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Faithful DNA Replication Requires Regulation of CDK Activity by Checkpoint Kinases

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1. Introduction

The most fundamental aspect of cell division is the precise transfer of genetic material to daughter cells. In order to maintain genome stability the daughter cells need to receive an exact copy of the genetic material from the original cell. This is achieved mainly through two processes. First, the genetic material is carefully copied during the process of DNA replication in S phase of the cell cycle, and thereafter it is precisely segregated into two identical daughter cells during mitosis (M phase). Additional control and preparation for DNA replication occurs in G1 phase, which is the gap phase between M and S phases, and DNA repair processes and preparation for mitosis occurs in G2 phase, which is the gap phase between S and M phases.

A major obstacle for genome stability is endogenous sources of DNA damage during S phase, which can lead to mutations or chromosome rearrangements if left unrepaired. Such mutations and chromosome rearrangements can again cause cell death or lead to the development of diseases associated with genomic instability such as cancer and neurodegenerative disorders (Jackson and Bartek, 2009). The cellular mechanisms that protect against induction of endogenous DNA damage during S phase are therefore particularly important.

DNA replication is tightly regulated by a number of signaling pathways including regulators of Cyclin-Dependent Kinase (CDK) activity. Following exposure to external sources of DNA damage CDK activity is restrained due to activation of the checkpoint kinases Ataxia Telangiactasia-like Rad3 kinase (ATR), Checkpoint kinase 1 (CHK1) and WEE1 kinase (Cliby et al., 1998; Rowley et al., 1992; Sørensen et al., 2003). Importantly, recent work have suggested that proper control of CDK activity by these checkpoint kinases is also required during normal S phase to protect cells against the induction of harmful DNA lesions (Beck et al., 2010; Lam et al., 2004; Syljuåsen et al., 2005). In this chapter we review the roles of ATR, CHK1, and WEE1 during normal DNA replication, and discuss their critical function in maintaining genome stability by preventing induction of harmful DNA lesions in S phase. We also discuss the links of ATR, CHK1, and WEE1 with cancer.

2. Regulation of key cellular processes by ATR, CHK1, and WEE1 kinases

2.1 Regulation of ATR and its biological roles

Human ATR is a Ser/Thr kinase first cloned as a member of the phosphatidylinositol-3-kinase related kinases (PIKK) family (Cimprich et al., 1996). Other family members are ATM and DNA-PKcs, which are all regulating DNA damage responses. Whereas ATM and DNA-PK are activated by DNA double strand breaks (DSBs), ATR is activated upon the generation of lesions containing single stranded DNA (ssDNA) (Lopez-Contreras and Fernandez-Capetillo, 2010).

ssDNA can evolve during normal replication, at stalled replication forks, and following DSB processing such as the DNA end resection required in the initial step of homologous recombination (HR) repair (Mimitou and Symington, 2011; Zou and Elledge, 2003). Coating of ssDNA by RPA helps loading of ATR to DNA damage sites (Bochkarev et al., 1997; Fanning et al., 2006; Zhou and Elledge, 2000). ATR recognition of RPA-coated ssDNA is dependent on the ATR-interacting protein (ATRIP) (Cortez et al., 2001), which binds RPA directly (Ball et al., 2007). ATR and ATRIP are constitutively associated, it is assumed that none of them exists freely (Cortez et al., 2001). However, the binding of ATR/ATRIP to RPA is not sufficient for ATR activation (Byun et al., 2005; MacDougall et al., 2007; Stokes et al., 2002). It also needs to be activated by TOPBP1, and this occurs via an independent mechanism dependent on the RAD17 clamp loader and the 9-1-1 (RAD9-RAD1-HUS1) complex. RAD17 is recruited by RPA-coated ssDNA and loads the 9-1-1 complex, which subsequently recruits TOPBP1 and brings it in close proximity to ATR so that TOPBP1 can activate ATR (Kumagai et al., 2006). ssDNA may in itself not elicit strong ATR activation as evidenced from recent work in *Xenopus* extracts. High level ATR activation were observed at areas of ssDNA with 5'-primed ends, which greatly exceeded activation by naked ssDNA pieces (MacDougall et al., 2007). It has been suggested that these ends may be the loading site for the 9-1-1 complex (Majka et al., 2006) which can ensure that small pieces of ssDNA generated during replication does not lead to high levels of checkpoint activation.

