2. Molecular Mechanism Controlling the G1/S Transition of Mammalian Cells

2.1. Factors Regulating G1 Phase of the Cell Cycle

2.1.1. G1 Phase CDK/Cyclin Complexes

The progression through the cell cycle is governed by the periodic activation and inactivation of cyclin-dependent kinase (CDK) complexes. The CDK proteins are Ser/Thr protein kinases, and their kinase activities are controlled by their association partners, which are called cyclins *(22)*. The protein levels of the CDKs remain constant through the cell cycle, whereas the levels of the cyclins vary during the cell cycle, owing to periodic expression and degradation. The timely regulation of different CDK/cyclin complexes is responsible for well-organized cell cycle progression, as these complexes act in G1 to initiate S phase and in G2 to initiate mitosis. These mechanisms are conserved from yeast to mammals *(23)*. The kinase activity of the CDKs is also tightly controlled by the binding of inhibitors and phosphorylation events.

In the middle of the cyclin proteins is a domain of well-conserved amino acid sequences called the *cyclin box*. While cyclins were originally characterized as being the regulatory subunit of CDK that is periodically expressed and degraded during the cell cycle *(24)*, it was later found that many cyclins do not cycle and that they can regulate cellular functions other than the cell cycle *(25)*. These include: cyclin G *(26)*, which is a regulatory subunit of protein phosphatase 1A *(27)*; cyclin H, which forms a complex with CDK7 that regulates not only other CDKs as a CDK-activating kinase (CAK) *(28)* but also transcription and DNA repair *(29)*; cyclin L, which is a regulatory subunit of CDK11 and promotes pre-mRNA splicing *(30)*; and cyclin T, which forms a complex with CDK9 and activates transcription by hyperphosphorylation of the carboxyl-terminal domain of the large subunit of RNA polymerase II *(31)*.

Eleven CDK proteins (Cdc2 = CDK1, CDK2, . . . CDK11) have been discovered and examined in mammalian cells to date. Of these, CDK2, CDK3, CDK4, and CDK6 are principally responsible for G1 progression and entry into S phase. CDK4 and CDK6 are activated in mid G1, whereas CDK2 is activated in late G1. While CDK4 and CDK6 are co-expressed in many cell types, CDK6 does not fully compensate for the function of CDK4 in most cells *(32,33)*. The CDK4/cyclin D and CDK 6/cyclin D complexes play pivotal roles in early to mid G1, whereas CDK2/cyclin E and possibly CDK2/cyclin A function at the late stage of G1 *(22)*. These cyclins are comprehensively termed

the G1 cyclins *(34)*. Three types of cyclin Ds (D1, D2, and D3) have been identified, each of which functions as a regulatory subunit of either CDK4 or CDK6 *(35)*. In mid G1 phase, the CDK4/cyclin D complexes phosphorylate the pRB (retinoblastoma) family of nuclear phosphoproteins (*see* **Subheading 2.1.4.**), which are the key regulators of the G1/S transition *(36)*, whereas CDK2/ cyclin A and CDK2/cyclin E phosphorylate pRB at the G1 to S transition *(34,37)*.

When quiescent cells enter the cell cycle owing to mitogenic signals, the expression of the cyclin Ds is induced and the CDK4/cyclin D and CDK6/ cyclin D complexes are formed as the cells progress through the G1 phase *(38)*. The kinase activity of the complexes is then activated when they enter the cell nucleus and are phosphorylated by CAK. This allows the complexes to phosphorylate target proteins such as pRBs *(39,38)*. Thus, the cyclin D protein types play a pivotal role in G1 by transmitting the mitogenic signal to the pRB/E2F pathway. Notably, cyclin D1 also seems to play a CDK-independent role as a modulator of transcription factors because it interacts with histone acetylases and components of the transcriptional machinery. The cyclin D1–deficient mouse is viable but does have developmental abnormalities that are limited to restricted tissues *(38)*. Proteasomal degradation of cyclin D1 is triggered by its phosphorylation on a single threonine residue (Thr-286) by glycogen synthase kinase-3β *(40)*.

Cyclin E, which regulates CDK2 and possibly also CDK3, is expressed in late G1 and early S phase *(34)*. The level of cyclin E is abruptly decreased by proteolysis after polyubiquitination mediated by SCF (Skp2) ubiquitin ligase *(41)*. Cyclin E regulates the initiation of DNA replication by phosphorylating components of the DNA replication machinery *(39)*. The CDK2/cyclin E complex also triggers the duplication of centrosomes at G1/S phase by phosphorylating the multifunctional protein nucleophosmin (also known as B23) *(42,43)*. CDK2/cyclin E also targets NPAT (nuclear protein mapped to the AT locus) as a phosphorylation substrate, which may explain why CDK activity is linked to the periodic synthesis of histones *(44–46)*.

Cyclin A can activate two different CDKs and functions in both S phase and mitosis *(47)*. Cyclin A starts to accumulate during S phase and is abruptly destroyed before metaphase. The synthesis of cyclin A is mainly controlled at the transcription level and involves E2F and other transcription factors. It is still unknown why CDK2/cyclin A and CDK2/cyclin E complexes are both required for the initiation of DNA replication and why their order of activation is tightly regulated. Using a cell-free system, it has been shown that cyclin E stimulates replication complex assembly by cooperating with Cdc6, a regulator of the initiation of DNA replication, whereas cyclin A has dual functions: first, it activates DNA synthesis by the replication complexes that are already as-

sembled, and second, it inhibits the assembly of new complexes *(48)*. This regulatory mechanism allows cyclin E to promote replication complex assembly while cyclin A blocks this assembly. Thus, the dual functions of cyclin A ensure that the assembly phase (G1) ends before DNA synthesis (S) begins, thereby preventing re-initiation until the next cell cycle.

2.1.2. INK4 Family of CDK Inhibitors

CDK activity is negatively controlled by association with CDK inhibitors (CKIs), which inactivate CDK/cyclin complexes and thereby cause growth arrest *(39,17)*. CKIs are grouped into either the INK4 (inhibitors of CDK4) family or the CIP/KIP family based on their structure and which CDK they target. There are four INK4 CKIs and three CIP/KIP CKIs (*see* **Subheading 2.1.3.**). The first class of inhibitors includes the INK4 proteins, which specifically inhibit CDK4/cyclin D1-associated kinase activity and are therefore specific for early G1 phase *(49)*. Four such proteins have been identified{\\}$p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ *(39)*. These INK4 CKIs compete with cyclin D for binding to CDK4 and consequently cause CDK4/cyclin D complexes to dissociate. Note that the major portion of these molecules is composed of four ($p16^{INK4a}$ and $p15^{INK4b}$) or five ($p18^{INK4c}$ and $p19^{INK4d}$) tandem ankyrin (ANK) repeats (**Fig. 1**). This 33-residue repeat was first discovered in ANK, a membrane protein of red cells. Later this motif was found in a wide variety of proteins *(50)*. The beta hairpin helix-loop-helix folds formed by the multiple tandem ANK repeats stack in a linear manner to produce an elongated structure that is considered to be involved in macromolecular recognition. $p16^{INK4a}$, which consists of four ANK repeats (**Fig. 1**), represents the minimal ANK folding unit *(51)*.

Of these CKIs, only the $p16^{INK4a}$ gene (also known as major tumor suppressor 1 or *MTS-1*) has been classified as a tumor suppressor by the genetic criteria of loss of heterozygosity (LOH) *(49)*. Unlike other tumor suppressor genes, *p16* is often silenced by homozygous deletions at the $p16^{INK4a}$ locus (9p21) that also often inactivate two other important genes nearby, namely *p15INK4b* and *p14ARF* (*see* **Subheading 2.3.**).

