATR takes on the front-line signaling responsibilities when cells are challenged with other forms of genotoxic stress, such as UV light exposure or treatment with agents that interfere with DNA replication (aphidicolin, hydroxyurea). Deciphering the functional consequences of p53 phosphorylation by ATM and ATR proved far more challenging than identification of the phosphorylation. The location of Ser 15 at the p53 amino terminus suggested that modification of this residue might trigger the dissociation of p53 from MDM2, a protein that targets p53 for ubiquitination, nuclear export, and proteosomal degradation (Freedman et al., 1999; Juven-Gershon and Oren, 1999). Therefore, if the model were correct, ATM/ATR-dependent phosphorylation of Ser 15 would free p53 from its destabilizing binding partner, thereby favoring p53 accumulation. It turns out, however, that Ser 15 phosphorylation is not sufficient to disrupt the p53–MDM2 interaction; rather, this modification stimulates the transactivating function of p53 by enhancing the binding of this protein to the transcriptional coactivator, p300 (Dumaz and Meek, 1999). Studies have reinforced the notion that ATM and ATR enhance p53 accumulation by triggering the release of this protein from MDM2. One mechanism for p53 stabilization involves an intermediate protein kinase, hChk2.
(also named hCds1) and hChk1, which relays ATM and ATR dependent signals to p53 and many other downstream target proteins in IR or replication challenged cells. ATM activates hChk2 by phosphorylating an amino terminal threonine residue (Thr 68), while ATR similarly activates Chk1 by phosphorylating at serine 345 (Melchionna et al., 2000). hChk2 and hChk1, in turn, phosphorylate yet another amino-terminal Ser residue (Ser 20) in p53 (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). Unlike the Ser 15 modification mentioned above, phosphorylation at Ser 20 interferes directly with the binding of p53 to MDM2, thereby favors p53 accumulation in response to IR-induced DNA damage. The physiological relevance of hChk2 in the regulation of p53 is supported by the finding that loss of function mutations in hChk2 can give rise to a variant form of Li-Fraumeni syndrome, a heritable, cancer-prone disorder typically associated with germ-line mutations in p53 (Bell et al., 1999).

2.11 The S-phase checkpoint

During DNA replication, mammalian cells must be on high alert for DNA structural abnormalities, such as strand breaks or base modifications that interfere with the accurate copying of the genome. In addition to the usual array of environmental insults, the process of DNA replication itself adds intrinsic risks, such as base mis-incorporation errors and stalled replication forks, which demand an immediate response from the checkpoint machinery if genome integrity is to be preserved. Fortunately, DNA damage detected during S-phase is apt to be repaired precisely via homologous recombination mechanisms involving sister chromatids (Johnson and Jasin, 2000).
Fig 13. Roles of ATM and ATR in signaling through S-phase checkpoints:
The ATM-dependent pathway shown in the left panel is initiated by the presence of a DNA DSB in an S-phase cell. The checkpoint response leads to the proteosome-mediated degradation of Cdc25A, and, in turn, the failure to maintain activation of cyclin-Cdk2 complexes and a resultant inhibition of DNA synthesis. Disruption of this pathway yields the radio-resistant DNA synthesis (RDS) phenotype. In the right panel, the checkpoint pathway is triggered by intrinsic events or environmental insults that impair replication fork progression during S phase. The pathway is governed primarily by ATR, and may use members of the Rad family of checkpoint proteins as damage sensors and as scaffolds for the assembly of checkpoint signaling complexes. The operation of this pathway prevents mitotic catastrophe that results from incomplete or inaccurate DNA replication, and orchestrates high-fidelity DNA repair through homologous recombination. Complete loss of this pathway is likely incompatible with viability, even in the absence of genotoxic agent-induced DNA damage.
Indeed, studies in bacteria suggest that S-phase cells rely heavily on homologous recombination to restart stalled replication forks, even in the absence of genotoxic agents (Cox et al., 2000). Non-homologous repair mechanisms also play very prominent roles in DNA DSB repair during all phases of the cell cycle; however, these mechanisms are inherently less precise, and therefore confer an increased risk that inaccurately repaired DNA will be carried forward into M phase. Although the G2 checkpoint should, in principle, catch any cells that have exited S phase with damaged DNA, these cells may have already missed their best opportunity to perform error-free repair via homologous recombination.