Activated ATR regulates a plethora of cellular responses among DNA repair and cell cycle effects, and deletion of ATR in mice causes embryonic lethality (Brown and Baltimore, 2000; de Klein et al., 2000). ATR plays a major role in maintaining genome stability during S phase where it is essential for stabilizing stalled replication forks (Paulsen and Cimprich, 2007) and prevents excessive origin firing (Friedel et al., 2009). Consistent with these findings, deletion of ATR in mice causes embryonic lethality associated with loss of genome integrity (Brown and Baltimore, 2000; de Klein et al., 2000). In response to DNA damaging agents ATR regulates the S and G2/M checkpoints (Cliby et al., 1998). ATR also controls postreplicative DNA repair (Gohler et al., 2011) and homologous recombination repair (Wang et al., 2004), and contributes to promote telomere maintenance (McNees et al., 2010; Pennarun et al., 2010).

In a large scale proteomic analysis more than 700 ATM/ATR targets have been identified; most of the targets can be recognized by both ATM and ATR (Matsuoka et al., 2007). The major target of ATR activation is CHK1, which couples the recognition of ssDNA with cell cycle effects in S and G2/M phases (Liu et al., 2000). Among other regulators of DNA damage signaling, ATR also phosphorylates histone H2AX (Ward and Chen, 2001), the Bloom's syndrome helicase (BLM) (Davies et al., 2007) and p53 (Tibbetts et al., 1999).

2.2 Regulation of CHK1 and its biological roles

CHK1 is a Ser/Thr kinase and was first discovered in fission yeast (Walworth et al., 1993). Human and murine CHK1 was identified to by Sanchez et al. (1997) and Flaggs et al. (1997).

CHK1 is a constitutively active kinase that is further phosphorylated by ATR upon several stimuli like replication stress, DSBs, UV and other DNA damaging agents. Upon the generation of ssDNA containing lesions, active ATR further phosphorylates CHK1 on Ser 317 and Ser345 and stimulates its function (Guo et al., 2000; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). Claspin is required for the CHK1 phosphorylation by ATR (Kumagai and Dunphy, 2000). After the ATR induced phosphorylation, CHK1 undergoes autophosphorylation at Ser296 (Clarke and Clarke, 2005; Kasahara et al., 2010). Only a few CHK1 molecules are phosphorylated simultaneously on the ATR sites and on Ser296. CHK1-pSer296 was found only in the soluble fraction, whereas CHK1-pSer317 and CHK1-pSer345 were found both on chromatin and in the soluble fraction. It is likely that the autophosphorylation leads to dephosphorylation at the ATR sites (Kasahara et al., 2010). Given that CHK1 is a constitutively active kinase, the DNA damage induced ATR phosphorylation likely does not upregulate CHK1 kinase activity per se. Rather, it was reported that phosphorylated CHK1 dissociates from chromatin (Smits et al., 2006; Zhang et al., 2005) and ATR regulation of CHK1 may thereby control transition of DNA damage signals from chromatin to its targets.

