irectly inhibit DNA synthesis. For example, HU stalls replication forks by epleting the deoxynucleotide triphosphate (dNTP) pool, while aphidicolin ac-vates the checkpoint by inhibiting DNA synthesis by blocking the activities f polymerases (**Fig. 4**) . In addition, DNA-modifying agents that block repli-ation can elicit the S-phase checkpoint. These agents include methyl iethanesulfonate (MMS) and UV-induced DNA lesions, which slow down ie rate of DNA-replication-fork progression in budding yeast *(211)*.

The study of the DNA-replication checkpoint is most advanced in yeasts. ;owever, the checkpoint mechanisms that were unveiled in yeasts also seem) be conserved in mammalian cells *(2,149)*. The central checkpoint kinases Iec1 (ATR in humans) and Rad53 (CHK2 in humans) play an essential role in iaintaining DNA replication fork stability in response to DNA damage and ;plication fork blockage, and they inhibit the activation of late-firing replica-on origins after HU and MMS exposure *(4)*. The DNA replication forks ap-ear to function both as the activator and as the primary effector of the S-phase ieckpoint pathway, since the recruitment of Ddc2 (ATRIP in humans) to uclear foci and the subsequent activation of the Rad53 kinase occurs only uring S phase and requires the assembly of the replication forks *(212)*.

In budding yeast, proteins that are essential for DNA replication, such as 'NA polymerase ε and its interacting partners Dpb11 and Drc1/Sld2, are also ;quired for efficient checkpoint activation *(213)*. Dpb11 and its human ho-iolog TopBP1 associate with the PCNA-like protein Ddc1 and human Rad9,

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ig. 4. *(continued)* ATR/ATRIP (Rad3/Rad26) complex and the Polα–primase com-.ex and several other replication proteins are also recruited to the unwound DNA. fter this, the RAD1/RAD9/HUS1 complex binds to chromatin, an event that requires .e RAD17/RFC2-5 complex.

Two kinds of S-phase checkpoint mechanisms are known. One monitors the stalled ;plication forks (DNA-replication checkpoint) while the other monitors the replica-on block induced by DSBs during S phase (intra-S-phase checkpoint). In contrast to .e checkpoints at the G1/S and G2/M transitions that arrest the cell cycle, these S iase checkpoints can only delay the progression of S phase. Proteins involved in the gulation of DNA replication such as DNA polymerase ε, Dpb11 (TopBP1 in hu-ans), Drc1/Sld2 (budding yeast), Ddc1 (budding yeast), and RPA are also required ır the S-phase checkpoint in response to replication blockage. Claspin (Mrc1 in yeast) at is phosphorylated at Ser-864 and Ser-895 by CHK1 also regulates the S-phase ieckpoint. ATM is the master transducer of the S-phase checkpoint and phosphory-tes BRCA1 and BRCA2 as well as NBS1 (at Ser-343), which is a component of the BS1/MRE11/RAD50 complex. CHK1 also phosphorylates TLK (at Ser-695), a pro-in kinase that is potentially involved in regulation of chromatin assembly. Acetyla-on of nucleosomal histone H3 or H4, which regulates the chromatin structure, and ;ne expression also play a role in the S-phase checkpoint.

respectively, and seem to collaborate in monitoring the progression of replication forks *(214,215)*. The Polα–primase complex and RPA (replication protein A) are also required for the S-phase checkpoint in response to replication blocks *(216)*.