2.1.3. CIP/KIP Family of CDK Inhibitors

The second family of CDK inhibitors is composed of the more broadly acting CIP/KIP proteins, such as $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which inhibit the activities of cyclin D-, E-, and A-dependent kinases and induce cell cycle arrest *(39)*. Thus, these CKIs are not specific for a particular phase of the cell cycle. Unlike the INK4 proteins, the CIP/KIP proteins associate with the CDK-cyclin complexes and thus do not dissociate these complexes *(39,52)*. The CIP/KIP proteins share a homologous domain at their N-termini that is believed to
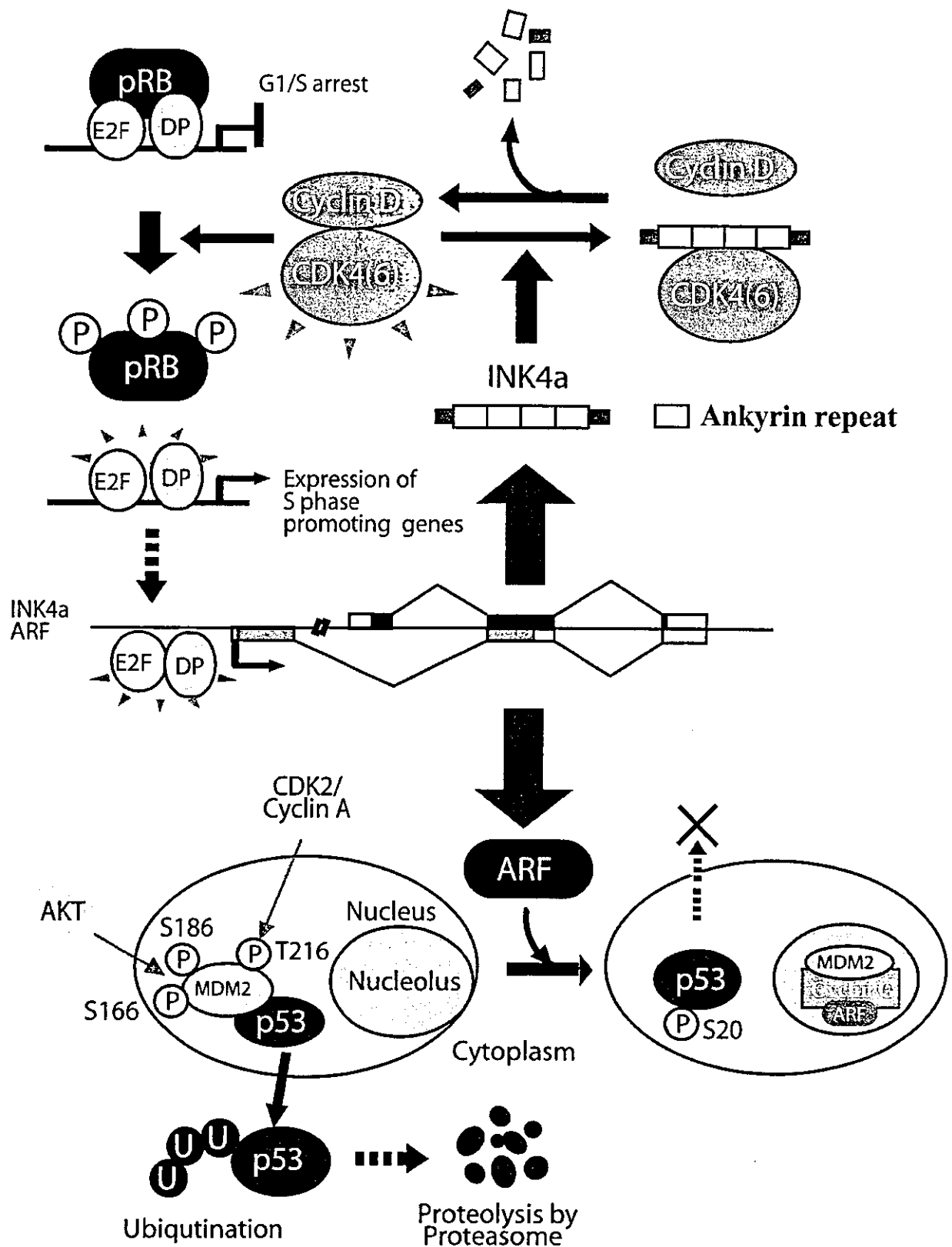
Fig. 1. The *INK4a/ARF* locus encodes p16^{INK4a} and p19^{ARF}, two degenerated tumor suppressor proteins. Absence of p16^{INK4a} (composed of ANK) activates CDK4(6)/cyclin D kinase, which phosphorylates pRB, thereby activating the E2F/DP1 transcription factor. Since the transcription of the *ARF* gene is regulated by E2F, expression of ARF is induced. ARF separates MDM2 from p53 by recruiting MDM2 into the nucleolus, possibly in collaboration with cyclin G1 and cyclin G2. This stabilizes p53 in the nucleus and allows it to activate the expression of the target genes that function in cell cycle checkpoint, DNA repair, and apoptosis. Thus, the *INK4a/ARF* locus influences both the pRB-E2F and p53 pathways.

participate in the CKI role of these proteins. This amino-terminal domain contains characteristic motifs that are used for binding to both the CDK and cyclin subunits. This association of CIP/KIP proteins with CDK/cyclin complexes has an inhibitory effect. For example, it inhibits CDK2 activity by preventing its Thr-160 phosphorylation by CAK.

p21^{WAF1} was discovered almost simultaneously by several studies, each of which employed different approaches. It was discovered as a CDK2-associated protein by a two-hybrid system *(53)*, as a senescent cell-derived inhibitor (Sdi1) of cellular growth *(54)*, as a potential mediator of p53 tumor suppression that was named wild-type p53-activated fragment (WAF1) *(55)*, and as a biochemically isolated CDK2/cyclin-binding protein *(56)*. Transcription of the *p21WAF1* gene can be induced by the tumor suppressor p53 *(55)*, by the antimitogenic cytokine transforming growth factor (TGF)-β *(57)*, and by the phorbol ester tetradecanoyl-phorbol acetate (TPA), which is a protein kinase C activator *(58)*.

Apart from their role as CDK inhibitors, the CIP/KIP proteins may also act to bridge the bond between CDK4 (or CDK6) and cyclin D protein types (but not other CDKs or cyclins), thereby enhancing this association and promoting the recruitment of the CDK/cyclin D complexes to the nucleus *(52,59,60)*. A fact supporting this notion is that mouse fibroblasts that lack both p21^{WAF1} and p27^{KIP1} are unable to assemble detectable amounts of CDK/cyclin D complexes, and fail to efficiently direct cyclin D proteins into the nucleus *(61)*. These effects were reversed by returning the CKIs to these cells. Thus, unlike the INK4 family proteins, the CIP/KIP CKIs promote the formation of CDK4/cyclin D complexes *(62)*.

In the nucleus, p21^{WAF1} binds to proliferating cell nuclear antigen (PCNA) and blocks DNA replication *(63–65)*. p21^{WAF1} may also regulate the transcription of the genes involved in growth arrest, senescence, aging, or apoptosis after DNA damage *(52)*.

2.1.4. Retinoblastoma Family of Proteins and E2F

The tumor suppressor pRB is the protein product of the retinoblastoma (Rb) susceptibility gene that is required for the arrest in the G1 phase of the cell cycle *(66,67)*. pRB, and the related p107 and p130 proteins, share structural and functional properties and interact with a number of common cellular targets. Consequently, they form together the "pocket protein family" *(36,68)* (**Fig. 1**). They function as transcriptional repressors in the nucleus and inhibit the activity of the E2F (early gene 2 factor) transcription factor that regulates the expression of the many genes required for S phase entry and DNA synthesis *(69)*. Although the pRB proteins do not interact directly with DNA, they repress E2F-regulated genes in two ways. First, they directly bind to the transactivation domain of E2F proteins to repress their transcriptional activity.

Second, they recruit chromatin remodeling enzymes such as histone deacetylases (HDACs) or methyl transferases to the nearby surrounding nucleosome structure *(46,70)*.

In the early G1 phase, pRBs are not phosphorylated and can associate with more than eighty proteins, including the E2F family of transcription factors. As G1 progresses, however, the pRB proteins become phosphorylated on multiple serine and threonine residues, primarily by the CDK4/cyclin D or CDK6/cyclin D complexes, but also partly by CDK2/cyclin E *(71)*. This hyperphosphorylation inactivates the pRB proteins and causes them to release their cargo proteins, which activates the cargo proteins and allows them to mediate the events that are required for further cell cycle progression *(39,72)*. The phosphorylation of pRB by the CDK4(6)/cyclin D complexes releases histone deacetylase, which alleviates transcriptional repression, whereas the phosphorylation by CDK2/cyclin E disrupts the pocket domain of pRB, causing the pRB–E2F complex to dissociate *(73)*. Recently, it was suggested that the acetylation of pRB proteins may also influence their activity *(74)*.