CHK1 deficiency is embryonic lethal in mice (Liu et al., 2000; Takai et al., 2000) demonstrating that CHK1 is an essential kinase. Similar to ATR, CHK1 is essential for maintaining genome integrity during S phase. CHK1 controls S phase progression both in the absence and presence of DNA damaging agents (Sørensen et al., 2003) and inhibition of CHK1 in normal S phase causes DNA damage (Syljuåsen et al., 2005). CHK1 controls replication initiation and is required for normal replication fork progression (Petermann et al., 2006; Petermann et al., 2010) and stabilizes stalled replication forks (Feijoo et al., 2001). When DNA synthesis is blocked, a fraction of CHK1 depleted cells enter mitosis prematurely with incompletely replicated DNA (Zachos et al., 2005). CHK1 also controls mitotic entry in unperturbed cells (Kramer et al., 2004) and the G2/M checkpoint after DNA damage (Sanchez et al., 1997) as well as homologous recombination repair (Sørensen et al., 2005). Moreover, CHK1 is also involved in control of transcription (Shimada et al., 2008) and was reported to play a role in mitotic spindle checkpoint function (Zachos et al., 2007). The cell cycle regulatory role of ATR/CHK1 in S and G2 phases is thought to be largely due to CHK1-mediated control of the CDC25 phosphatases (Beck et al., 2010). Among other substrates of CHK1 are RAD51 (Sørensen et al., 2005) and FANCE (Wang et al., 2007).

CHK1 is constitutively targeted by ATR in S-phase, and the cell cycle regulatory role of CHK1 in S phase is largely to restrain the activity of the CDC25A phosphatase. CDC25A dephosphorylates the tyrosine 15 residue of CDK1 and CDK2 and thereby activates CDK1 and CDK2. Following CHK1 activation, CHK1 phosphorylation of CDC25A, facilitated by 14-3-3γ (Kasahara et al., 2010) leads to ubiquitin dependent degradation of CDC25A. The NEK11 kinase is also activated by CHK1, and NEK11-mediated phosphorylation of CDC25A promotes its degradation (Melixetian et al., 2009). The degradation of CDC25A leads to increased phosphorylation of the tyrosine 15 residue and inhibition of CDK activity, thus inducing cell cycle arrest (Bartek and Lukas, 2003; Zhou and Elledge, 2000). CHK1 can also phosphorylate CDC25B and CDC25C (Sanchez et al., 1997; Schmitt et al., 2006) which may also contribute to restrain CDK activity, although CDC25C is dispensable for activation of the G2 checkpoint (Chen et al., 2001). Interestingly, CHK1 constitutively phosphorylates CDC25B (Schmitt et al., 2006) and the effects of CDC25B overexpression seems to resemble those of CHK1-inhibition, as CDC25B overexpression also results in increased recruitment of CDC45 to chromatin and subsequent DNA damage in S phase cells (Bugler et al., 2010).
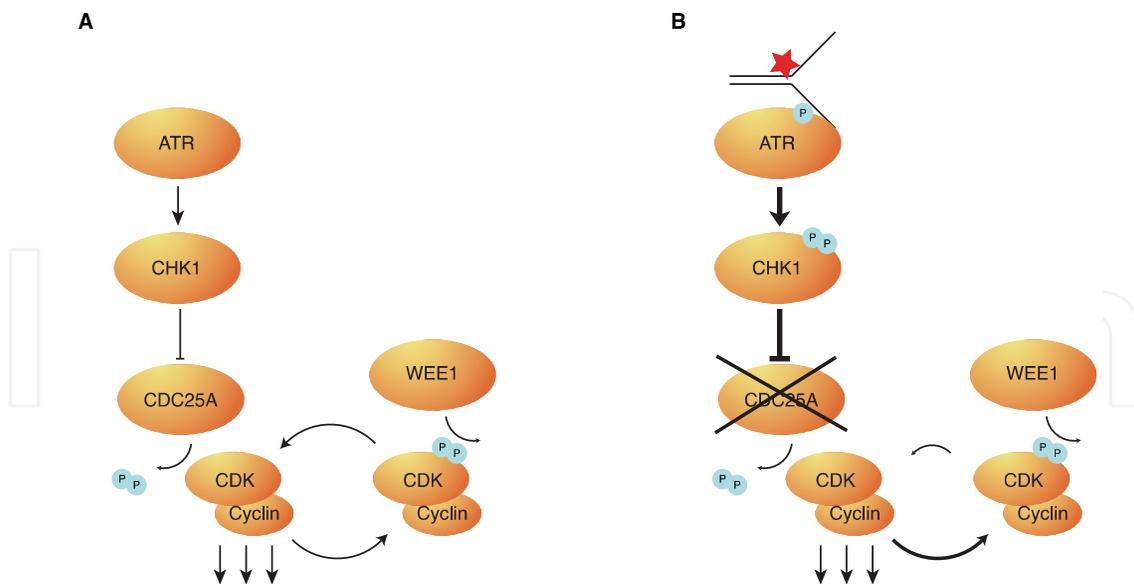
Fig. 1. Regulation of CDK activity by ATR, CHK1 and WEE1. (A) CDK activity is regulated by phosphorylation and dephosphorylation. WEE1 kinase inhibits CDK activity by phosphorylating Tyrosine 15, and WEE1 activity is counteracted by the CDC25A phosphatase. During replication in unperturbed S phase CHK1 controls CDC25A levels by a phosphorylation that signals proteolytic degradation. (B) Replication stress is sensed by ATR, which in turn stimulates CHK1. This mediates CDC25A degradation and inhibits CDK activity by shifting the equilibrium towards phosphorylated CDK.