Claspin, a CHK1-interacting protein, is required for the ATR-dependent activation of CHK1 in *Xenopus* egg extracts that contain incompletely replicated DNA *(217)*. Claspin, ATR, and Rad17 bind to chromatin independently and appear to collaborate in checkpoint regulation by detecting different aspects of the DNA replication fork *(218)*. *Xenopus* Claspin may be phosphorylated at Ser-864 and Ser-895 by CHK1 *(219)*. Human Claspin is a cell cycle-regulated nuclear protein whose levels peak at S/G2 phase and that is phosphorylated in response to replication stress or other types of DNA damage. It appears to work as an adaptor molecule that brings the ATR/CHK1 and RAD9/RAD1/HUS1 complexes together to regulate the S-phase checkpoint *(220)*. These observations suggest that the activation of CHK1 by ATR may be regulated by Claspin in a similar way in budding yeast: Rad9 is phosphorylated by Mec1 in response to DNA damage and subsequently serves as a scaffold protein for Rad53, thus allowing Rad53 to autophosphorylate and self-activate *(221)*. Mrc1, a yeast homolog of Claspin, is also important for the activation of Rad53 and Cds1 in response to HU, and thus may mediate the checkpoint response to replication blockage in a similar manner to Claspin *(222,223)*.

In budding yeast, the S-phase checkpoint activates the ATM-like Mec1 and the CHK2-related Rad53 kinases in response to stalled replication forks that arise owing to replication stress or DNA damage in S phase. These kinases in turn inhibit spindle elongation and late origin firing, which stabilize the DNA polymerases at the arrested forks *(4)*. Orc 2 (origin recognition complex 2) plays a pivotal role in maintaining the number of functional replication forks, and the amount of DNA damage required for Rad53 activation is higher in S phase than in G2 *(224)*. For the S-phase checkpoint, acetylation of the nucleosomal histone H3 or H4 that regulates chromatin structure and gene expression also appears to be important *(225)*. Studies in fission yeast suggest that the signal activating the S-phase checkpoint is generated only when replication forks encounter DNA damage *(226)*.

5.2. S-Phase Checkpoint in Response to DSBs

After DNA damage, proliferating cells actively slow down their DNA replication by activating a checkpoint. This gives the cell time to repair the damage. This checkpoint is often called the intra-S-phase checkpoint (**Fig. 4**) *(4,21)*. The intra-S-phase checkpoint consists of regulatory networks that sense DNA damage and coordinate DNA replication, cell cycle arrest, and DNA repair.

'he above-mentioned Cdc25A degradation pathway also appears to induce the ransient intra-S-phase response. Here, IR-induced formation of DSBs triggers legradation of Cdc25A, which in turn inhibits the S-phase promoting activity of CDK2/cyclin E and induces the transient blockade of DNA replication, vhich delays S-phase progression for several hours *(227)*. As described above, 'dc25A destruction involves the phosphorylation of Cdc25A on Ser-123 by oth CHK1 and CHK2 in response to IR, and on Ser-75 by CHK1 in response o UV irradiation *(99)*. Supporting the involvement in the S-phase checkpoint of ATM, its phosphorylation targets including CHK2, and the CHK2-reguated Cdc25A-CDK2 cascade, is the fact that mutants of ATM, CDK2, or the other proteins in the CHK2-regulated Cdc25A-CDK2 cascade fail to inhibit S-hase progression when they are irradiated. Consequently, these cells undergo adio-resistant DNA synthesis (RDS), which is a phenomenon of persistent)NA synthesis after irradiation *(127,199)*.

Another phosphorylation target of ATM, the master transducer of the S-hase checkpoint, plays a key role in the intra-S-phase checkpoint, namely, 3RCA1 (breast cancer susceptibility gene 1). BRCA2 may also be an imporant target of ATM *(228,229)*. Mutations in the *BRCA1* and *BRCA2* tumor supressor genes are responsible for the great majority of familial breast and varian cancers. These proteins form nuclear foci with Rad51 during S phase nd after DNA damage *(230)*. *BRCA1*- and *BRCA2*-mutant cells exhibit deects in the homologous repair of chromosomal DSBs. *BRCA1* or *BRCA2* defiiency in mice results in early embryonic lethality, but conditional deletions eveal that mice with *BRCA1* or *BRCA2* mutations suffer a wide range of carciomas *(231)*. Moreover, a mammary epithelium whose *BRCA1* or *BRCA2* gene as been deleted is highly susceptible to mammary tumorigenesis *(232)*. *RCA1* is omnipresent and plays broad roles in transcriptional regulation that nclude both p53-dependent and -independent responses. It also has ubiquitin gase activity when dimerized to Bard1, and undergoes damage-associated hosphorylation by multiple kinases that precedes repair-complex formation *230)*. In contrast, *BRCA2* has a more straightforward function{\}it is central) homology-directed repair (HDR) because of its interaction with Rad51 and s direct binding to single-stranded DNA *(233)*.