Given this state of affairs, it comes as no surprise that the S-phase checkpoint is considerably more multifaceted than this blanket descriptor implies. The canonical checkpoint defect displayed by A-T cells is radio-resistant DNA synthesis (RDS) (Painter and Young, 1980). In normal cells, exposure to IR provokes a rapid but reversible decrease in DNA synthesis, which reflects decreases in the rates of both replication origin firing and DNA strand elongation (Painter and Young, 1980). In the absence of ATM, the IR-induced decrease in DNA synthesis is dampened significantly, giving rise to the RDS phenotype. Treatment of cells with Wortmannin or caffeine at drug concentrations that abrogate ATM and ATR kinase activity, also induces RDS in normal (ATM and ATR-proficient) cells (Sarkaria et al., 1998). Although loss of ATM function is causally related to RDS, the downstream effectors in this S-phase checkpoint pathway have largely eluded identification. IR exposure during S phase activates the same pathway, except that degradation of Cdc25A, a protein tyrosine phosphatase that activates cyclin A-Cdk2 complexes as cells transit from G1 to S phase. Cdc25A is phosphorylated at Ser 123 by Chk2 and this modification targets Cdc25A for ubiquitin-dependent degradation (Falck et al., 2001). The down-regulation of Cdc25A coincides with the timely activation of cyclin A-Cdk2, which is essential for the firing of early origins of replication during S phase (Donaldson and Blow, 1999; Takisawa et al., 2000). Genetic manipulations that disrupt any step in the pathway from hChk2 to cyclin A-Cdk2 also give rise to the RDS phenotype. Remarkably, mutant hChk2 alleles associated with a variant form of Li-Fraumeni syndrome (Bell et al., 1999) fails bind and/or phosphorylate Cdc25A (Falck et al., 2001), which implies that genetic lesions in
the RDS pathway promote genome instability and cancer development. Overexpression of the ATRKI in SV40-transformed (but ATM-positive) human fibroblasts also induces RDS (Cliby et al., 1998). Considering that overexpressed ATRKI may nonspecifically cross-inhibit ATM function, these results suggest either that ATR also resides upstream of hChk2, or that ATR regulates a parallel pathway that contributes to the suppression of DNA synthesis in IR-damaged cells. By analogy to the p53 phosphorylation mechanism discussed earlier, it is possible that the down-regulation of Cdc25A by UV light or HU is mediated through an ATR-dependent pathway involving the hChk1 kinase.

A second participant in the DNA damage induced S-phase checkpoint is NBS1 (also termed nibrin), the product of the gene mutated in the human chromosomal instability disorder, Nijmegen breakage syndrome (NBS) (Carney, 1998; Petrini, 1999). The clinical features of NBS show considerable but not complete overlap with those displayed by A-T patients. Interestingly, NBS cells also display the RDS phenotype suggesting that the NBS1 protein is an upstream regulator of Cdc25A stability in IR-treated cells. The NBS1 protein is found in a complex with two other genome maintenance proteins, Mre11 and Rad50, which play important roles in the recombinational repair of DNA DSBs. Treatment of cells with IR induces the rapid formation of nuclear foci containing the NBS1–Mre11–Rad50 complex in close proximity to DNA DSB (Nelms et al., 1998). The appearance of these foci is dependent on the expression of NBS1; however, the absence of major DNA repair defects in cells from NBS patients indicates that the Mre11–Rad50 complex carries out its repair functions quite capably in NBS1-deficient cells (Petrini, 1999). The overlapping clinical phenotypes of A-T and NBS prompted speculation that the two proteins might be functionally interconnected. This prediction was validated by reports that ATM phosphorylates NBS1 on up to three serine residues (Ser 343, Ser 397, and Ser 615), and that Ser to Ala substitutions at any one of these sites generated a mutant NBS1 protein that failed to complement the checkpoint defects in NBS cells (Gatei et al., 2000; Lim et al., 2000). The exact roles of these phosphorylation events in the function of NBS1 are not fully understood.