The E2F transcription factor was first identified as a transcriptional activity that influences the adenovirus E2 gene promoter *(75)*. The family of E2F-related proteins include E2F1–E2F6. These proteins have conserved DNA-binding and dimerization domains, and three heterodimeric partners, namely DP1, DP2, and DP3 *(76,77)*. Of the six E2Fs, E2F6 is exceptional in that it lacks the domains required for transactivation and pRB binding that are normally in the E2F carboxy-termini. E2F6 appears to play a pRB-independent role in gene silencing and modulation of G0 phase *(78,79)*. Recent studies have expanded the roles that are played by the E2F family of transcription factors. It now appears that apart from being transcriptional regulators of genes involved in DNA metabolism and DNA synthesis, these proteins also seem to play contrasting roles in transcriptional activation and repression, proliferation and apoptosis, tumor suppression and oncogenesis, and possibly differentiation and DNA repair *(66,69,76,80,81)*.

2.1.5. Cdc25A Phosphatase

Cdc25, a dual-specificity phosphatase, removes inhibitory phosphates from the tyrosine and threonine residues of CDKs and thereby promotes cell cycle progression *(82)*. Three Cdc25 homologs, namely Cdc25A, Cdc25B, and Cdc25C, have been identified in mammalian cells. Cdc25C promotes the G2/M transition by activating CDK1 (Cdc2), whereas Cdc25B is proposed to act as a "starter phosphatase" that initiates the positive feedback loop at the entry into M phase *(83,84)*. In contrast, Cdc25A plays an important role in the G1/S transition *(85)*. Overexpression of Cdc25A activates CDK2/cyclin E or CDK2/cyclin A by inducing CDK2 tyrosine dephosphorylation. The activated CDK2/

cyclin complexes then abrogate checkpoint-induced arrest in S phase *(86,104,199)*. Without Cdc25A activity, the inhibitory tyrosine phosphorylation of CDK2 would persist, which would maintain the block of entry into S phase and DNA replication. Cdc25A and Cdc25B (but not Cdc25C) are potential human oncogenes that have been found to be overexpressed in a subset of aggressive human cancers *(87,88)*.

To activate the protein kinase activity of CDK2, it must be phosphorylated on Thr-160 but not on Thr-14 and Tyr-15 *(89)*. A multi-subunit enzyme CAK, which consists of cyclin H and CDK7, phosphorylates CDK2 on Thr-160 *(90,91)*, whereas the KAP phosphatase dephosphorylates Thr-160 in the absence of cyclin, thereby rendering CDK2 inactive *(92)*. CDK2 is phosphorylated on Thr-14 and Tyr-15 by Wee1/Mik1-related protein kinases, whereas the Tyr-15 residue and possibly also the Thr-14 residue are dephosphorylated by Cdc25A *(93)*. Thus, downregulation of Cdc25A leads to growth arrest in late G1 *(94)*. Transcription of the *cdc25A* gene is inhibited by the E2F-4/p130 complex, which recruits histone deacetylase to the E2F site of the *cdc25A* promoter in response to TGF-β *(95)*.

As a cellular response to ultraviolet light (UV)-induced DNA damage, Cdc25A is highly degraded by ubiquitin- and proteasome-dependent proteolysis *(96)*. The same degradation also occurs after hydroxyurea (HU)-triggered stalling of replication forks *(97)* as well as during the midblastula transition in *Xenopus* embryos under physiological conditions *(98)*. Following DNA damage, Cdc25A is phosphorylated by checkpoint kinase 1 (CHK1) or checkpoint kinase 2 (CHK2) **(Fig. 2B)**. This phosphorylation is recognized as a tag by the proteolysis system and Cdc25A is degraded. Thus, CHK1 or CHK2 induces the G1/S phase checkpoint by phosphorylating Cdc25A *(see* **Subheading 4.2.**). Supporting this is the finding that the elimination of CHK1 expression through the use of siRNA not only abrogated the S or G2 arrest, it also protected Cdc25A from degradation*(88)*. During the basal turnover in unperturbed S phase, CHK1 phosphorylates serines 75, 123, 178, 278, and 292 of Cdc25A. In contrast, ionizing radiation (IR)-induced Cdc25A proteolysis is mediated by a combined action of CHK1 and CHK2. Thus, a CHK1-CHK2 inhibitor may be useful in cancer chemotherapy, as it may potentiate the cytotoxicity caused not only by DNA-damaging drugs that induce G2 arrest but also by agents that promote S arrest *(99,100)*.

2.1.6. Myc

The c-*myc* protooncogene is a pivotal regulator of cellular proliferation, growth, differentiation, and apoptosis *(101)*. The Myc family proteins (Myc, N-Myc, and L-Myc) are transcription factors with basic helix-loop-helix leucine zipper protein (bHLH-ZIP) motifs that bind to the DNA sequence

