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Cell Cycle Checkpoints: Preventing an Identity Crisis

Stephen J. Elledge

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells. Recent advances have revealed signal transduction pathways that transmit checkpoint signals in response to DNA damage, replication blocks, and spindle damage. Checkpoint pathways have components shared among all eukaryotes, underscoring the conservation of cell cycle regulatory machinery.

The cell cycle is a collection of highly ordered processes that result in the duplication of a cell. As cells progress through the cell cycle, they undergo several discrete transitions. A cell cycle transition is a unidirectional change of state in which a cell that was performing one set of processes shifts its activity to perform a different set of processes. A current focus of cell cycle research concerns how these transitions are coordinated to occur at a precise time and in a defined order. In principle, the ordering of cell cycle events could be accomplished by requiring the next event to physically require the completion of the previous event, much like building a house—the roof cannot go up until the walls are built. This has been referred to as a substrate-product relationship (*1*). Alternatively, dependency could be established by positive or negative regulatory circuits, and this appears to be the predominant mechanism. An example of a pathway of cell cycle events that is subject to positive and negative control is shown in Fig. 1A. A negative circuit is shown leading from b to a step in the d to e pathway. A positive circuit, shown linking events b and c, cannot be easily distinguished from a substrate-product relationship and depends upon the biochemical function of the step in question. These regulatory circuits are surveillance mechanisms that monitor the completion of critical cell cycle events and allow subsequent cell cycle transitions to occur. There are two classes of regulatory circuits, termed here intrinsic and extrinsic. Intrinsic mechanisms act in each cell cycle to order events. Extrinsic mechanisms are induced to act only when a defect is detected. Both mechanisms may use the same components to enforce cell cycle arrest. An example of how some of these circuits are integrated into a typical cell cycle is shown in Fig. 1B. These pathways are of considerable interest because their loss leads to reduced fidelity of cell cycle events such as chromosome duplication and segregation. Such alterations decrease the reproductive fitness of unicellular organisms and in multicellular organisms may lead to uncontrolled proliferation and cancer.

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Checkpoint is the name given to a particular subset of these intrinsic or extrinsic mechanisms (*1*). A checkpoint is a biochemical pathway that ensures dependence of one process upon another process that is otherwise biochemically unrelated. A null allele in a checkpoint gene results in a loss of this dependency and, thus, checkpoints are inhibitory pathways. This definition of a checkpoint is broad and can apply to many situations that occur in multicellular organisms, particularly during development. However, its most common usage is in reference to control of cell cycle transitions. The word checkpoint conjures visions of both a place (a border) and a process (examination) and this duality has led to some confusion. The word is often used in a manner suggesting that checkpoints are points in the cell cycle or are cell cycle transitions, but the usage is best restricted to refer to the biochemical pathway that ensures dependency. For example, the DNA-damage checkpoint is the mechanism that detects damaged DNA and generates a signal that arrests cells in the G1 phase of the cell cycle, slows down S phase (DNA synthesis), arrests cells in the G2 phase, and induces the transcription of repair genes. The position of arrest within the cell cycle varies depending upon the phase in which the damage is sensed. Whether the loss of a checkpoint has an immediate consequence for an organism during a normal cycle depends on the particular pathway and the inherent timing of the processes themselves. Timing and checkpoints can act as redundant controls to ensure the proper order of events. Thus, there are no constraints on whether checkpoints are essential or inducible (extrinsic).

The first indications that the cell cycle was not controlled strictly by a substrate-product relationship came from cell fusion experiments in *Physarium polycephalum* that showed that timing of mitotic entry could be influenced by the ratio of the nuclear volume to cytoplasmic volume (*2*). Similar experiments with mammalian cells showed that when cells in S and G2 phases of the cycle were fused, the G2 nucleus delayed mitotic entry until the S-phase nucleus finished DNA replication; then both nuclei synchronously entered mitosis (*3*). This was interpreted to mean that S-phase nuclei produced an inhibitor of mitosis. The first example of a dependency relationship relieved by mutation was from bacterial studies. DNA damage and certain mutations cause a block to septation resulting in filamentation of *Escherichia coli* (*4*), and mutations in the *recA*, *lexA* and *sulA*(*sfiA*) genes relieve this septation block (*5*). SulA is an inhibitor of septation induced in response to DNA damage as part of the SOS response (*6*) controlled by *recA* and the repressor *lexA*. In eukaryotes, cells from hu-

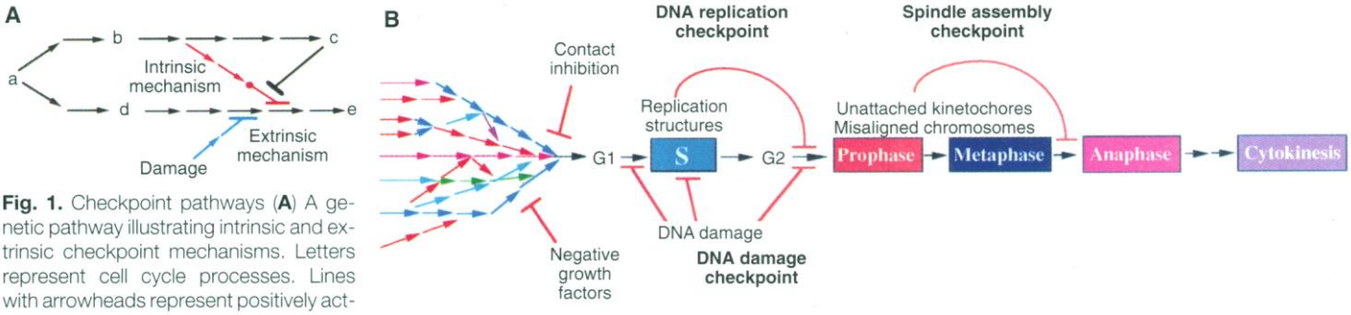

Fig. 1. Checkpoint pathways (**A**) A genetic pathway illustrating intrinsic and extrinsic checkpoint mechanisms. Letters represent cell cycle processes. Lines with arrowheads represent positively acting steps. Lines with perpendicular ends represent inhibitory steps. A dot at the beginning of a symbol indicates that once activated, it maintains its function without the need for upstream signals. The pathway shown as red symbols indicates an intrinsic checkpoint mechanism that operates to ensure that event C is completed before event E. After event B is completed, an inhibitory signal is activated that blocks completion of event E. After event C is completed, a signal is sent to turn off the inhibitory signal from B, thereby allowing completion of E. The blue symbols represent an extrinsic mechanism that is activated when defects such as DNA damage or spindle errors are detected. It is arbitrarily located on the D to E pathway but could also function by inhibiting a later step in the B to C pathway. In that case, the extrinsic pathway would utilize the intrinsic mechanism for cell cycle arrest. Mutations in any of the red or blue symbols would result in a checkpoint-effective phenotype. (**B**) Schematic representation of several cell cycle checkpoints. The colored arrows depict complex signaling pathways that operate in G1 to transmit information regarding cell proliferation. The red lines connecting particular events and cell cycle transitions represent the inhibitory signals generated by checkpoint pathways in response to those events. The points of contact of the negative growth factor and contact inhibition pathways with the cell cycle are arbitrary and meant to indicate arrest in G1.