Recent findings position ATM and ATR as critical upstream modulators of the breast cancer susceptibility protein, BRCA1. Like the NBS1 complex, BRCA1 participates in both checkpoint and repair pathways in DNA-damaged
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cells (Scully and Livingston, 2000; Scully et al., 2000). Studies of the DNA damage responses in BRCA1-deficient cells are focusing increasing attention on the role of BRCA1 in promoting high fidelity DNA repair through homologous recombination between sister chromatids (Moynahan et al., 1999; Scully et al., 2000). Several groups have documented that BRCA1 is phosphorylated in vitro on multiple sites by the ATM and ATR kinases (Cortez et al., 1999; Gatei et al., 2000; Tibbetts et al., 2000). The carboxy-terminal region of BRCA1 contains numerous S-Q motifs (total of 14) and of these potential ATM/ATR target sequences, 10 are localized in an ~300 amino acid stretch (residues1250–1550) termed the SQ cluster domain (SCD) (Cortez et al., 1999). The phosphorylation of BRCA1 in IR-damaged cells is significantly impaired in the absence of ATM, and demonstrated that at least five of the predicted S-Q target sites in the SCD were phosphorylated by ATM in vitro. Three of these sites, Ser 1387, Ser 1423, and Ser 1524, were identified as major IR-induced phosphorylation sites in intact cells (Cortez et al., 1999; Gatei et al., 2000). The earlier studies clearly hinted that at least an additional BRCA1-directed kinase was activated by DNA damaging agents, because significant BRCA1 phosphorylation was observed in cells that lacked either ATM or DNA-PK (Scully et al., 1997; Cortez et al., 1999; Tibbetts et al., 2000). ATR was the obvious suspect, and subsequent studies showed that ATR phosphorylated the carboxyl terminus of BRCA1 at six Ser/Thr residues in immune complex kinase assays (Tibbetts et al., 2000). The ATR phosphorylation sites partially overlap with those modified in vitro by ATM. Studies with phospho-Ser 1423-specific antibodies indicate that ATM and ATR share responsibilities for the modification of BRCA1 during IR-, UV- and replication inhibitor-induced genotoxic stress.

2.12 The G2- checkpoint

The final gatekeeper that blocks the entry of DNA-damaged cells into mitosis is the G2 checkpoint. Although relatively detailed view of the distal events that link this checkpoint pathway to the machinery that controls mitotic entry is known (Abraham, 2001), the contributions of ATM and ATR to the early stages of G2-checkpoint activation remain unclear. When A-T cells are exposed to IR during G2- or S-phase, any cells that reach G2- phase are
effectively arrested before they initiate mitosis. In fact, these cells show a more protracted G2-arrest than do ATM-expressing cells. On the other hand, if A-T cells are irradiated while in G2-phase, then the cells fail to arrest, and proceed on into mitosis (Beamish and Lavin, 1994). These results indicate that ATM is dispensable for activation of checkpoint-mediated G2-arrest, unless the DNA damage occurs during G2-phase itself. The ATM independent pathway that initiates G2-arrest in cells that have experienced a genotoxic insult before G2-phase has not been defined. However, virtually it is certain that ATR and Chk1 are central players in this alternative pathway of G2-checkpoint activation. Accumulating evidence supports the idea that ATR is primarily responsible for the activation of the Chk1 kinase by DNA damaging agents in vertebrate cells. Studies in the Xenopus model system identified four S/T-Q sites in Xchk1 that were phosphorylated by ATR in vitro, and whose modification in oocyte extracts is ATR-dependent (Guo et al., 2000). Moreover, mutated Xchk1 containing Ser to Ala substitutions at all four sites failed to complement the DNA replication checkpoint defect in extracts that were depleted of endogenous Xchk1. Similarly, overexpression of a kinase-inactive ATR mutant inhibited DNA damage-induced hChk1 phosphorylation in a human fibroblast cell line (Liu et al., 2000). The regulatory linkage between ATR and Chk1 strongly implicates ATR as a proximal component of the DNA damage-induced G2 checkpoint in mammalian cells. Studies in yeast have defined an elegant mechanism for the negative regulation of mitotic entry by Chk1, and the fundamental characteristics of this pathway seem to be conserved during vertebrate evolution. The current model posits that DNA damage leads to the activation of Chk1, which, in turn, phosphorylates the mitosis-promoting phosphatase, Cdc25C. Phosphorylation of Cdc25C by hChk1 creates a binding site for 14-3-3 proteins. The 14-3-3-bound Cdc25C is either catalytically inhibited or sequestered in the cytoplasm (or both) and is prohibited from dephosphorylating and activating the mitotic cyclin B-Cdc2 kinase. The damaged cells are thus, effectively blocked from entering mitosis. The critical role of Chk1 in the implementation of the G2 checkpoint is underscored by the finding that Chkl-/- embryonic stem cells display a substantial reduction in G2 arrest following IR exposure (Liu et al., 2000). In light of the ATR-Chk1 pathway outlined earlier, it is difficult to explain the fact the ATM-deficient cells fail to activate the G2 checkpoint when DNA damage
occurs during G2 phase? One possibility is that ATM also contributes to the inhibition of Cdc25C activity, particularly in IR-damaged cells, by activating Chk2 since it has been shown to phosphorylate Ser 216 of Cdc25C in vitro (Brown et al., 1999). In cells that express both ATM and ATR, ATM may activate Chk2 to reinforce the block to cyclin B-Cdc2 activation imposed by the ATR-hChk1 pathway. A defect in the maintenance of the checkpoint induced G2- arrest has been observed in Chk2--/- embryonic stem cells (Hirao et al., 2000). Hence, the ATM-Chk2 pathway may play a secondary role in G2 checkpoint activation when cells incur DNA DSB during G1- or S-phase. On the other hand, the cell cycle phase-specific defect in G2- checkpoint function observed in ATM-null cells suggests that ATM is essential for G2- checkpoint activation after cells have traversed G1- and S- phase. The failure of ATR to engage the G2 checkpoint machinery in cells that have completed DNA replication suggests that the sensor and/or effector apparatus through which ATR operates is fully functional only during S-phase.