A

Double strand break

γ-Ray

Double strand break
DNA replication stress

HU

T68
ⓟ
CHK2

S31 ⓟ
E2F
S1423   S1457
S1387 ⓟⓟⓟ S1524
BRCA1
T99 ⓟ
BLM
S15 ⓟ
p53
S635 ⓟ ⓟ S645
RAD17
S140 ⓟ ⓟ S139
H2AX

ATM

ATRIP
ATR ⓟ

S317 ⓟ ⓟ S345
CHK1

S272 ⓟ
NC ⓟ
S343 ⓟ
S957 ⓥ S966
ⓟ ⓟ
MRE11 NBS MC
RAD50
ⓟ S25
BP
S1
ⓟ
LK
ⓟ
DC

DNA
replication

Apoptosis

Repair/
recombination

Cell cycle
arrest/delay

Damage induced
transcription

B

ATM

p53 S46
kinase

ATRIP
ATR

T387 ⓟ
CHK2 ⓟ
T383 ⓟ ⓟ T68
CHK2 ⓟ
ⓟ

S20
ⓟ
p53 ⓟ S46

S317 ⓟ ⓟ S345
CHK1

S364
ⓟ
E2F S988
ⓟ
BRCA1 S117 ⓟ
PML

S216
ⓟ
CDC25C

ⓟ
PLK3

S178
ⓟ ⓟ ⓟ S292
S123 CDC25A

S75 ⓟ ⓟ S278
CDC25A

S864 ⓟ ⓟ S895
Claspin

S695
ⓟ
TLK

Damage induced
transcription

Apoptosis

Repair/
recombination

Cell cycle
arrest/delay
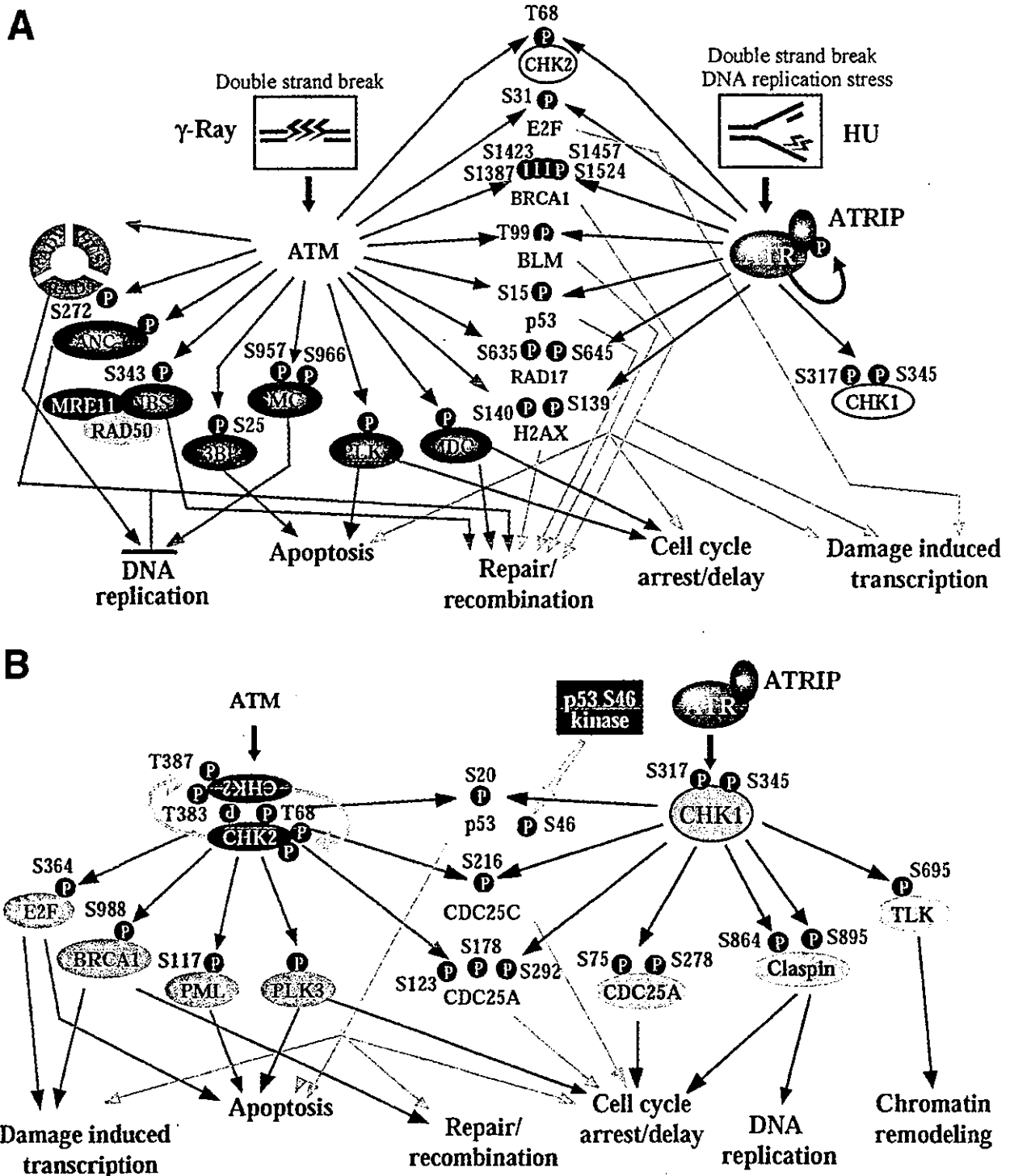
DNA
replication

Chromatin
remodeling

Fig. 2. ATM/ATR and CHK1/CHK2 kinases mediate the signaling network of the DNA damage and DNA replication checkpoints. (A) Phosphorylation target proteins of ATM and ATR kinases. There are two parallel pathways that respond to DNA damaging stress in mammalian cells. The ATM pathway responds to the presence of DSBs acting at all phases of the cell cycle. The ATR pathway not only responds to DSBs but also to the agents that disturb the function of replication forks. Following their activation by DSBs or replication stress, ATM/ATR kinases phosphorylate unique (red and black, respectively) or overlapping (green) target proteins at specific serine (S) or threonine (T) residues of indicated (if known) numbers. (B) Phosphorylation target

CACGTG (E-box) when dimerized with Max, another bHLH-ZIP. A head-to-tail pair of Myc-Max dimers form a heterotetramer that is capable of bridging distant E-boxes. Mitogen exposure promptly induces the expression of c-*myc*. Ectopic expression of c-*myc* also encourages quiescent cells to enter into S phase *(102)*. Myc not only targets genes that encode cyclins D2, D1 and E, and Cdc25A as a transcription factor, but also sequesters $p27^{KIP1}$ into CDK4(6)/cyclin D complexes away CDK2/cyclin E to cause phosphorylation and subsequent ubiquitination and proteasome-mediated degradation of the $p27^{KIP1}$, thereby realizing at least three distinct regulatory functions of CDK2/Cyclin E activity, E2F-dependent transcription, and cell growth *(103)*.

In association with Max, Myc binds to the E-boxes in a variety of gene promoters and thus orchestrates the transcriptional activation of a diverse set of genes. However, Myc on its own inhibits the transcription of other genes, including *p21WAF1* *(104)* and another cyclin-dependent kinase inhibitor, *p15INK4b* *(105,106)*. The DNA-binding protein Miz-1 directly recruits Myc to the *p21WAF1* promoter, where Myc selectively inhibits bound p53 from activating *p21WAF1* transcription and favors the initiation of apoptosis *(107)*. Thus, Myc can influence the outcome of a p53 response in favor of cell death.

2.2. The p53-pRB Pathway Controls the G1/S Transition

The *p53* tumor suppressor gene (TP53) is the most frequently mutated gene (about 50%) in human tumors, and encodes a 53 kDa transcription factor (p53) that directly induces the expression of a substantial number of genes that are important for cell cycle regulation, DNA damage repair, and apoptosis *(108,109)*. Of the genes that are induced by p53, $p21^{WAF1}$ plays a pivotal role in G1 arrest by inhibiting CDK4(6)/cyclin D1 activity, thereby reducing the phosphorylation of pRB and promoting G1 arrest of the cell cycle. This interconnecting signaling pathway involving p53, pRB, and E2F plays an essential role in G1/S transition of the cell cycle. $p21^{WAF1}$ is also known to inhibit S phase progression (G1 arrest) by binding to PCNA, a ring protein that promotes DNA replication *(63)*. The expression level of p53 is low in the absence

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Fig. 2. *(continued)* proteins of CHK1 and CHK2 kinases. CHK2 is primarily phosphorylated by ATM (and partially by ATR), whereas CHK1 is phosphorylated by ATR. Then, CHK1/CHK2 kinases transmit the checkpoint signals by phosphorylating unique (red and black, respectively) or overlapping (green) target proteins at specific serine (S) or threonine (T) residues of indicated numbers. CHK2, phosphorylated on Thr-68 by ATM, is activated to autophosphorylate on Thr-383 and Thr-387 (blue arrows), further enhancing its kinase activity. Ser-46 of p53 is presumed to be phosphorylated by putative p53 S46 kinase. These phosphorylated proteins further propagate the signal to the downstream targets, thereby regulating various cellular events.

of cellular stress. However, various types of stress, including DNA damage, induce p53 expression and cause G1 arrest. In cases where the DNA damage is too severe to be repaired, p53 induces apoptosis as a desperate attempt to protect the organism (19,110, 111). This essential role of p53 as a critical brake on tumor development explains why it is so frequently found in cancer cells (112,113).

Other genes that are upregulated by p53 (112) include cyclin G1 (27), MDM2 (murine double murine 2), BAX (bcl2-associated X protein), GADD45 (114), 14-3-3σ (115), CDK4 (116), p53R2 (117,118), p53AIP1 (119), p53DINP1 (120), and p53RDL1 (121). Cyclin G1 and MDM2 regulate the stability of the p53 protein (see Subheading 2.3.). Bax forms a homodimer or heterodimer with Bcl2, and increasing amounts of the Bax homodimer trigger cytochrome-c release from mitochondria, thus promoting apoptosis (122). Gadd45 (induced after growth arrest and DNA damage) is involved in regulating nucleotide excision repair of UV-damage together with p53 and another p53-downstream gene, p48XPE (123). The 14-3-3σ protein associates with and recruits Cdc25C from the nucleus to inhibit the activation of CDK1/cyclin B, thus causing G2 arrest. p53R2 is a homolog of ribonucleotide reductase small subunit (R2). Expression of p53R2, but not that of R2, is induced by DNA damage and serves to supply the cell with the deoxyribonucleotides needed for DNA repair. p53RDL1 (p53-regulated receptor for death and life) interacts with its ligand Netrin-1 and promotes the survival of damaged cells against apoptosis.

At least some of the eleven phosphorylation sites identified on p53 seem to play pivotal roles in its regulation. Three functionally important domains have been identified in the p53 molecule, and phosphorylation at these sites is considered to influence the structural changes of these domains. The middle domain constitutes the core domain that associates with the specific nucletotide sequences at the promoter regions of its target genes. This domain harbors the vast majority of the p53 "hot spot" mutations found in human cancers. In cells with damaged DNA, Ser-15 of p53 is phosphorylated by ATM (ataxia telangiectasia mutated) or ATR (ATM-Rad3-related) (124–126). The ATM gene was first isolated from patients with the autosomal recessive disorder ataxia telangiectasia (A-T). These patients exhibit cerebellar degeneration, immunodeficiency, radiation sensitivity, and predisposition to cancer (127). Phosphorylation of p53 at Ser-20 by CHK1 or CHK2 may also be important for regulating the interaction between p53 and MDM2 (128,129). Upon severe DNA damage, Ser-46 on p53 is phosphorylated and apoptosis is induced. As p53AIP1 (p53-regulated apoptosis-inducing protein 1) is selectively induced by p53 molecules that have been phosphorylated at Ser-46, it may be that p53AIP1 mediates this p53-dependent apoptosis by inducing the release of cytochrome-c from mitochondria (130). p53DINP1 (p53-dependent damage-

nducible nuclear protein 1) functions as a cofactor of the putative p53-Ser46 kinase that promotes phosphorylation of p53 at Ser-46 *(120)*.