mans with the recessive disorder ataxia telangiectasia (AT) fail to show the reduction in rate of DNA synthesis and mitotic delay in response to DNA damage characteristic of normal cells (*7*). This was interpreted to mean that these cells were defective in the ability to coordinate cell cycle transitions in response to DNA damage, an interpretation that stands today. However, it was not until studies in the yeast *Saccharomyces cerevisiae* revealed the effects of the *rad9* mutation on cell cycle progression in response to DNA damage that the significance of the earlier studies began to be fully realized for eukaryotes (*8*). The identification of checkpoints in a genetically tractable organism known for its cell cycle genetics facilitated the generalization of these concepts to other aspects of the cell cycle and provided a basis for understanding much of its higher-order regulation. Furthermore, the connection between checkpoint failure, DNA damage sensitivity, and genomic instability in AT and *rad9* mutants provided an important insight into processes contributing to cellular dysfunction in cancer (*9*).

The identity of cell cycle phases is established not only by what they are in terms of the genes that are expressed and the processes that are executed, but also by what they are not. Cells in one phase often actively inhibit the processes of other phases through checkpoints (*3*). One notion concerning the origin of these inhibitory pathways derives from ideas about how cells evolved distinct S and M phases. Primitive cells may have initially performed S and M phases simultaneously like bacteria, but then evolved into simple oscillators that alternated between DNA replication and mitosis as genomes grew more complex. As these two states became biochemically incompatible, strong selective pressure would have existed for a mechanism to inhibit the function of the previous state during a transition. If an inhibitory mechanism persists until the next change of state, it provides an inhibitory barrier that must be overcome, a checkpoint. Such molecular logic whereby a transition turns off the previous state while promoting the future state is a hallmark of cell cycle transitions (*10, 11*).

Cdks as Key Regulators of Cell Cycle Transitions and Effectors of Checkpoints

Several cell cycle transitions are dependent upon the activity of cyclin-dependent kinases (Cdks), and inhibition of these kinases is a mechanism by which some checkpoint pathways cause cell cycle arrest. These enzymes are composed of a kinase subunit, Cdk, and an activating subunit, cyclin, in a complex that is subject to many levels of regulation (Fig. 2) (*12*). There appears to be a single cell cycle Cdk in *S. cerevisiae*, Cdc28, and *Schizosaccharomyces pombe*, Cdc2, whereas mammals have several—Cdc2, Cdk2, Cdk3, Cdk4, and Cdk6—that are specialized for different transitions. Cyclins are absolutely required for kinase activity and also contribute to substrate specificity. The Cdk-cyclin complex is both positively and negatively regulated by phosphorylation. In addition, Cdks are further regulated by binding to inhibitors (CKIs) and other proteins such as Suc1 (Cks1) that might modify their specificity or accessibility to regulators (*13*). Each of the proteins that influence Cdk activity are potential interfaces for signal transduction pathways that regulate cell cycle transitions and thus are potential targets for manipulation by checkpoint pathways.

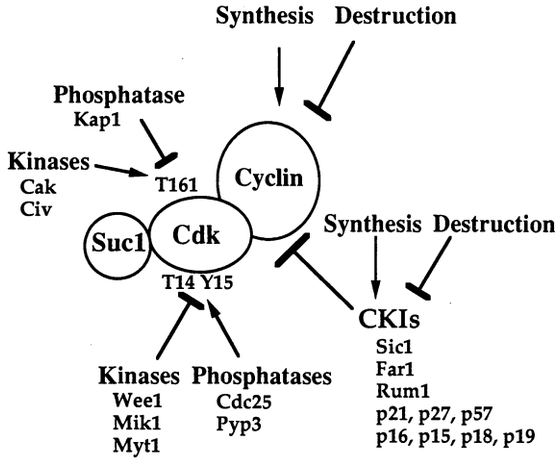

Fig. 2. Regulation of cyclin-dependent kinases. Arrowheads represent activating events and perpendicular ends represent inhibitory events. Genes known to perform the indicated functions are listed below. Both cyclins and some CKIs (Cdk inhibitors) are regulated by synthesis and ubiquitin-mediated proteolysis. Checkpoint pathways could act to promote inhibitory pathways or inhibit activating pathways to cause cell cycle arrest.

DNA Damage and DNA Replication Checkpoints in *S. cerevisiae*

Many checkpoint pathways have been identified primarily through the analysis of *cdc* (cell division cycle) mutants in yeast. Among these are checkpoints that sense mating partners, coordinate cell size and cell cycle progression, inhibit mitosis while in G1, make nuclear division dependent upon budding, restrict DNA replication to once per cell cycle, and make DNA synthesis dependent upon G1 cyclins. In this review, I focus on the best defined pathways, the DNA damage and DNA replication checkpoints and the spindle-assembly checkpoint (Fig. 1B).

In response to DNA damage and blocks of DNA replication, cells from both prokaryotes and eukaryotes induce a set of physiological responses thought to facilitate DNA repair processes. Among these responses are cell cycle arrest in G1, S phase, and G2, a slowing of DNA replication, and increased transcription of genes encoding proteins that participate in DNA replication and repair (*1*). In some organisms, an additional response, apoptosis, exists but is not explored in this review. Checkpoint-dependent arrest is thought to prevent the replication of damaged templates and the segregation of broken chromosomes. Since checkpoints are signal transduction pathways, they will be discussed in terms of their initiating signals, sensors, transducers, and effectors. A current view of the genetic organization of these pathways in *S. cerevisiae*, *S. pombe*, and mammals is shown in Fig. 3. The most striking feature of these pathways is that they share at least one common component in the signal transduction branch of the pathway, a phosphoinositide (PI) kinase superfamily member, indicating evolutionary conservation. Secondly, in the yeast pathways, the same signal transduction conduit is used both for the DNA damage checkpoint and arrest in response to replication blocks. I will use the budding yeast (*S. cerevisiae*) pathway as the primary example and discuss fission yeast and mammals when there are important differences.