Another important phosphorylation target of ATM that plays a role in the ntra-S-phase checkpoint is NBS1 (Nijmegen breakage syndrome gene 1) *(234–36)*. NBS 1 (Xrs2 in yeast) forms a multimeric complex with the MRE11/ AD50 nuclease, MDC1 (mediator of DNA damage checkpoint protein 1), nd other unidentified proteins, and recruits them to the vicinity of DNA damge sites by direct binding to the phosphorylated histone H2AX *(237)*. ATM hosphorylates NBS1 at Ser-343 in response to IR *(238)*. Cells harboring a oint mutation of NBS1 at this phosphorylation site failed to engage in the S-

phase checkpoint induced by IR *(239)*. Moreover, in collaboration with the BRCA1 C-terminus domain, the highly conserved NBS1 forkhead-associated domain plays a crucial role in the recognition of damaged sites *(240)*. After recognizing the DNA damage, the NBS1 complex proceeds to rejoin the DSBs predominantly by homologous recombination repair in vertebrates. This process collaborates with the cell cycle checkpoints at S and G2 phase to facilitate DNA repair.

Mutations in the MRE11-complex genes result in sensitivity to DNA damage, genomic instability, telomere shortening, aberrant meiosis, and abnormal checkpoint signaling in S phase. Blockade of NBS1-MRE11 function and the CHK2-Cdc25A-CDK2 pathway entirely abolishes the inhibition of DNA synthesis that is normally induced by IR. This results in the complete RDS that is also seen when cells harbor a defective ATM gene *(227)*. However, the phosphorylation of NBS1 and CHK2 by ATM seems to trigger two distinct branches of the intra-S-phase checkpoint because CDK2-dependent loading of Cdc45 onto replication origins, a prerequisite for the recruitment of DNA polymerase, is prevented in normal or NBS1/MRE11-defective cells when they are irradiated but not in irradiated cells that harbor a defective ATM protein *(227)*. 53BP1, which plays a partially redundant role in the phosphorylation of the downstream checkpoint effector proteins BRCA1 and CHK2, is also a key transducer of the intra-S-phase and G2-M checkpoint arrests that occur in response to IR *(241)*.

CHK1 may also be necessary for the intra-S-phase checkpoint when DNA synthesis is inhibited by DNA damage *(242)*. Supporting this is that chemical or genetic ablation of human CHK1 triggers the accumulation of Cdc25A, prevents the IR-induced degradation of Cdc25A, and causes RDS *(87)*. Moreover, the basal turnover of Cdc25A operating in unperturbed S phase requires CHK1-dependent phosphorylation of its Ser-123, Ser-178, Ser-278, and Ser-292 residues *(100)*. The ATR-CHK1 pathway may also play an important role in the intra-S-phase checkpoint that is induced by replication-associated DSBs caused by application of the topoisomerase I inhibitor topotecan (TPT) *(243)*, although it has no relationship with DNA-PK activity *(244)*. However, in budding yeast, the intra-S-phase checkpoint control is not activated by another topoisomerase I inhibitor, camptothecin (CPT), and the CPT-hypersensitive mutant strain that fails histone 2A (H2A) Ser-129 phosphorylation is an essential component for the efficient repair of DSBs that do not induce the intra-S-phase checkpoint *(245)*. In *Xenopus* egg extracts, DNA lesions generated by exonuclease or etoposide, a DNA topoisomerase II inhibitor, activate a DNA damage checkpoint that blocks the initiation of DNA replication *(246)*. TLK, a protein kinase that is potentially involved in regulating chromatin assembly and that is phos-

ɔhorylated by CHK1 on its Ser-695 residue, also appears to be involved in the ⁱTM/CHK1-dependent intra-S-phase checkpoint *(172)*.