2.3. Regulation of p53 Stability by ARF and MDM2

The *INK4a* locus that generates p16^{INK4a} also encodes a degenerated gene product called ARF (after alternative reading frame) *(131)*. Thus, the locus encodes two tumor suppressor proteins, p16^{INK4a} and p19^{ARF} (p14^{ARF} in humans), which activate the growth suppressive functions of pRB and p53, respectively *(67)*. ARF is a highly basic (pI > 12), arginine-rich nucleolar protein *(132)*. Deletion of the *ARF* gene can inactivate p53 function in tumors where p53 itself remains intact. Transcription of the *ARF* gene is regulated by E2F, and thus the *INK4a/ARF* locus influences both the pRB-E2F and p53 pathways.

Overexpression in the same tumor lines of MDM2 (murine double murine 2; Hdm2 in humans), a protein whose expression is upregulated by p53 (*see* **Subheading 2.2.**), has the same effect. This is because ARF binds to MDM2 and abrogates its p53-inhibitory activity. MDM2 destabilizes p53 by catalyzing its ubiquitination by acting as an E3 ubiquitin ligase. This promotes the nuclear export of p53, thereby allowing it to be targeted for proteasomal degradation *(133,134)*. Actually, MDM2 is frequently overexpressed in human tumors, and this leads to the rapid degradation of p53 *(135)*. Since MDM2 directly binds to the N-terminus of p53, phosphorylations of p53 at Ser-15, Ser-20, and Thr-18 are important for the dissociation of MDM2 from p53 *(128,129)*. MDM2 is itself transcriptionally activated by p53, which thus creates a negative feedback loop. Consequently, inhibiting the interaction between p53 and MDM2 by the application of synthetic molecules may serve as an effective cancer treatment because it may lead to cell cycle arrest or apoptosis in p53-positive tumor cells *(109)*.

Other proteins also modulate MDM2 activity. Mitogen-induced activation of phosphatidylinositol 3-kinase (PI3-kinase) and its downstream target, the AKT/PKB serine-threonine kinase, results in the phosphorylation of MDM2 on Ser-166 and Ser-186. CDK2/cyclin A also phosphorylates MDM2 on Thr-216 *(136)*. These phosphorylation events are necessary for the translocation of MDM2 from the cytoplasm into the nucleus and thus serve to promote the p53-inhibitory activity of MDM2 as a ubiquitin ligase *(134)*. Cyclin G1 directly binds to MDM2 *(137)*, recruits PP2A (protein phosphatase 2A) to dephosphorylate MDM2 at Thr-216, and releases MDM2 from p53, thereby cooperating with ARF to restrict the ability of MDM2 to negatively regulate p53 *(138)* (**Fig. 1**). Indeed, cyclin G1^{-/-} mouse embryo fibroblasts show enhanced accumulation of p53 and are partially deficient in an irradiation-induced G2/M-phase checkpoint *(139)*. Cyclin G2 may also have redundant or compensatory

functions, because it associates with many of the same proteins to which cyclin G1 binds, including p53, PP2A, MDM2, and ARF *(137)*. This p53-stabilizing effect of PP2A/cyclin G complexes may also influence the malignancy of cancer cells, considering that enhanced expression of a truncated form of PP2A was observed in highly metastatic melanoma cells *(140)*. Cells overexpressed with this truncated form of PP2A show irradiation-induced checkpoint defects and appear to elevate genetic instability, which may promote tumor progression *(141)*. These data suggest that cyclin G1 is a positive feedback regulator of p53, since it downregulates the activity of MDM2, which would otherwise restrain the accumulation of p53 *(142)*.

3. DNA Damage Checkpoints

DNA damage caused by IR, chemical reagents, or similar environmental insults induces cell cycle arrest at G1, S, or G2, thereby preventing the replication of damaged DNA or aberrant mitosis until the damage is properly repaired. The molecular mechanism in mammalian cells that detects the presence of double-strand breaks (DSBs) is not well understood. Research in the budding yeast *Saccharomyces cerevisiae,* however, tells us that a quintet complex composed of RAD24, RFC2, RFC3, RFC4, and RFC5 acts in this organism as a sensor of DSBs *(143)*. Disruption of these components causes defects in the damage checkpoint machinery of *S. cerevisiae (144)*. The same DSB-sensing mechanism is also used in another useful yeast strain, *Schizosaccharomyces pombe (145,146)*. In *S. cerevisiae,* the signal of the DSB abnormality is transmitted to the ring-shaped hetero-trimer that is composed of Ddc1/Rad17/Mec3 (Rad9/Rad1/Hus1 in fission yeast and mammals). This hetero-trimer resembles the replication factor PCNA *(147)*. In fission yeast, this complex activates Rad3 kinase, which then phosphorylates CHK1 *(148)*. The activated CHK1 then targets Cdc25C for phosphorylation. Cdc25C is subsequently recognized by Rad24, a 14-3-3 protein (*see* **Subheading 2.2.**), which recruits it out from the nucleus into the cytoplasm, where it inactivates the CDK1/cyclin B complex (Cdc2/Cdc13 in *S. pombe*), which results in G2/M arrest *(149)*. The 14-3-3 proteins bind to serine/threonine-phosphorylated residues in a specific manner and regulate key proteins involved in various physiological processes such as the cell cycle, intracellular signaling, apoptosis, and transcription regulation *(150)*. Similar checkpoint regulatory mechanisms involving 14-3-3 proteins are also employed in vertebrate cells *(2,86,151)*. For example, human CHK1 is activated by phosphorylation and thereby phosphorylates Cdc25C on Ser-216, which is recognized by the 14-3-3σ protein. The 14-3-3σ protein then removes Cdc25C from the nucleus to the cytoplasm, thereby preventing the activation of the CDK1/cyclin B complex and entry into mitosis *(3,152–154)*.

In mammalian cells, there are two parallel pathways that respond to DNA-damaging stresses (**Fig. 2**). The first pathway is the ATM pathway, which responds to the presence of DSBs at all phases of the cell cycle. The second pathway is the ATR (ATM-Rad3-related) pathway, which responds not only to DSBs but also to the agents that interfere with the function of replication forks *(126,4,127)*. A third pathway may involve the newly identified ATX (ATM-related X protein), which phosphorylates and activates CHK1 and/or CHK2 *(126,127,155)*. As shown in **Fig. 2**, ATM phosphorylates many target proteins at their specific serine or threonine residues and activates their functions. In response to IR, for example, ATM phosphorylates RAD9 on Ser-272 *(156)*; PLK3, which further phosphorylates CHK2, contributing to its full activation *(157)*; SMC1 (the cohesin protein) on Ser-957 and Ser-966 *(158,159)*; H2AX on Ser-140, which is required for 53BP1 accumulation at DNA break areas *(160)*; 53BP1 on Ser-6, Ser-25/Ser-29, and Ser-784 *(161)*; and MDC1 *(162)*.

The human ATR protein complexes stably with a protein called ATRIP (ATR-interacting protein). These complexes localize in nuclear foci after damage and thus appear to be recruited to the sites of DNA damage *(163)*. The ATR homologs in fission yeast (Rad3) and budding yeast (Mec1) also form similar complexes with the ATRIP-related factors Rad26 and Ddc2/Lcd2/Pie1, respectively, which are also recruited to the sites of DNA damage *(164–167)*. ATR phosphorylates H2AX on Ser-139 *(168)*, whereas ATM/ATR phosphorylate E2F on Ser-31 *(169)*. The checkpoint functions of ATM in response to IR are primarily mediated by the effector kinase CHK2, whereas those of ATR in response to replication inhibition and UV-induced damage are mediated by CHK1. Thus, the structurally unrelated CHK2 and CHK1 proteins channel the DNA damage signals from ATM and ATR, respectively *(21,170)*. However, recent observations suggest the existence of various "crosstalks" among these kinases *(100,171)*, and the presence of a novel checkpoint cascade signaling by way of ATM-CHK1 to Tousled-like kinases (TLKs) that causes chromatin remodeling in response to various stresses *(172)*.