DNA damage sensors and signal modifiers. Once DNA damage occurs, it can be processed through various repair pathways. These modifications may be required to produce the actual checkpoint signal. In

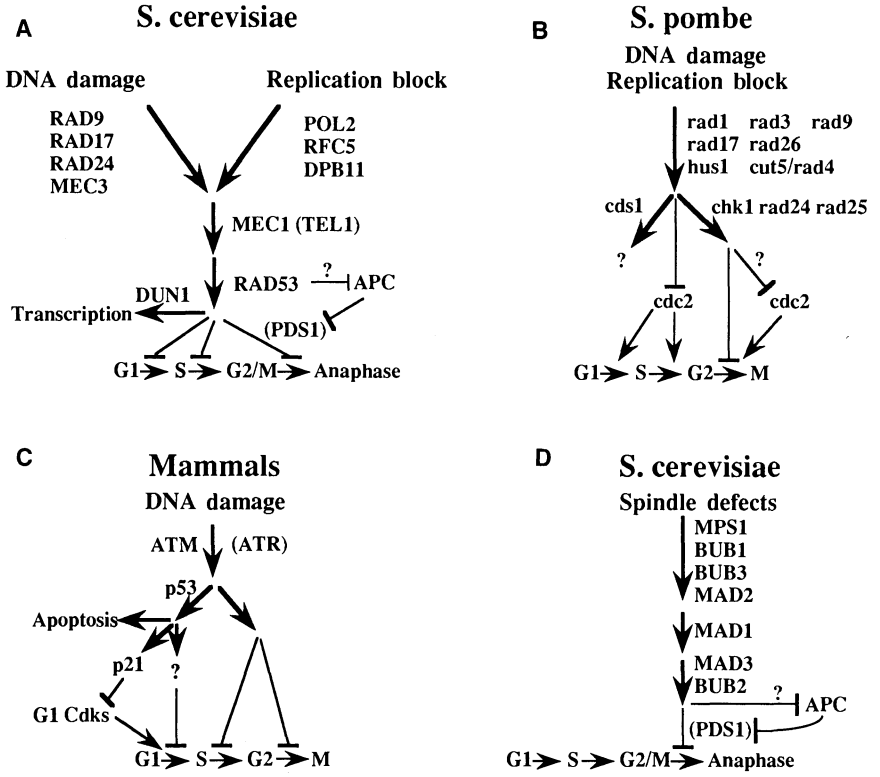

Fig. 3. A current view of the genetic organization of checkpoint pathways. The order of function of genes in groups along a single arrow is unknown and the order listed is arbitrary. (**A**) The DNA damage and DNA replication checkpoint in *S. cerevisiae*. *RFC5* and *DPB11* have not been examined for their G1 and G2 checkpoints and are therefore tentatively placed with *POL2*. *PDS1* has not been ordered genetically relative to *MEC1* and *RAD53* but is tentatively placed in parentheses at the end of the pathway because it alone is involved in both the DNA damage and the spindle checkpoints. The inhibitory connection between *RAD53* and the APC is hypothetical and is meant to indicate that *PDS1* might be regulated indirectly through regulation of the APC. Thus, this branch is an alternative to the inhibitory connection between *RAD53* and the G2/M to anaphase transition arrow. *TEL1* is in parentheses to indicate a minor redundant role with *MEC1*. G2/M is meant to indicate that arrest prior to anaphase can be considered either G2 or metaphase. (**B**) The DNA damage and DNA replication checkpoint in *S. pombe*. Arrows leading away from *cds1* indicates its function in an as yet undefined checkpoint function. The inhibitory circuit between *chk1* and *cdc2* is hypothetical and is based upon the putative role of tyrosine phosphorylation in the DNA damage checkpoint. It is meant as a possible alternative to the direct inhibition shown, not as a redundant pathway. The branch affecting the G1/S transition is assumed to exist but has not been demonstrated experimentally. Although the order of gene function shown is accurate, it is not clear that replication blocks and damage activate the pathway to equivalent degrees because modification of *chk1* is not observed in response to replication blocks (*53*). (**C**) The DNA damage checkpoint in mammals. ATM is shown transducing a signal to activate p53 which in turn activates the Cdk inhibitor p21 and a second unknown pathway. Little is known about the genes involved in the S phase slowdown and G2 arrest. (**D**) The *S. cerevisiae* spindle assembly checkpoint. *PDS1* is tentatively placed at the bottom of the pathway but has not been ordered genetically relative to the other genes shown.

Table 1. Homologs of DNA replication and damage checkpoint genes in yeast and humans. Genes are aligned on the basis of structural as opposed to functional similarities. Assumed biochemical activities are based on sequence similarity in some cases. Genes marked with an asterisk have not yet been shown to have checkpoint defects. Abbreviations: RFC, replication factor C; PIK, phosphoinositide kinase; PK, protein kinase; TF, transcription factor; CKI, cyclin-kinase inhibitor. Dashes indicate that a homolog has not yet been identified. In the case of *S. cerevisiae* which is completely sequenced, dashes indicate that highly related sequences have not been detected in the database. In the case of 14-3-3 proteins in humans, at least 7 genes have been identified. Of the *S. cerevisiae* genes listed only *CHK1* and *BMH1* and *2* have not yet been shown to have checkpoint function.

S. cerevisiae	Activity	*S. pombe*	Human
RAD9		–	–
RAD24	RFC-related	*rad17*	–
RAD17	Nuclease	*rad1*	–
MEC3		–	–
MEC1	PIK	*rad3*	ATR*
TEL1	PIK	–	ATM
RAD53	PK	*cds1*	–
POL2	Polymerase	*cdc30**	Pol ε*
DPB11		*cut5*	–
*CHK1**	PK	*chk1*	–
PDS1		–	–
DUN1	PK	–	–
*BMH1,2**	14-3-3	*rad24, 25*	14-3-3*
–		*rad26*	–
–		*rad9*	HRAD9*
–	TF	–	p53
–	CKI	–	p21

E. coli, there is considerable evidence to suggest that some types of DNA damage are converted to single-stranded DNA (ssDNA) that is then bound by RecA; it is this recombination complex that is sensed by LexA. In *S. cerevisiae*, it is not clear whether there are several sensors that can detect different types of damage, whether all damage is processed to one or a few forms that can be sensed by a limited number of sensors, or whether it is the actual process of repair that is sensed. We know that the presence of ssDNA correlates with arrest through analysis of *CDC13*. *CDC13* encodes a protein that binds to telomeres and protects them from degradation (*18*). Temperature-sensitive *cdc13* mutants accumulate ssDNA at the telomeres and arrest in the G2 phase of the cell cycle (*14*). Four genes, *RAD9*, *RAD17*, *RAD24*, and *MEC3* have properties consistent with a role as signal modifiers or sensors (*15*, *16*). They are required for G2 arrest of *cdc13* mutants and also for arrest in G1 and G2 in response to DNA damage, but not in response to a blockade of replication. Interestingly, *rad17*, *rad24*, and *mec3* mutants decrease the amount of ssDNA that accumulates in *cdc13* mutants, whereas *rad9* mutants increase the amount of ssDNA accumulation. Furthermore, *rad17* is structurally related to the *U. maydis* checkpoint gene *rec1*, a 3′-5′ exonuclease (*16*, *17*), suggesting that *rad17* is involved in modifying damage to generate a signal. (However, if *cdc13* mutants degrade their DNA from the telomere, they would use a 5′-3′ exonuclease to generate the single strand observed in vivo, not a 3′-5′ exonuclease.) Rad24 and its *S. pombe* counterpart Rad17 are related to RFC, a protein that binds gapped DNA. Thus, they are implicated in damage recognition although no biochemical function has been demonstrated (*19*). Because ssDNA is present in *cdc13rad9* mutants but fails to cause arrest, Rad9 must be a sensor or transducer of the DNA damage signal.