### 2.13 Checkpoint Regulation of Replication Initiation

Cellular checkpoint mechanisms ensure the normal order of cell cycle events. Aberrant DNA replication or DNA damage in S-phase immediately leads to the activation of an intra-S-phase checkpoint. In addition, checkpoint-mediated pathways are critical for the control of rereplication. The absence of proteins like p53 or geminin in yeast ensures that some of these pathways are unique to metazoans. In addition not all of these pathways have been examined in yeast. Cdc45 accumulates on chromatin when the checkpoint pathways are abrogated by caffeine-mediated inhibition of ATM and ATR, (Costanzo et al., 2000; 2003; Falck et al., 2002), indicating that checkpoint pathways inhibit firing of origin before Cdc45 loading. Among the many proteins involved in Cdc45 loading, Cdc7 and Cdk2 are critical targets of the intra-S-phase checkpoint. The ATM-mediated checkpoint inhibits Cdk2 activity (Costanzo et al., 2000), whereas the ATR-mediated checkpoint inhibits Cdc7 activity (Costanzo et al., 2003) (Figure 14).
Fig 14. Inhibition of origin firing by checkpoint pathways: Checkpoint pathways that suppress origin firing following fork stalling or DNA damage are shown. The MRN-dependent pathway inhibits origin firing in parallel with Chkl- and Chk2-dependent pathways. The mechanism by which the ATR-dependent pathway inhibits Cdc7 is not known. The downstream target of MRN-dependent pathway for inhibition of origin firing is also unknown. The MRN complex may be required upstream from ATM, but could also be a target downstream from ATM. Other targets of regulation by ATM include Chk2 (which inhibits Cdc25A) and p53 (which activates p21) to control Cdk2. DSB; double-stranded DNA break, MRN; Mre11-Rad50-Nbs1 complex.

Cdk2 is inhibited by a phosphorylation at Tyr15, which is mediated through Cdc25A degradation (Costanzo et al., 2000; Falck et al., 2001). In contrast, the exact mechanism for Cdc7 inhibition is not known, but impaired association between Cdc7 and its regulatory subunit, Dbf4, was observed (Costanzo et al., 2003). In addition to the ATM-Chk2-Cdc25A-Cdk2 pathway, the Mre11/Rad50/Nbs1 (MRN) complex also inhibits DNA synthesis (Falck et al., 2002) (Figure 14). The MRN complex may be required upstream from ATM but could also have targets downstream from and independent of ATM. Subunits
of MCM2-7 are phosphorylated directly by ATM/ATR after DNA damage (Cortez et al., 2004; Yoo et al., 2004). The consequences of this phosphorylation are presently unknown. However, if a large fraction of MCM2-7 is phosphorylated, it might prevent firing of new origin.