The expression of the labile CHK1 protein is restricted to the S and G2 phases *(173)*. Although it is active even in unperturbed cell cycles, it is further activated in response to DNA damage or stalled replication *(100,174)*. Following a checkpoint signal, CHK1 is phosphorylated on Ser-317 and Ser-345 by ATR in cooperation with the sensor complexes, which include the mammalian homologs of Rad17 and Hus1. The phosphorylation at the Ser-345 site is required for nuclear retention of CHK1 following an HU-induced checkpoint signal *(175–177)*. CHK1 not only stimulates the kinase activity of DNA-dependent protein kinase (DNA-PK) complexes, which leads to increased phosphorylation of p53 on Ser-15 and Ser-37; it also elevates the DNA-PK-

dependent end-joining reactions, thereby promoting the repair of DSBs *(178)*. *CHK1⁻/⁻* mice show a severe proliferation defect and death in embryonic stem (ES) cells and peri-implantation embryonic lethality. The ES cells lacking CHK1 have also been shown to have a defective G2/M DNA damage checkpoint in response to IR *(179,180)*. In contrast, CHK1-deficient cells called DT40 are viable, but they fail to arrest at G2/M in response to IR and fail to maintain viable replication forks when DNA polymerase is inhibited *(181)*.

In contrast, the other Ser/Thr protein CHK2 kinase (also known as hCds1) must be phosphorylated at Thr-68 by ATM to activate it in response to IR-induced DNA damage (this is not the case for damage owing to UV or HU) *(170,182)*. Unlike CHK1, CHK2 is a stable protein that is expressed throughout the cell cycle and that seems to be inactive in the absence of DNA damage *(173)*. Its activation involves its dimerization and autophosphorylation. Unlike the catalytically inactive form of CHK2, wild-type CHK2 leads to G1 arrest after DNA damage by phosphorylating p53 on Ser-20, which causes the preformed p53/MDM2 complexes to dissociate and increases the stability of p53 *(128)*. Unlike *ATM⁻/⁻* and *p53⁻/⁻* mice, *CHK2⁻/⁻* mice do not spontaneously develop tumors, although the IR-induced G1/S cell cycle checkpoint—but not the G2/M or S phase checkpoints—was impaired in primary mouse embryonic fibroblasts (MEFs) derived from *CHK2⁻/⁻* mice *(183,184)*.

That the fission yeast homolog of CHK2, Cds1, may participate in repair is suggested by the finding that it interacts with the Mus81–Eme1 endonuclease complex, which can resolve the Holliday junction *(185,186)*. The human Mus81–Eme1 complex also has a similar function as a flap/fork endonuclease that is likely to play a role in the processing of stalled replication fork intermediates *(187)*.

CHK1 and CHK2 share partly redundant roles in that they target common downstream effector proteins such as the Polo-like kinase 3 (PLK3) *(188)*, the promyelocytic leukemia (PML) protein *(189)*, the E2F1 transcription factor *(190)*, or the TLKs *(172)*. PLK3 binds to and phosphorylates p53 on Ser-20. Through this direct regulation of p53 activity, PLK3 is at least partly involved in regulating the DNA damage checkpoint as well as M-phase function. The *PML* gene is translocated in most acute promyelocytic leukemias and encodes a tumor suppressor protein that plays a pivotal role in gamma irradiation–induced apoptosis. It is proposed that CHK2 mediates gamma irradiation–induced apoptosis in a p53-independent manner through an ATM-CHK2-PML pathway *(189)*. PML also recruits CHK2 and p53 into the PML-nuclear bodies, and enhances the p53/CHK2 interaction to protect p53 from MDM2-mediated ubiquitination and degradation *(191)*. Mutations in the prototypic member of the Tousled (Tsl) kinase from the plant *Arabidopsis thaliana* lead to a pleiotropic phenotype *(192)*. In mammals, however, the TLKs are regulated in a cell

cycle-dependent manner that peaks at S phase and are involved in chromatin assembly by phosphorylating the chromatin assembly factors Asf1a and Asf1b *(193)*. CHK1 phosphorylates TLK1 on Ser-695 in vitro, and substitution of Ser-695 with alanine impairs the efficient downregulation of TLK1 after DNA damage *(172)*.

4. G1 Checkpoint Response

In mid-to-late G1, and if the cellular environment is favorable for proliferation, a binary decision—whether to commit to the mitotic cell cycle and enter S-phase, or whether to not commit to the cell cycle and remain in a quiescent, non-proliferative state—is made at the "restriction point" *(194,195)*. As described above, many proteins are involved in making this critical decision and in ensuring proper progression of the G1/S transition. Although cyclin D meets the criteria of the critical restriction point factor, the system seems to be far more complex than just relying on a single factor. Moreover, the relationship between the restriction point and DNA damage checkpoints remains elusive *(196)*. The cell cycle checkpoints that monitor the proper G1/S transition and S phase progression during potentially hazardous genotoxic stress *(103, 197)* will be discussed in the following sections.

4.1. The ATM(ATR)/p53-Mediated Pathway

The ATM(ATR)/p53 pathway plays a pivotal role in one of the checkpoint mechanisms that arrest the cell cycle at G1 phase following DNA damage (G1 checkpoint) (**Fig. 3**). As described in **Subheading 3**, ATM is activated in response to IR, whereas ATR is activated in response to replication inhibition or UV-induced damage. The activated ATM or ATR then phosphorylates p53 (on Ser-15), and this phosphorylation causes MDM2 to dissociate from p53, which stabilizes p53 and leads to its accumulation *(128,129)*. Increased expression of ARF owing to E2F1 stabilization in response to DNA damage also blocks the inhibitory function of MDM2, thereby increasing the nuclear amount of p53.