2.3 Regulation of WEE1 and its biological roles

Wee1 was first discovered in fission yeast. Wee1 deficiency led to the 'wee' phenotype because premature mitosis was induced which led to a smaller cell size (Russell and Nurse, 1987). Wee1 is a Ser/Thr and Tyr protein kinase which negatively regulates cell cycle progression by phosphorylating and inhibiting CDKs thereby enabling the completion of DNA replication and the timely entry into mitosis (Heald et al., 1993). WEE1 kinase catalyzes the inhibitory Tyrosine 15 phosphorylation of CDK1 and CDK2 and thereby inhibits CDK activity (Parker and Piwnica-Worms, 1992; Watanabe et al., 1995). At entry into mitosis WEE1 is inhibited by phosphorylation as well as degraded by ubiquitin-dependent proteolysis, thus boosting CDK activity to promote mitosis. CDK phosphorylation primes WEE1 for ubiquitylation via the beta-TRCP SCF type of ubiquitin ligase, and this activity may be further supported by the Tome-1 SCF ubiquitin ligase (Ayad et al., 2003). In *Xenopus*, activated XCHK1 also phosphorylates the XWee1 kinase, contributing to increased Tyrosine 15 phosphorylation and inhibition of CDK activity upon CHK1 activation (Lee et al., 2001).

In mice Wee1 is essential for embryonic survival. Wee1 deficient MEFs display growth defects, chromosome aneuploidy, gamma-H2AX foci formation and CHK2 activation (Tominaga et al., 2006). Most previous reports suggest that human WEE1 mainly functions in the G2 phase to restrain mitotic entry. However, recent data revealed that inhibition of WEE1 in S phase leads to induction of DNA damage in a manner dependent on CDK1 and CDK2 and the replication proteins MCM2 and CDT1 (Beck et al., 2010). These data suggest that human WEE1 also has an important function in regulation of normal S phase progression.