DNA replication sensors and signal transducers. Structures in the replication complex or unreplicated DNA may send signals to inhibit mitotic entry (*3*). A persuasive argument against signaling by unreplicated DNA is that several mutants that block the initiation of DNA replication, such as deletions of *Pol* α (*20*), *cut5* (*21*), or *cdc18* (*11*) in *S. pombe* and CDC6 in *S. cerevisiae* (*22*) allow mitosis to proceed with unreplicated DNA. However, it is a formal possibility that the unreplicated DNA in these mutants is in a different "non-signaling state" than the unreplicated DNA in cells whose cycles have already progressed into S phase. Furthermore, it is not certain whether the replication checkpoint is active constitutively once DNA replication has initiated, or merely becomes activated when replication is blocked. There is an inducible transcriptional component to the pathway that is not constitutively active during S phase (*23*), suggesting that the arrest mechanism is also inducible. So far none of the mutants defective for the checkpoint pathway allow mitosis to occur sooner than it normally does in an unperturbed cycle, but this may reflect the inherent timing of DNA replication and mitosis.

Three DNA replication genes required for the DNA replication checkpoint in *S. cerevisiae*, *POL2* (*24*), *DPB11* (*25*), and *RFC5* (*26*) are candidate sensors of DNA replication. *POL2* encodes DNA polymerase ε (Pol ε), which is required for chromosomal DNA replication (*27*). The *pol2* checkpoint-defective mutants are proficient for G1 and G2 arrest in response to DNA damage, but are defective in the transcriptional response and the ability to prevent mitotic entry when replication is blocked by hydroxyurea (HU), an inhibitor of ribonucleotide reductase. *DPB11* is the *S. cerevisiae* homolog of *S. pombe cut5*, which is required for DNA replication and for arrest in response to HU (*28*). *RFC5* is a component of replication factor C that binds to gapped DNA, such as that present on the lagging strand during replication, and recruits proliferating cell nuclear antigen (PCNA), which in turn recruits DNA Pol δ and Pol ε. A polymerase could function as a sensor of DNA replication because it is located at the replication fork. However, at the current level of resolution, it is impossible to distinguish between a sensory role versus a signal transduction role for any of these proteins and it is possible that it is the activity of an entire complex that must be intact to properly sense replication.

POL2 and *RAD9* (and *RAD17*, *RAD24*, *MEC3*) participate in temporally alternative branches of the pathway for sensing DNA damage. DNA damage incurred during S phase is sensed largely in a *POL2*-dependent manner, whereas damage incurred in G1 and G2 is primarily dependent upon *RAD9* (*29*), and *pol2rad9* double mutants are completely defective for the transcriptional response in all phases of the cycle. This is consistent with their complementary roles in activating cell cycle arrest. However, *rad9* mutants do show reduced slowing of DNA replication in response to the methylating agent methyl methane sulfonate (*30*) and, although *rad9* mutants alone are not HU-sensitive, they greatly enhance the HU-sensitivity of *pol2* mutants. This suggests a minor or redundant role for *RAD9* in the S-phase checkpoint pathway.

Signal transducers. Two essential genes form the central conduit for checkpoint signal transduction in *S. cerevisiae*, *MEC1* (*ESR1*, *SAD3*) (*15*, *23*, *31*) and *RAD53* (*SPK1*, *MEC2*, *SAD1*) (*15*, *23*, *32*). All cell cycle arrest, reduction in the rate of DNA replication, and transcriptional responses to DNA damage and incomplete replication are dependent upon these two genes (*23*, *29*, *33*). *MEC1* is a member of the PI kinase superfamily, some members of which are protein kinases. The Mec1 homolog in *S. pombe*, Rad3, has an associated protein kinase activity that is dependent upon a functional kinase domain within Rad3 (*34*). While short of formal proof, these results strongly suggest that Rad3 and Mec1 are protein kinases. *TEL1*, which is required for telomere length maintenance (*35*), is a structural homolog of *MEC1* (*36*, *37*). Although *tel1* mutants have functional checkpoints, mutations in *TEL1* enhance the sensitivity of *mec1* mutants to DNA damage and therefore *TEL1* has a minor checkpoint role.

To date, *MEC1* (*rad3*) and *TEL1* are the only checkpoint genes conserved in a functional sense among higher eukaryotes (see Table 1). Homologs include *mei-41* (*32*) in *Drosophila melanogaster*, and ATM (ataxia telangiectasia mutated, *39*) and ATR (AT and rad-related, *34*) in mammals. ATM mutant cells have defective G1 and G2 DNA damage checkpoints and show radiation-resistant DNA synthesis, for example, they do not slow replication in response to damage, and therefore share a subset of the *mec1* and *rad3* phenotypes (*7*, *40*). ATM is more closely related to *TEL1*, whereas ATR, also known as FRP (FRAP-related) (*41*), *MEC1*, and *rad3* form a separate subfamily. Although the function of ATR is unknown, ATR and ATM bind to distinct and complementary portions of meiotic chromosomes suggesting a possible role in signaling different stages of meiotic progression or perhaps in the recombination process itself (*43*). Mutants in *mec1* (*esr1*) have been reported to be defective in meiotic recombination (*31*, but see *42*) and in cell cycle arrest when recombination is blocked (*42*).

RAD53 is a protein kinase that is phosphorylated and activated in response to DNA damage. Phosphorylation of Rad53 is dependent upon *POL2*, *RAD9*, and *MEC1* (*29*, *37*, *44*). This and other data (*37*) indicate that *RAD53* functions downstream of *MEC1*, *POL2*, and *RAD9* to transduce the signal from DNA damage and incomplete replication and may be a substrate of Mec1. Modification of Rad53 in response to DNA damaging agents is much more pronounced than that achieved in response to HU, indicating complexity in the upstream signaling process (*37*). Although checkpoint defective, *rad53* mutants are much less sensitive to UV and HU than *mec1* mutants, indicating that Mec1 controls processes that are not solely dependent upon Rad53.

Effectors of the transcriptional response. In parallel to the cell cycle arrest response to DNA damage and replication blocks is a separate transcriptional response specifically controlled by the protein kinase Dun1 (*29*, *45*). Although there is currently no evidence that the transcriptional response is involved in cell cycle arrest in *S. cerevisiae*, it is clearly involved in arrest in mammals and for this reason is included here. Dun1 kinase activity is increased by DNA damage in a *RAD53*- and *MEC1*-dependent manner, and this activation is required for transcriptional activation of the genes encoding ribonucleotide reductase, *RNR1*, *RNR2*, and *RNR3*. However, *DUN1* does not appear to have a unique role in cell cycle arrest. Presumably there will be a series of transcription factors analogous to p53 that are altered in a Dun1-dependent fashion to activate transcription. Other mutants in this portion of the pathway include the *crt* (constitutive *RNR3* transcription) mutants (*46*), three of which—*SSN6*, *TUP1*, and *CRT1*—are epistatic to *dun1* and are therefore likely to function downstream of *DUN1* or in a separate pathway. Because *TUP1* and *SSN6* are general components of transcriptional repressors, part of the transcriptional response may be accomplished by alleviating repression.