The principal kinases relaying ATM(ATR)-initiated checkpoint signaling are preferentially CHK2 for ATM and CHK1 for ATR. In response to IR or DNA replication stress, ATR phosphorylates CHK1 at Ser-317 and Ser-345 *(175,177,198)*, which moderately increases its kinase activity and allows it to propagate the signal to downstream effectors, including p53, which CHK1 phosphorylates on Ser-20 *(129)*. In response to IR, ATM phosphorylates CHK2 at Thr-68 *(199)*, followed by CHK2 autophosphorylation on Thr-383 and Thr-387 and the activation of several target proteins, including p53, which CHK2 also phosphorylates on Ser-20 *(128,129)*. These Ser-20 phosphorylation events both induce MDM2 to dissociate from p53.
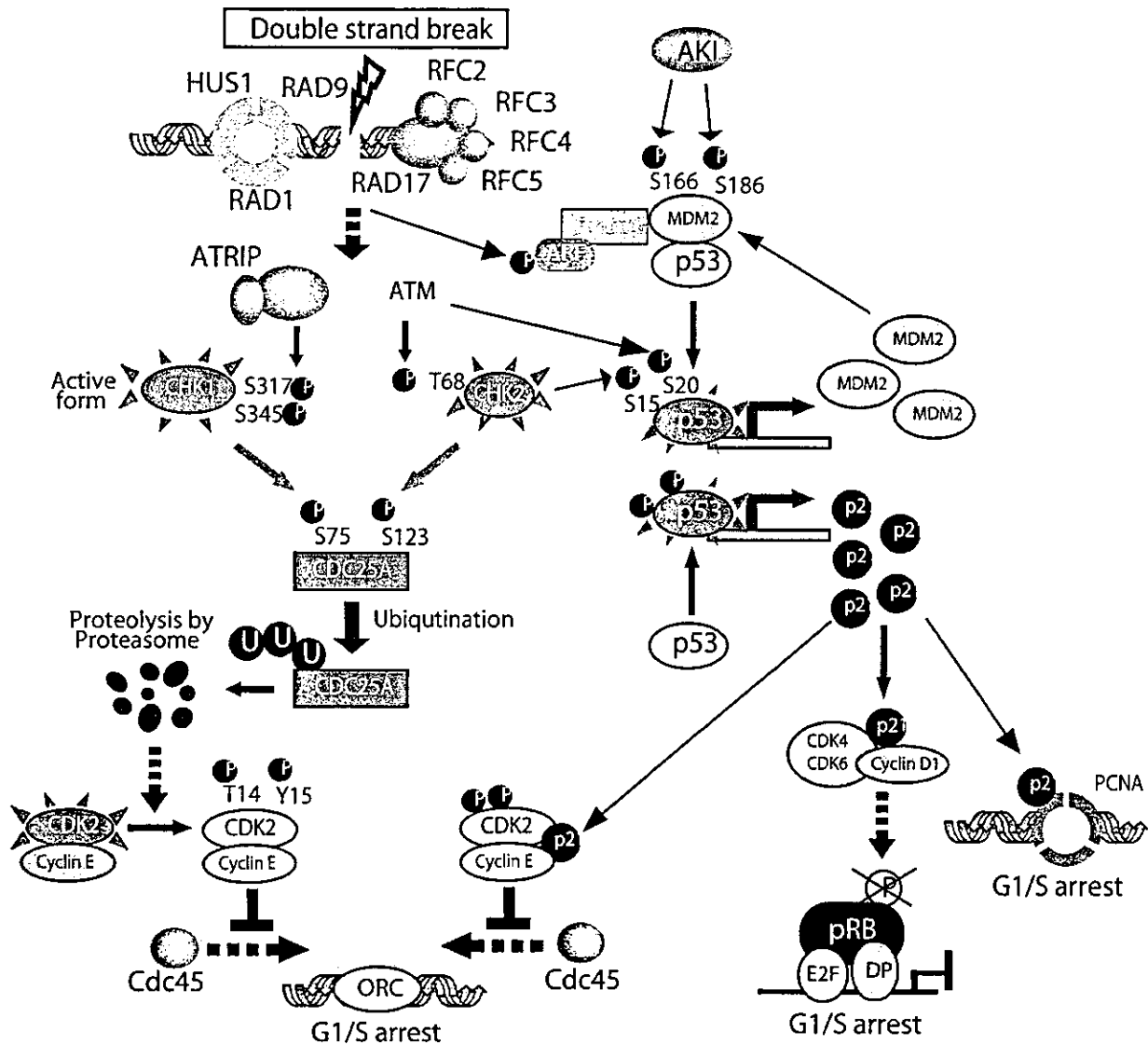
Fig. 3. ATM(ATR)-mediated G1/S checkpoint pathways. DNA damage triggers a rapid cascade of phosphorylation events involving either the ATM and CHK2 (upon IR) or the ATR and CHK1 (upon UV light) kinases. These phosphorylation events activate the target protein kinases to trap and phosphorylate the next target proteins, thereby transmitting the DNA damage signals. It has been determined that in response to IR, ATM phosphorylates CHK2 at Thr-68, whereas ATR (or ATM) phosphorylates CHK1 at Ser-317 and Ser-345. In one pathway (left), the CHK2 or CHK1 kinase phosphorylates Cdc25A phosphatase at serines 75, 123, 178, 278, and 292 (100). Of these, the Ser-123 residue that is targeted by CHK2 and the Ser-75 residue that is phosphorylated by CHK1 seem to be particularly critical residues of Cdc25A (202, 99). The phosphorylated Ser-123 or Ser-75 residue is recognized by the ubiquitination (Ub) enzyme, and this promotes the rapid degradation of Cdc25A by the proteasome. Due to the disappearance of Cdc25A phosphatase activity that this degradation causes, the CDK2/cyclin E complex is locked in its inactive form because of the presence of the inhibitory phosphorylation on the Thr-14 and Tyr-15 residues of CDK2. Thus, the CDK2/cyclin E complex fails to load Cdc45 onto chromatin and the blockade of the initiation of the DNA replication origins is maintained.

The stabilized and activated p53 protein that results from CHK1/CHK2-mediated phosphorylation induces the transcription of a large number of genes, including *p21*^{WAF1}, which silences the kinase activities of the CDK2/cyclin E, CDK2/cyclin A, or CDK4(6)/cyclin D complexes. This prevents the complexes from loading the Cdc45 origin binding factor onto chromatin, which precludes the recruitment of DNA polymerases, thereby blocking initiation of DNA replication from the unfired origins *(200,201)*. Another important consequence of inhibiting both the CDK2 and CDK4(6) kinase complexes is that these complexes cannot then phosphorylate pRB, which allows pRB to maintain its inhibition of the E2F-dependent transcription of S-phase genes that are essential for S-phase entry as described in **Subheading 2.1.4.** These effects all result in G1 arrest. Maintenance of the G1/S arrest by way of this pathway after DNA damage is a delayed response that requires the transcription, translation, and/or protein stabilization of key checkpoint transducers. However, once initiated, this pathway provides a long-lasting G1 arrest, and the entry into S phase is prevented as long as a single unrepaired DNA lesion is detected by the checkpoint machinery.

1.2. The ATR(ATM)/Cdc25A-Mediated Pathway

The human Cdc25A phosphatase plays a pivotal role at the G1/S transition because it enhances the kinase activities of the CDK2/cyclin E and CDK2/cyclin A complexes by dephosphorylating the inhibitory phosphorylated Thr-14 and Tyr-15 residues of CDK2 *(102197)*. After UV and IR exposure, Cdc25A is ubiquinated because it is phosphorylated by CHK1 (in the case of UV)

Fig. 3. *(continued)* In the other pathway (right), Ser-15 of p53 is directly phosphorylated by ATM or ATR in cells with damaged DNA. The phosphorylation of p53 at Ser-20 by CHK1 or CHK2 induces the dissociation of the p53/MDM2 complex, which increases the stability of p53 because MDM2 primes p53 for ubiquitination and proteasomal degradation (*see* **Fig. 1**). CHK1 also stimulates the kinase activity of DNA-PK complexes, which increases the phosphorylation of p53 on Ser-15 and Ser-37. Furthermore, DNA damage can also upregulate ARF, which specifically inhibits MDM2, putatively in collaboration with the cyclin G proteins. The collective result is that stable and transcriptionally active p53 transcription factor accumulates in the cell nucleus and induces the expression of a large number of target genes, including the p21 CDK inhibitor. The increased p21 levels inhibit the CDK2/cyclin E complex or the CDK4(6)/cyclin D complexes, thus arresting the cell cycle at G1/S phase. Upon severe DNA damage, Ser-46 on p53 is phosphorylated by the putative p53-Ser46 kinase with the aid of p53DINP1, which selectively induces the expression of p53AIP1, which is a mediator of apoptosis because it induces the release of cytochrome-*c* from mitochondria.

(87,96,) and CHK2 (in the case of IR) *(202)*. The critical residue of Cdc25A that is targeted by CHK2 is Ser-123 *(202)*. Cdc25A is also phosphorylated on Ser-75 by CHK1 *(109)*. The phosphorylated Ser-123 residue (and possibly also the phosphorylated Ser-75 residue) is recognized by the ubiquitination (Ub) enzyme, which promotes the rapid degradation of Cdc25A by the proteasome system. Removal of Cdc25A in turn keeps the CDK2-associated kinase complexes in their inactive form due to the persisting inhibitory phosphorylation of their Thr-14 and Tyr-15 residues. This results in G1 arrest. The important target of this cascade is the inhibition of CDK2-dependent loading of Cdc45 onto DNA pre-replication complexes. Thus, the ATM(ATR)–CHK2(CHK1)–Cdc25A–CDK2 pathway accounts for the rapid and p53-independent initiation of the G1 checkpoint, where the abundance and activity of Cdc25A decreases without delay in response to IR- or UV-mediated DNA damage **(Fig. 3)**. It is likely that this regulatory mechanism is conserved among vertebrates and operates in every cell type.

During interphase, CDK2 appears to phosphorylate Cdc25A, which constitutes a Cdc25A-CDK2 autoamplification feedback loop *(203)*. Cdc25A also seems to be involved in the G2/M transition, besides its commonly accepted effect on G1/S progression *(87)*. Proteolysis of Cdc25A is also linked with the intra-S-phase checkpoint, which guards against premature entry into mitosis in the presence of stalled replication forks.