2.14 Interface between checkpoint activation and DNA damage and repair

Human cancer(s) can be viewed as a disease of underlying genetic instability. Most if not all human tumors display some form of genomic instability, including subtle DNA sequence alterations, gross chromosomal rearrangements, aneuploidy and gene amplifications. These alterations have the potential to affect the function of growth-regulating genes that are associated with the malignant transformation of cells. Hence to elucidate the early events in carcinogenesis it is imperative to understand the origin of genetic alterations that are found in tumors. The basis of genomic instability is unfaithful transmission of genetic information from a mother cell to its daughters. DNA damage is a relatively common event in genomic DNA. If left unrepaired, the myriad types of DNA damage can pose a serious threat to the faithful transmission of the genetic material. Defects in DNA damage signaling and/or repair lead to failure in repair process, and genetic instability and ultimately inaccurate DNA transactions or mitotic chromosome segregation. Therefore, loss of rapid detection of DNA lesions poses a serious problem for the normal functioning of cells. Endogenous DNA damage occurs at high frequency (Rouse and Jackson, 2002) that induces several biochemical processes to enable the cell either to cope with the damage or to activate a programmed cell death response, presumably to avert potentially deleterious mutations. The DNA damage responses include: (a) activation of DNA damage checkpoint to arrest cell cycle progression and allow more time for repair, (b) removal of the damaged DNA and restoration of continuity of the DNA duplex and (c) changes in the transcription profile that may be beneficial to the cell.

Detection of DNA damage is a complex issue for two reasons: (a) an array of physically dissimilar DNA lesions needs to be recognized and (b) even a single damage needs to be repaired. Studies in budding yeast have indicated that even a single persistent DSB can be detected and can trigger a global DNA
damage response (Lee et al., 1998). UV, IR or reactive oxygen intermediates that stall replication forks (Featherstone and Jackson, 1999) and reared through homologous recombination (HR) or non-homologous end joining (NHEJ) (Abraham, 2001). Although the repair of different types of DNA lesions relies on different sets of proteins, nevertheless they trigger common signal transduction pathways, referred to as checkpoint pathways. These events point towards the critical role played by sensors and transducers of DNA damage response towards initial detection and their ability to connect the initial damage detection with repair mechanisms (Lowndes and Murguia et al., 2000). The rapidity and potency of the DNA damage response indicates that the signaling pathways are highly sensitive and have the ability to greatly amplify the initial stimulus (Rouse and Jackson, 2002). Many different proteins have been shown to get localized to sites of DNA damage called “repair foci” with some of them having a role in both detection and repair of damage. In mammalian cells, immunofluorescence analyses suggest this and includes ATR, ATRIP (Zou et al., 2002), the MRE11-RAD50-NBS1 (MRN) complex (D’amours et al., 2002), activated forms of hRad17 (Zou et al., 2002) and hCHK2 (Melchionna et al., 2000), BRCA1 (Scully et al., 1997) etc. Although these foci are used as a read out for DNA damage response in a range of contexts, the exact composition and their biological importance remain unclear. IR induced damage appears to cause transient immobilization of HR proteins RAD51, RAD52, RAD54 at the site of damage (Essers et al., 2002). Similarly for nucleotide excision repair (NER) endonuclease complex, ERCC1-XPF was found to be recruited and immobilized along with factors of sensing components on the templates (Houtsmuller et al., 1999). Nuclear focus formation by the human MRN complex in response to IR has revealed a role for functional ATM in the formation of some the foci arising after DSBs (Mirzoeva and Petrini, 2001). Replication associated DSBs arise primarily because of processing of DNA structures at damaged forks by the enzymes involved in HR, leading to the formation of Holliday junction (HJ) (McGlynn et al, 2001). HR proteins in yeast and other eukaryotes also play a role in maintaining replication fidelity and preventing the accumulation of damaged structures (Muris et al., 1996) when challenged with replicational stresses in form of HU (Sogo et al., 2002). It is hypothesized that in mammalian genome if limited DNA processing is necessary during S phase to restart the stalled
replication fork then this process is crucially controlled through ATR. Any disturbance in the ATR dependent pathways can induce and generate DSBs and allow the checkpoint proteins to bind to sites of stalled replication (Boddy et al., 2000). Phosphorylation of these HR proteins should make them functional (Bashkirov et al., 2000). Thus, checkpoint pathways work not only through their ability to halt cell cycle but also their fundamental role in DNA damage response by averting genetic instability.