Besides its function with H2AX (a histone H2A variant), Mdc1 (mediator of ƆNA damage checkpoint protein 1) controls damage-induced checkpoints by ɔromoting the recruitment of repair proteins to the sites of DNA breaks *(247)*. Cells that lack the *MDC1* gene are sensitive to IR because they fail to activate he intra-S-phase and G2/M checkpoints properly, probably due to an inability o regulate CHK1 properly. Thus, MDC1 facilitates the establishment of the ntra-S-phase cell cycle checkpoint *(248)*. Notably, MDC1 is hyperphosɔhorylated in an ATM-dependent manner, and rapidly relocalizes to nuclear ʼoci at sites of DNA damage, which appears to be crucial for the efficient acti/ation of the intra-S-phase checkpoint *(249)*.

The ATR/ATRIP complex requires the RFC (replication factor C) and ᵓCNA-like proteins to fully activate the replication-stress response because ʀFC recognizes the primer-template junction and recruits PCNA onto DNA to ʼunction as a sliding clamp that tethers DNA polymerases *(4,250)*. In fission /east and humans, the PCNA-like complex (Rad1/Rad9/Hus1 or RAD1/RAD9/ ꟷIUS1) is recruited in a RAD17-dependent manner onto the chromatin after lamage *(149,251)*. In budding yeast, the homologous PCNA-like complex Rad17/Mec3/Ddc1) is recruited to DSBs and the sites of DNA damage in a ʀad24-dependent manner *(252,253)*. Thus, it is possible that the Rad17 comɔlex recognizes DNA damage and loads the PCNA-like complex onto DNA, hereby responding to DNA damage independently of ATR/ATRIP *(254)*.

As with fission yeast, RAD17 and HUS1 are required for the phosphorylaion of CHK1 by ATR in mammals *(254,255)*. ATR also phosphorylates Rad17 ıt its Ser-635 and Ser-645 residues *(256)*. This phosphorylation is significantly ꜱtimulated by the increased amounts of PCNA-like complexes that were re:ruited onto the chromatin after damage. Unlike the *hus1*-null fission yeast :ells, which are defective for the G2/M DNA-damage checkpoint, mouse cells hat lack the mouse homolog of the fission yeast protein Hus1 enter mitosis ıormally after DNA damage but display an S-phase checkpoint defect *(257)*. Γhe mouse Hus1 protein also seems to play a role in the NBS1-independent :heckpoint-mediated inhibition of DNA synthesis that is generated by the ꞡenotoxin benzo(a)pyrene dihydrodiol epoxide (BPDE), which causes bulky ƆNA adducts. However, the *hus1*-null mouse cells displayed intact S-phase :heckpoint responses in response to IR-induced DSBs *(257)*.

›. Defects in G1/S Checkpoint and Cancer

Defects in the genome maintenance mechanisms, including DNA repair and :ell cycle checkpoint pathways, are believed to enhance genetic instability and

cause the accumulation of mutations and chromosomal aberrations that is a hallmark of cancer cells *(155)*. Most of the G1/S checkpoint transducers and effectors are classified as either tumor suppressors or proto-oncogenes, and their loss-of-function mutations or overexpression appear to play pivotal roles in many types of human tumors. Mouse models that mimic the defects of these genes display similar phenotypes to human patients, which suggests that these checkpoint regulators are important in the surveillance of genomic destabilization and the prevention of tumor development.