Effectors of the cell cycle arrest response. Organisms may differ in their requirements for blocking mitotic entry depending upon how their cell cycles are organized. *S. pombe*, *Xenopus laevis*, and mammals can effectively prevent the G2-to-metaphase transition by blocking Cdk activity. However, *S. cerevisiae* starts spindle assembly during S phase, effectively initiating mitosis, and in order to inhibit cell cycle progression they must block entry into anaphase. This is likely to be accomplished by a mechanism distinct from that used to block entry into metaphase because they are different biochemical steps (see the discussion of *cdc55* mutants in the spindle assembly checkpoint). The best candidate for an effector of cell cycle arrest in response to DNA damage is *PDS1*, an anaphase inhibitor. The *pds1* mutants fail to arrest in G2 in response to gamma irradiation (γ-IR) or in the presence of *cdc13* mutations (*47*). Furthermore, *pds1* mutants are also defective in the spindle assembly checkpoint, indicating a potentially common target for these two checkpoint pathways. Pds1 is degraded by ubiquitin-mediated proteolysis by a set of proteins that promote anaphase, the anaphase-promoting complex (APC). *PDS1* mutants resistant to destruction cause a pre-anaphase arrest (*48*), so blocking Pds1 destruction is one mechanism by which cells could respond to DNA damage to prevent mitosis and allow time for repair. Whether Pds1 is directly modified in response to DNA damage, or whether it is indirectly regulated through control of the APC remains to be determined. Because failure to degrade mitotic cyclins causes arrest after anaphase, it is unlikely that arrest in response to DNA damage is mediated by complete inhibition of APC function. Indeed, γ-IR of *pds1* mutants allows progression through telophase into the next cycle. In addition, *pds1* mutants do arrest if DNA replication is blocked, indicating the existence of a distinct S phase–specific effector. Pds1 could have an indirect role in checkpoint function. For example, Pds1 may be required for prolonged cohesion of newly replicated sisters during checkpoint arrest. If the process of sister separation, once begun, sends a positive signal for progression through the cell cycle, *pds1* mutants could simply bypass the checkpoint signal by initiating anaphase.

The *S. pombe* Checkpoint Pathway

The organization of the *S. pombe* checkpoint pathway (Fig. 3B) is similar to that of the *S. cerevisiae* pathway, and these pathways share several conserved genes (Table 1). A group of 7 genes, *rad1*, *rad3*, *rad9*, *rad17*, *rad26*, *hus1*, and *cut5* are required for cell cycle arrest in response to both damage and replication blocks (*49*). The protein kinase *cds1* is very similar in sequence with the first 70% of the *S. cerevisiae* Rad53 protein, is required for survival during cell cycle arrest with HU (*50*). Cell cycle arrest by DNA damage but not blocked replication requires the function of the Chk1 (Rad27) protein kinase (*51*) and the 14-3-3 proteins encoded by *rad24* and *rad25* (*52*). Chk1 becomes phosphorylated in response to DNA damage in a Chk1-dependent manner (*53*). This presumed autophosphorylation is dependent upon the *rad1*, *rad3*, *rad9*, *rad17*, *rad24*, and *hus1* genes and *chk1* is therefore placed downstream of these genes. Major similarities between the yeasts include structural similarity between checkpoint genes: *MEC1* and *rad3* (*34*, *54*), *RAD17* and *rad1* (*16*), *RAD53* and *cds1* (*50*), *RAD24* and *rad17* (*19*), and *DPB11* and *cut5* (*25*). Furthermore, signals from both DNA damage and blocked replication are transduced through a common pathway in both organisms.

There are also significant differences in checkpoint control: (i) unlike *MEC1* and *RAD53*, *rad3* and *cds1* are not essential, (ii) *rad53* mutants behave differently than *cds1* mutants when DNA replication is blocked, (iii) *S. pombe rad17* is required for both the damage and replication checkpoints whereas *S. cerevisiae RAD24* is required only for arrest by DNA damage, (iv) cell cycle arrest in response to blocked replication (*55*) and possibly damage (*56*) requires inhibitory phosphorylation of a tyrosine on *S. pombe* Cdc2 but this is not the case in *S. cerevisiae* (*49*).

Without knowledge of the essential roles of *RAD53* and *MEC1*, it is difficult to explain why the two yeasts differ in this respect. One explanation may be that the timing of mitosis relative to the end of DNA synthesis differs in the two yeasts. Unlike *S. cerevisiae* which exerts its size control in G1, *S. pombe* integrates its size control primarily during G2 to provide an additional, perhaps redundant, delay before mitotic entry. Support for this hypothesis is that *wee1-50* mutants, which accelerate mitosis to produce smaller *S. pombe* cells, are lethal in combination with mutants of the *rad3* group or *chk1* (*51*, *57*). However, an alternative explanation that cannot be eliminated is that *wee1-50* mutants cause a DNA replication problem that requires *rad3* function.

The fact that the *S. pombe rad17* mutant is defective for both the damage and replication checkpoints while *S. cerevisiae RAD24* is required only for arrest by DNA damage may mean that they are not true homologs, although they are structurally related. Alternatively, this discrepancy may underscore differences in how replication blocks are sensed in the two organisms.

Unlike *rad53* mutants, *cds1* mutants do not enter mitosis in the presence of HU (*50*). However, once HU is removed, *cds1* mutants complete the bulk of DNA synthesis and undergo a mitotic catastrophe in which chromosomes fail to properly segregate prior to septation. While the difference in cell cycle arrest remains unclear, the delayed mitotic catastrophe is potentially revealing and raises the possibility that the response to DNA replication blocks may include essential functions other than simply preventing mitosis. Support for this also comes from the fact that the loss of viability of *rad53* mutants in HU cannot be suppressed by blocking mitosis with microtubule inhibitors (*23*). One such additional function might be controlling the integrity of stalled replication complexes. A partial loss of replication fork integrity could result in the disassembly of replication forks. While loss of a few forks would not necessarily be a catastrophic event, if two converging forks collapse (CFC), the intervening DNA is not replicated. Failure to prevent or repair CFC will lead to segregation of partially replicated chromosomes and catastrophe.

In *S. pombe*, tyrosine phosphorylation of Cdc2 is necessary for proper arrest in response to blocked replication (*55*). It is not

known whether this phosphorylation is directly regulated by the checkpoint pathway or merely required for proper checkpoint function. Recent experiments concerning the replication checkpoint in *X. laevis* extracts indicate that tyrosine phosphorylation alone cannot explain the observed cell cycle arrest. An undefined Cdk inhibitor activated by replication blocks was detected (*58*). Experiments in *Aspergillus nidulans* have implicated redundancy between Cdc2 tyrosine phosphorylation and a *bimE*-dependent pathway (*59*). The *bimE* protein is a component of the APC and *bimE* mutants are likely to have unusually large amounts of cyclin B. Tyrosine phosphorylation is required for arrest in response to DNA damage in *A. nidulans* (*60*). Because the proteins that control tyrosine phosphorylation are known, it is clear that the next step is to determine how (and if) DNA damage and interference with DNA replication affects the activities of these enzymes and to what extent the APC is involved in arrest.