4.3. Other Potential G1 Checkpoint Pathways

It has been reported that there is another G1 checkpoint induced by IR exposure, which is characterized by enhanced protein degradation *(204)*. In this checkpoint, DNA damage unmasks a cryptic "destruction box" (RxxL) within the cyclin D1 amino-terminus that is then recognized by the anaphase-promoting complex (APC) ubiquitin ligase, which primes cyclin D1 for rapid proteasomal destruction *(197)*. This causes the p21^{WAF1} protein, which served as an assembly factor of the CDK4(6)/cyclin D1 complexes, to be released. p21^{WAF1} is then free to bind to another of its targets, the CDK2/cyclin E complex. This binding inactivates the kinase activity of the complex **(Fig. 3)**. Since the proliferation of many mammalian somatic cells depends on the presence of abundant CDK4(6)/cyclin D1 complexes, the destruction of cyclin D1 together with the inactivation of the S-phase-promoting CDK2/cyclin E strongly induces G1 arrest.

Exposure of epithelial cells to UV light can also lead to yet another G1 checkpoint mechanism. This mechanism involves the gradual accumulation of p16^{INK4a}, which selectively disrupts the CDK4(6)/cyclin D1 complexes. This again causes the release of p21^{WAF1}, which can then bind to and inhibit CDK2/cyclin E, thereby resulting in G1 arrest. If these mechanisms are confirmed as

cell cycle checkpoints, they would each serve as examples of an ATM-independent, cell-type-restricted response. Note that because cyclins D2 and D3 are not degraded upon DNA damage, these pathways would have little effect in cell types that express several D-type cyclins or lack cyclin D1.

To ensure the exact duplication of the genome during every cell division, which is a basic requirement of every proliferating cell, eukaryotes adopt a strategy that temporally separates the assembly of the pre-replication complex (pre-RC) from the initiation of DNA synthesis **(Fig. 4)** *(201,205)*. A key component of the pre-RC is the hexameric minichromosome maintenance (MCM) protein complex, which consists of the six Mcm2–Mcm7 proteins *(206)*. The MCM complex is presumed to be a helicase functioning in the growing forks, and like other helicase proteins *(207)*, it actually adopts a toroidal structure when observed under a microscope *(208)*. The MCM complex is recruited to the replication origins, where the two protein kinase complexes Cdc7–Dbf4 (in budding yeast) and CDK2/cyclin E trigger a chain reaction that results in the phosphorylation and activation of the MCM complex and finally in the initiation of DNA synthesis *(201,209)*. At the onset of S phase, S-phase kinases promote the association of Cdc45 with MCM at the origins. Upon the formation of the MCM–Cdc45 complex at the origins, the duplex DNA is unwound and various replication proteins, including DNA polymerases, are recruited onto the unwound DNA *(200)*. A "licensing checkpoint" that prevents passage into S phase in the absence of sufficient origin licensing may also exist in mammalian cells *(210)*.

5. The S-Phase Checkpoint

Proliferating cells are always exposed to life-threatening insults that disturb the proper replication and segregation of their genomes into daughter cells. In response to these genotoxic insults, eukaryotic cells have evolved checkpoint mechanisms that monitor the progression of DNA replication at S phase and halt replication if an abnormality is observed **(Fig. 4)** . At least two distinct S-phase checkpoints seem to exist. One of these occurs in response to DNA-replication stress that interferes with the proper progression of the replication forks. The other is an intra-S-phase checkpoint that functions in response to DSBs *(2,4)*. The S-phase checkpoint may also have a function during an unperturbed S phase, because even in the absence of exogenous agents, mutants of the many genes that are involved in this checkpoint show aberrant checkpoint signaling, and some mutants also cause checkpoint induction *(2)*.

5.1. S-Phase Checkpoint in Response to DNA Replication Stress

Several types of agents are known to interfere with the function of replication forks and to elicit the S-phase checkpoint. These include agents that
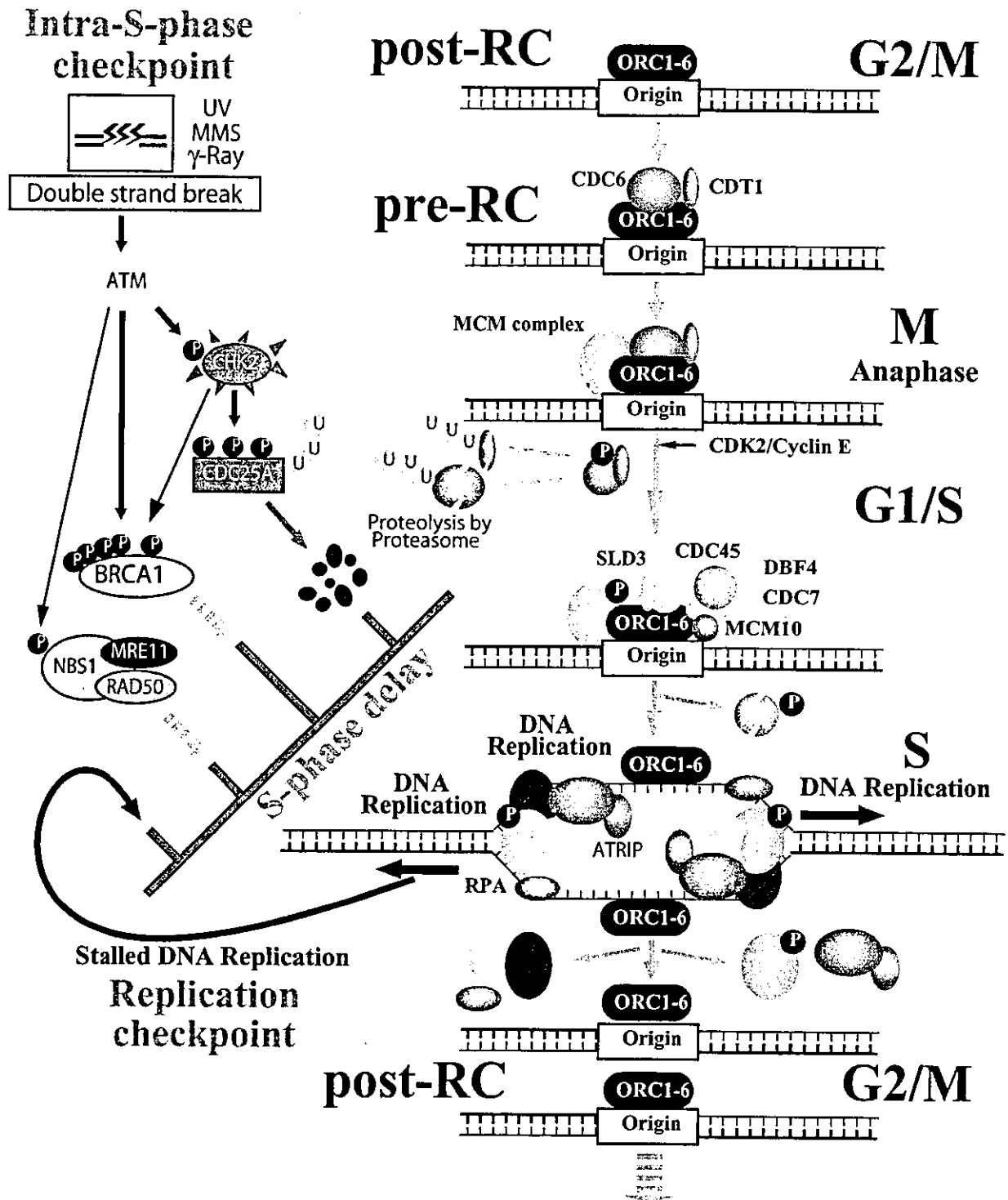
Fig. 4. Molecular mechanism of S-phase progression and the S-phase checkpoints. Upon initiation of DNA replication, DNA replication initiation factors such as MCM10, CDC45–Sld3 (budding yeast) and RPA and checkpoint complexes bind to the pre-replicative complex (pre-RC) on chromatin and trigger the unwinding of DNA. The hexameric MCM2-7 complex is recruited to the replication origins by a number of proteins, including MCM10, RPA, and CDC45–Sld3. The MCM complex is a putative helicase of the growing forks. Two protein kinase complexes, Cdc7/Dbf4 (budding yeast) and CDK2/cyclin E, trigger a chain reaction that results in the phosphorylation of the Mcm complex and finally the initiation of DNA synthesis. The