Mutations in the p53 gene are responsible for the large majority of sporadic human cancers, and thus p53 is a key target for cancer therapy *(67,108,110,135)*. p53 gene mutations can also be inherited in a subset of families with the Li-Fraumeni syndrome (LFS), which is characterized by a predisposition to sarcomas, brain and breast tumors, and childhood adrenocortical carcinoma *(258)*. The inactivation of the *INK4a/ARF* (or *CDKN2a*) locus, which engages the pRB and p53 tumor suppressor pathways through its capacity to encode the two distinct gene products $p16^{INK4a}$ and $p14^{ARF}$, is also a common genetic event in the development of human melanoma *(259)*. Human cells harboring pRB and p53 mutations also cause telomere dysfunction that results in the chromosomal end-end joining and fusion-bridge-breakage cycles that trigger the aneuploidy observed in most cancer cells *(67)*. Both p53- and ARF-deficient mice spontaneously develop tumors and die of cancers early in life, and the primary MEFs cultured from p53- and ARF-deficient mice do not senesce in culture but instead yield immortal cell lines *(67)*. Moreover, many Burkitt lymphomas (BL) carry point mutations in the p53 tumor suppressor gene, bear other defects in the $p14^{ARF}$-MDM2-p53 pathway, or the $p16^{INK4a}$ gene is inactivated by promoter methylation or homozygous deletion *(260)*. Thus, disruption of both the pRB and p53 pathways is also critical for BL development. Overexpression of cyclin E, which deregulates the G1/S checkpoint and contributes to genomic instability, is also observed in several types of human tumors, including carcinomas of the lung, breast, and head and neck *(21)*. Furthermore, overexpression of Cdc25A in a subset of breast cancers is associated with poor patient survival, which suggests that both Cdc25A and its downstream target CDK2 might represent suitable therapeutic targets in early-stage breast cancer *(261)*.

ATM is the gene responsible for the rare disorder A-T, which is a genomic instability syndrome that causes cancer predisposition, radiation sensitivity, neurodegeneration, and immunodeficiency. The cells of A-T patients show markedly abnormal cell cycle checkpoint responses at G1, S, and G2 *(127,199,262)*. Moreover, while LFS, the highly penetrant familial cancer phenotype, is usually associated with inherited mutations in the p53 gene, some LFS families that do not have germline mutations of p53 have instead het-

rozygous germline mutations in CHK2 *(258,263)*. This suggests that human CHK2 is a tumor suppressor gene whose mutation confers a predisposition to arcomas, breast cancers, and brain tumors. Supporting this is the fact that oc-asional sporadic cancer-associated mutations have been detected in both the CHK1 and CHK2 genes *(263)*. In certain patients with an A-T-like disorder A-TLD), mutations in MRE11, but not in ATM, are found, and the clinical resentations of these patients mutated in *hMRE11* genes are virtually identi-al to those seen in A-T patients *(237,264)*.

Fanconi's anemia (FA) is an autosomal recessive disease that is character-zed by bone marrow failure, developmental anomalies, a high incidence of nyelodysplasia and acute nonlymphocytic leukemia, cellular hypersensitivity o crosslinking agents, and a high risk of developing acute myeloid leukemia nd certain solid tumors *(265,266)*. The six known FA gene products (FANCA, FANCC, FANCD2, FANCE, FANCF, and FANCG proteins) interact in a ommon pathway, in which the mono-ubiquitination and nuclear foci forma-ion of FANCD2 are essential. Mono-ubiquitinated FANCD2 colocalizes with BRCA1 and hRad51 in S-phase-specific nuclear foci *(265,267)*. ATM phos-horylates FANCD2 on its Ser-222 residue in response to IR, and this is re-uired for the activation of an S-phase checkpoint. Thus, FANCD2 links the FA and ATM damage-response pathways *(268)*. Consequently, the FA pro-eins are involved in the cell cycle checkpoint and DNA-repair pathways, and lisruption of the FA genes results in chromosome instability, a common fea-ure of many human cancers *(232)*.