Mammalian DNA Damage Checkpoints

Mammals have the same cell cycle responses to DNA damage as yeast, but in addition may activate a cell death pathway. Cell elimination is a viable strategy for metazoans because their goal is not the survival of each damaged cell, which might harbor mutations, but the survival of the organism. I will not discuss the apoptotic response. Of the various mammalian checkpoints, only the G1 DNA damage checkpoint is understood in any detail. Three mammalian genes control the DNA damage checkpoint, mutated in ataxia telangiectasia (ATM) (*7*, *39*), p53 (*61*), and p21 (*62*). Of these, p53 is the most widely studied. The p53 gene is the tumor suppressor most frequently mutated in human cancers (*63*). It encodes a transcription factor that is activated in response to DNA damage and perturbation of nucleotide pools. Cells defective for p53 are unable to arrest in G1 in response to γ-irradiation and show reduced apoptosis. Part of p53's ability to arrest G1 cells results from activation of transcription of p21, a tight-binding inhibitor of Cdks that control entry into S phase (*64*). Mouse embryo fibroblasts lacking p21 show a partial defect in G1 arrest that is less severe than that of p53-defective fibroblasts, indicating that a second p53-dependent G1 arrest pathway exists. While the nature of this pathway is not known, experiments with mutant forms of Cdk4 have suggested that tyrosine phosphorylation of Cdk4 may be required for G1 arrest in response to UV irradiation (*65*) and is therefore a good candidate for the p21-independent pathway. The p21 protein has also been shown to control checkpoint function in human cells (*66*). It is not known how yeast cells arrest in G1, but failure to destroy inhibitors such as Sic1 (or Far1 in the case of α-factor arrested cells) could provide a mechanism similar to inhibition by p21.

How p53 is activated in response to DNA damage is still unknown. Both its stability and specific activity as a transcription factor appear to increase in response to DNA damage, but the precise mechanism has remained elusive despite intensive study (*63*). The ATM gene has been implicated in regulation of p53. Cells lacking ATM show a reduced and delayed activation of p53 in response to DNA damage (*67*). Given its relationship to *MEC1* and *rad3*, it is likely that ATM plays a role in transducing the DNA damage signal to p53. Although ATM is upstream of p53, ATM mutants die via p53-dependent apoptosis in response to DNA damage. Therefore, an ATM-independent mechanism for p53 activation must exist, perhaps controlled by ATR.

The fact that both p53 and ATM are frequently mutated in human cancers strongly implicates checkpoint function in the prevention of cancer. How much we can learn about cancer from the analysis of yeast checkpoints will depend upon the degree of conservation between the human and yeast pathways. So far, few human checkpoint genes have been identified and of these, only ATM has yeast homologs (Table 1). However, it appears that budding and fission yeasts share many conserved checkpoint genes, and it is often the case that genes shared among these organisms are common to all eukaryotes. With the rapid advances occurring in the identification of genes in various sequencing projects, a definitive answer to this question should be known in the very near future. In this regard, a human homolog of the *S. pombe rad9* gene has been identified, although its role in animal cells has yet to be determined (*68*). Furthermore, the genes that control cell cycle arrest in response to DNA damage also control other aspects of the DNA damage response, possibly specific DNA repair pathways and apoptosis. Therefore, although it is generally assumed that the loss of the ability to arrest the cell cycle leads to genomic instability and cancer, we are actually far from having definitively proven that fact. Proof will require specific elimination of the ability to arrest the cell cycle without affecting the rest of the signaling pathway. In the one case where this has been accomplished, loss of p21 in the mouse, cancer did not result (*62*), and p21 mutations are very rare in human tumors. Therefore, this question will require future scrutiny.

The Spindle Assembly Checkpoint

The proper segregation of chromosomes requires the execution of a number of processes during mitosis: a bipolar spindle must be assembled; chromosomes must attach to the spindles through the kinetochore, a protein structure that forms on the centromeres of chromosomes; kinetochores of sister chromatids must bind to spindle fibers attached to opposite poles; and properly attached chromosomes must arrive at the metaphase plate. The spindle assembly checkpoint prevents the onset of anaphase, the actual segregation of chromosomes, until these processes have been properly accomplished. Once these events take place, cells can execute anaphase and progress into the next cell cycle.

Spindle assembly signals and sensors. While it is premature to discuss the molecular identity of sensors, there is a lively debate as to the nature of the event being sensed. The assembly of spindles involves many different components any of which could (and may) be sensed. As noted in a recent review (*69*), the sensor could detect the amount of free tubulin, the function of the microtubule organizing center, the bipolarity of the spindle, the attachment of microtubules to the kinetochore, or the tension generated on the kinetochore by attachment to a bipolar spindle. Because many of these processes are interdependent, it is possible that defects in any one could result in the failure of a common event that could be the signal for all defects. The leading candidates for signals are lack of chromosome attachment to the spindle and the absence of tension generated on a chromosome attached to a bipolar spindle.

Support for the sensing of tension at the kinetochore comes from experiments in which chromosomes were micromanipulated with glass needles. By manipulating chromosomes of grasshopper spermatocytes in meiosis, it is possible to force both attachments of sister chromatids to the same pole. While normally unstable, such a mono-oriented chromosome pair can be made stable and will persist until anaphase if force toward the opposite pole is exerted on the chromosome by the needle (*70*). Furthermore, meiotic anaphase can be delayed by the presence of a chromosome lacking its synaptic mate. Applying force on that chromosome with a glass needle mimics tension from proper bipolar attachment to the spindle and causes rapid entry into anaphase (*71*).

Evidence supporting the sensing of unattached kinetochores derives from laser ablation of kinetochores in mammalian mitotic cells. If the unattached kinetochore of the last monoattached chromosome is destroyed by laser ablation, cells no longer delay an-

aphase entry even though there is a lack of tension on that chromosome (*72*). Additional evidence is that *S. cerevisiae* lacking *CDC6* and *S. pombe* lacking *cdc18* undergo mitosis with unreplicated chromosomes (*11*, *73*). The absence of bipolar spindle attachment in this case should prevent tension but does not prevent anaphase. Furthermore, the absence of sisters precludes the need for the APC suggesting that the only role of ubiquitin-mediated proteolysis in anaphase is to allow sister separation (*73*). It is also possible that one mechanism operates in meiotic cells (tension) and a second in mitotic cells (attachment). Alternatively, a lack of tension may produce free microtubule-binding sites in the kinetochore and these may activate the checkpoint (*69*). This would accommodate both sets of observations. Regardless of the model, it is clear that the spindle assembly checkpoint is capable of detecting signals generated at the kinetochore. Genetic evidence supporting this is that mutants in genes encoding kinetochore proteins such as Ctf13 or mutations in the centromere itself delay mitosis in budding yeast. Remarkably, higher eukaryotic cells can execute anaphase efficiently in the absence of kinetochores (*74*) or even chromosomes themselves (*75*)! This suggests that the presence of kinetochores establishes the checkpoint in the first place. It would be interesting to know whether spindle damage would prevent anaphase entry in the absence of chromosomes.