The *BRCA1* gene was cloned by positional cloning as one of the genes that onfers genetic predisposition to early-onset breast and ovarian cancer *(230)*. The *BRCA2* tumor-suppressor gene was also identified by a similar approach *230)*. Inherited mutations in *BRCA1* or *BRCA2* predispose people to develop reast, ovarian, and other cancers *(269)*. *BRCA2* has been identified as being he seventh FA gene, and mutated BRCA2 protein fails to bind to Rad51 in esponse to genotoxic stress, which prevents Rad51 from localizing to nuclear lamage foci *(231,270)*. It has been suggested that thet FA proteins FANCA, BRCA2, and FANCD2 act indirectly with the cellular defense machinery gainst oxidative stress by linking it with the defense machinery against DNA lamage *(271)*.

Nijmegen breakage syndrome (NBS) is a recessive genetic disorder that is haracterized by elevated sensitivity to IR that induces DSBs and a high fre-uency of malignancies *(240)*. Cells derived from NBS patients show chromo-ome fragility, IR sensitivity, and RDS (failure to suppress S-phase progression n the presence of IR-induced DSBs) *(239)*. These phenotypic features are remi-iscent of those in the cells established from A-T patients, although the clinical resentation of NBS differs considerably from that of A-T. RDS was first re-

ported for cells derived from A-T patients and was later found in NBS, A-TLD, and FA patients as well *(266,269)*. Moreover, cells derived from tumors with mutated *BRCA1* *(272)* and *CHK2* *(202)* genes also undergo RDS when they are irradiated. It has been proposed that in combination with defects in other cell cycle checkpoints, RDS may contribute to the destabilization of the genome, thereby predisposing individuals bearing these genetic aberrations to cancer.

Patients with the rare genetic disease Bloom's syndrome (BS) are predisposed to developing all the cancers that affect the general population. BS arises through mutations in both alleles of the BLM (Bloom's syndrome mutated) gene, which encodes a 3'-5' DNA helicase, a member of the RecQ family. Cells derived from BS patients exhibit marked genetic instability, and BLM protein is known to contribute to the cellular response to IR by acting as a downstream ATM kinase effector *(273)*. Notably, BLM-deficient cells exhibit a normal p53 response to IR, as well as an intact G1/S cell cycle checkpoint, which indicates that the ATM and p53 pathways are functional in BS cells *(273)*. BLM-deficient cells also exhibit an intact S-phase arrest, proper recovery from S-phase arrest, and intact p53 and p21 responses after HU treatment. However, BLM-deficient cells show a reduction in the number of replicative cells and a partial escape from the G2/M cell cycle checkpoint, and have an altered p21 response *(274)*.

Many tumors display numerical and structural centrosome aberrations. Recent evidence shows that the centrosome plays an active role not only in the regulation of microtubule nucleation activity, but also in the coordination of centrosome duplication with cell cycle progression, in the stress response, and in cell cycle checkpoint control *(275)*. The single centrosome in G1 phase is duplicated during S phase. The two centrosomes then set up the poles of the mitotic spindle, and each incipient daughter cell receives one centrosome *(276)*. Note that centrosome aberrations can give rise to chromosomal instability, and cells that lack a functional p53 pathway are proposed to acquire multiple centrosomes through the failure of a G1-phase checkpoint *(277)*. p53 controls centrosome duplication by either direct physical binding to the centrosomes or by enhancing $p21^{WAF1}$ expression, which regulates the timely activation of CDK2/cyclin E and ensures the coordinated initiation of centrosome and DNA duplication *(277)*. Thus, loss or mutational inactivation of p53 leads to abnormal amplification of centrosomes due to the deregulation of the centrosome duplication cycle, which increases the frequency of mitotic defects and unbalanced chromosome transmission to daughter cells.

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