A molecular correlate has been identified for a signal regulated by tension. The monoclonal antibody 3F3 recognizes a phosphoepitope on an unknown kinetochore protein that appears much more abundantly on unattached kinetochores (*76*). The large amount of signal on a monoattached chromosome can be diminished by exerting a force toward the unattached pole to generate tension (*77*). Furthermore, injection of antibodies to 3F3 delays anaphase and the dephosphorylation of the 3F3 epitope, suggesting that this dephosphorylation may be necessary to turn off the checkpoint after all the chromosomes have been aligned on the spindle (*78*).

Signal transducers in the spindle assembly checkpoint. The genetic pathway responsible for the spindle assembly checkpoint is shown in Fig. 3D. The majority of these genes were identified in two screens for the failure to arrest in the presence of the microtubule depolymerizing drugs. *MAD1*, *MAD2*, and *MAD3* (mitotic arrest defective) (*79*) and *BUB1*, *BUB2*, and *BUB3* (budding uninhibited by benimidazole) (*80*) are not essential genes but their mutants attempt aberrant mitoses in the presence of microtubule inhibitors and die. The *mad* and *bub* mutants also show an increased frequency of spontaneous chromosome loss reflecting a role in detecting endogenous errors. These proteins are also required for delaying anaphase entry in the presence of chromosomes carrying mutant centromeres, indicating a role in detecting kinetochore-generated signals. *MPS1* was identified as a gene required for spindle pole body assembly and later was found to have a checkpoint phenotype (*81*).

The spindle assembly checkpoint signals through two protein kinases, Mps1 and Bub1 (*82*). Overproduction of Mps1 arrests the cell cycle (*83*) and this arrest is dependent upon all of the *MAD/BUB* genes. This suggests that activation of Mps1 may be one of the initial signaling events in the checkpoint pathway. Mad1 becomes phosphorylated when the checkpoint is activated (*84*), and this event has been used to order the action of other genes in the pathway. Mps1 directly phosphorylates Mad1 in vitro and is required for its phosphorylation in vivo (*83*), indicating that this may be a critical signaling event. A complication exists in placing Mps1 directly adjacent to Mad1 in the signaling pathway because Mad1 phosphorylation is also dependent upon *BUB1*, *BUB3*, and *MAD2* when the checkpoint is activated, and these genes are also required for cell cycle arrest when Mps1 is overproduced. Clearly this is a complex signaling mechanism that cannot be easily organized using genetic analysis alone. Mad2 is a protein that binds to Mad1 and is required for its phosphorylation (*69*). Mad2 may be a central protein in this signaling cascade because its localization to kinetochores changes under conditions that activate the signaling pathway. Mad3 and Bub2 are not required for Mad1 phosphorylation and are therefore placed after Mad1 in the signal transduction pathway.

The *Xenopus* homolog of *MAD2*, *XMAD2*, is required for the spindle assembly checkpoint in vitro (*85*) and the human homolog is required for checkpoint function in vivo (*86*). Both human and *Xenopus* Mad2 localize to the kinetochores of unattached chromosomes. Once chromosomes attach to microtubules, Mad2 immunostaining is lost. While possibly due to epitope masking, the more exciting possibility is that Mad2 associates with unattached kinetochores and signals to activate the spindle checkpoint. Since in yeast Mad2 binds to Mad1 and is required for Mad1 phosphorylation, it is possible that *Xenopus* Mad1 is also localized to kinetochores and will have the phosphorylation properties attributed to the 3F3-reactive protein. A plausible model for how the spindle assembly checkpoint may operate is that in the absences of tension or presence of free microtubule binding sites, a protein kinase such as Mps1 or Bub1 is activated and phosphorylates a protein localized at the kinetochore. This phosphorylation leads to recruitment of the Mad2 protein which then connects the circuit allowing Mad1 phosphorylation and generation of the arrest signal, perhaps through Mad3 and Bub2. The binding of Xmad2 and the model of sensing kinetochore attachment suggests that the spindle assembly checkpoint may not be inducible (extrinsic) in the same sense as the DNA damage pathway, but is active during each cell cycle when kinetochores mature. Microtubule inhibitors would prevent the proper assembly of the kinetochore microtubules and would thereby maintain the checkpoint signal.

Effectors of the spindle checkpoint pathway. Significant advances have recently been made concerning the mechanism of spindle checkpoint-dependent cell cycle arrest. Interference with ubiquitin-mediated proteolysis either by mutations in components of the APC in vivo or by inhibition with methyl ubiquitin in vitro can arrest cells before anaphase (R. W. King *et al.*, p. 1652), consistent with a role for proteolysis. Yeast arrested via this checkpoint have stable cyclins and high Cdk activity. Although cyclins are degraded by the APC, cyclins are not the critical substrate for the anaphase transition (*87*). As described above, degradation of the Pds1 protein is dependent upon the APC and is required for anaphase entry. Furthermore, *pds1* mutants show checkpoint defects. Together, these observations provide evidence that Pds1 is a potential effector of the spindle assembly checkpoint. The same caveats apply here as for the role of Pds1 in DNA damage checkpoint. It remains to be determined precisely how Pds1 degradation is controlled. Is the activation of the APC blocked by checkpoint activation, or is Pds1 somehow protected from an activated APC?

A gene that is required for arrest by microtubule inhibitors but which may lie outside of the pathway shown is *CDC55*, a non-essential regulatory component of the PP2A phosphatase. Unlike *mad* and *bub* mutants which ignore the inhibitory signal of microtubule inhibitors, *cdc55* mutants allow the separation of sister chromatids in the presence of nocodazole by inhibiting Cdc28 kinase activity through tyrosine phosphorylation (*88*). Thus, Cdc28 inactivation may be a secondary pathway allowing separation of sister chromatids. Whether this represents anaphase or a return to a premitotic state could not be determined because the experiments were done in the presence of nocodazole. Whether this pathway is used during arrest in wild-type cells remains to be determined. If so, it may represent an adaptation response in which unicellular organisms unable to repair a checkpoint-

activating defect undertake a defective transition rather than remain terminally arrested. In this sense, an adaptation pathway is a method cells use for measuring time. Such an adaptation response has been suggested for DNA damage (*89*).

If the DNA damage and spindle assembly checkpoint arrest at the same position in the cell cycle as suggested by their dependency on the same effector, *PDS1*, this may explain why the DNA damage and replication checkpoints have evolved an arrest mechanism distinct from tyrosine phosphorylation in budding yeast. As noted for *cdc55* mutants, tyrosine phosphorylation of Cdc28 at that stage of the cell cycle may activate anaphase as opposed to preventing it.

The flexibility of checkpoint pathways. Once cell cycle arrest mechanisms are established it is possible that many signaling pathways can interface with a central pathway to utilize the same arrest mechanism. For example, the spindle assembly checkpoint in *X. laevis* requires the activity of a mitogen-activated protein (MAP) kinase, $p44^{ERK2}$ (*90*). The arrest of mature oocytes in the second meiotic metaphase by CSF (cytostatic factor) also requires $p44^{ERK2}$ and is likely to use the same arrest mechanism. Size control and DNA replication both require tyrosine phosphorylation in *S. pombe*. The DNA damage and replication checkpoints appear to use the same pathway in *S. cerevisiae*, *S. pombe*, and *A. nidulans*. *Drosophila melanogaster* uses tyrosine phosphorylation of Cdks to regulate cell cycle progression during development (*49*). These signal transduction pathways appear to be flexible modules that can be adapted to meet diverse evolutionary demands.

Summary

The last 8 years have seen a rapid increase in our knowledge of the regulation of cell cycle transitions. Many of the main cell cycle checkpoints have been identified and biochemical analysis of their signal transduction mechanisms are under way. For those that directly regulate Cdk activity, we have sufficient basic knowledge of Cdk regulation to uncover the mode of regulation. It is now a matter of connecting the signal transduction proteins to each other and to the direct effectors of Cdk function. For those pathways that operate through non-Cdk regulation such as *PDS1*, there is much to learn about how they carry out their inhibitory functions. Furthermore, we know very little about the mechanisms these pathways use to monitor cell cycle events.

Important questions remain as to the nature of checkpoints in mammals and the integration of checkpoint pathways with cell proliferation controls and development. Inappropriate expression of the proto-oncogene *cMyc* can activate the p53-dependent checkpoint pathway (*91*). Do growth promoting pathways generally become integrated into checkpoint pathways as a consequence of cellular differentiation? Is this a mechanism of cancer prevention? Furthermore, what are the relative contributions of DNA repair, cell cycle arrest, and apoptosis to cancer prevention by checkpoint pathways? Are other checkpoints such as the spindle assembly pathway disrupted in tumors? Checkpoints figure prominently in chemotherapeutic strategies to eliminate cancer cells. Most agents kill cancer cells by activating checkpoint-mediated apoptosis pathways or by exploiting chemical sensitivities due to loss of checkpoint function (*9*). In the future we should be able to exploit our increased understanding of checkpoints to further this cause. While we have learned much, we have only dipped beneath the surface of what we must know to fully understand checkpoints. Fortunately we now have the biochemical and genetic tools needed to address many of these interesting and important questions. These are stimulating times. So much so that it is virtually impossible to keep one's excitement in check, and that's the point.

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Cancer Cell Cycles

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Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired damage to genes that directly regulate their cell cycles. Genetic alterations affecting $p16^{INK4a}$ and cyclin D1, proteins that govern phosphorylation of the retinoblastoma protein (RB) and control exit from the G_1 phase of the cell cycle, are so frequent in human cancers that inactivation of this pathway may well be necessary for tumor development. Like the tumor suppressor protein p53, components of this "RB pathway," although not essential for the cell cycle per se, may participate in checkpoint functions that regulate homeostatic tissue renewal throughout life.

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 (*1*). The machinery for DNA replication and chromosome segregation is insulated from interruption by extracellular signals, and its essential and autonomous nature implies that damage to the pivotal components would be highly debilitating, if not fatal, to cells. Therefore, genes commanding these processes should not be frequent targets of mutation, deletion, or amplification in cancer.

Oncogenic processes exert their greatest effect by targeting particular regulators of G_1 phase progression (*2*, *3*). During the G_1 phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into a resting state (G_0) (*4*, *5*). Unlike transit through the S, G_2, and M phases, G_1 progression normally relies on stimulation by mitogens and can be blocked by antiproliferative cytokines. Cancer cells abandon these controls and tend to remain in cycle, and because cell cycle exit can facilitate maturation and terminal differentiation, these processes are subverted as well. The decision to divide occurs as cells pass a restriction point late in G_1, after which they become refractory to extracellular growth regulatory signals and instead commit to the autonomous program that carries them through to division (*4*, *5*). An appreciation of restriction point control is central to our understanding of how and why cancer cells continuously cycle.

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Restriction Point Control and the G_1-S Transition

Passage through the restriction point and entry into S phase is controlled by cyclin-dependent protein kinases (CDKs) that are sequentially regulated by cyclins D, E, and A (Fig. 1). In general, CDK activity requires cyclin binding, depends on both positive and negative regulatory phosphorylations (*6*), and can be constrained by at least two families of CDK inhibitory proteins (*7*).

D-type cyclins act as growth factor sensors, with their expression depending more on extracellular cues than on the cell's position in the cycle (*8*). As cells enter the cycle from quiescence (G_0), one or more D-type cyclins (D1, D2, and D3) are induced as part of the delayed early response to growth factor stimulation, and both their synthesis and assembly with their catalytic partners, CDK4 and CDK6, depend on mitogenic stimulation (*5*). The catalytic activities of the assembled holoenzymes are first manifest by mid-G_1, increase to a maximum near the G_1-S transition, and persist through the first and subsequent cycles as long as mitogenic stimulation continues. Conversely, mitogen withdrawal leads to cessation of cyclin D synthesis; the D cyclins are labile proteins, and because their holoenzyme activities decay rapidly, cells rapidly exit the cycle. Specific polypeptide inhibitors of CDK4 and CDK6—so-called INK4 proteins—can directly block cyclin D–dependent kinase activity and cause G_1 phase arrest (*9*). The four known 15- to 19-kD INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) bind and inhibit CDK4 and CDK6, but not other CDKs. Like the three D-type cyclins, the *INK4* genes are expressed in distinct tissue-specific patterns, suggesting that they are not strictly redundant.

A loss of cyclin D1–dependent kinase activity before the restriction point prevents many cultured cell lines from entering S phase, but its absence later in the cell cycle is without effect (*10*, *11*). Hence, cyclin D–dependent kinases must phosphorylate some substrate or substrates whose modification is required for G_1 exit, and the retinoblastoma tumor suppressor protein (RB) is one such target (*12*). Notably, cyclin D–dependent kinases are dispensable for passage through the restriction point in cultured cells that lack functional RB, and in this setting, ectopic expression of INK4 proteins does not induce G_1 phase arrest (*13*). Thus, INK4 proteins inhibit cyclin D–dependent kinases that, in turn, phosphorylate RB (Fig. 2). Disruption of this "RB pathway" is important in cancer.

RB and other RB-like proteins (p130, p107) control gene expression mediated by a family of heterodimeric transcriptional regulators, collectively termed the E2Fs (*14*, *15*), which can transactivate genes whose products are important for S phase entry (*14*, *16*) (Fig. 2). In its hypophosphorylated form, RB binds to a subset of E2F complexes, converting them to repressors that constrain expression of E2F target genes (*17*). Phosphorylation of RB frees these E2Fs, enabling them to transactivate the same genes, a process initially triggered by the cyclin D–dependent kinases (*5*, *12*, *13*) and then accelerated by the cyclin E–CDK2 complex (*18*–*20*) (Fig. 2).

In proliferating cells, the expression of cyclin E is normally periodic and maximal at the G_1-S transition (Fig. 1), and throughout this interval, cyclin E enters into active