3. Regulation of DNA replication by CDKs

3.1 Replication initiation

DNA replication is a tightly regulated process, where cells must secure that all parts of the genome are replicated precisely once during S-phase. Cells initiate replication from a large number of chromosomal loci known as replication origins. The activation of origins constitutes a very important means of replication control, because cells cannot regulate the speed of the DNA polymerases. In the budding yeast replication origins are specific DNA sequences, which are recognized by ORC (origin recognition complex) and additional series of protein recruitment. However, in metazoans the origins have no consensus sequence and the licensing appears to be a more stochastic event (Goren and Cedar, 2003; Zink, 2006). Activation of each replication origin leads to the assembly of a bi-directional replication fork (Bell and Dutta, 2002). Replication is further organized into clusters of origins that fire in near-synchrony (Goren and Cedar, 2003; Pope et al., 2010). These clusters are organized in replication factories, which contain between 5-50 forks (Berezney et al., 2000), and can be visualized as replication foci. After the initiation process, the protein complex at each origin changes to a post-replication state, thereby preventing further initiation events from the same origins for the rest of the cell cycle (Bell and Dutta, 2002; Blow and Dutta, 2005).
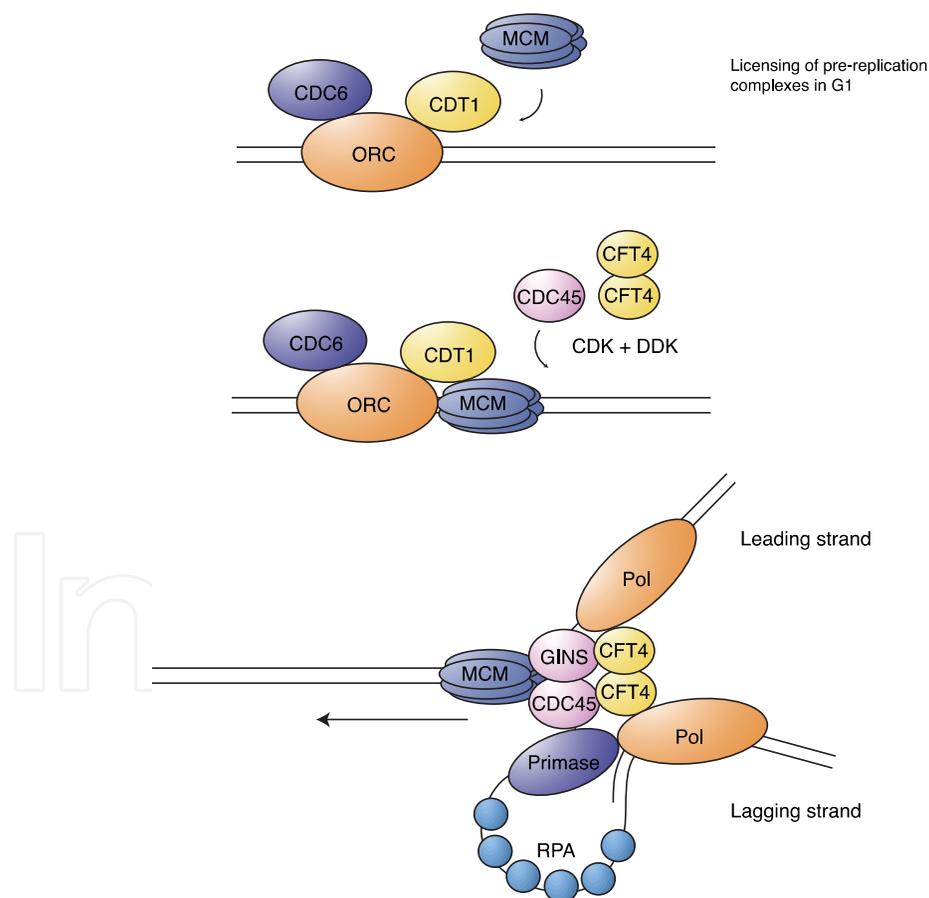

Fig. 2. Replication initiation. Origin licensing occurs in G1 by the MCM2-7 complex being recruited to ORC by CDT1 and CDC6. Loading of CDC45-GINS is crucial for replication initiation and is facilitated by AND-1/CTF4 in a CDK2 dependent manner. This allows the assembly of a replication fork with primase and polymerases onto the leading and lagging strand.

The formation of a replication fork occurs by the ordered assembly of several replication proteins during exit from mitosis and in G1 phase of the cell cycle. Binding of the ORC protein complex provides a molecular landing pad for the sequential assembly of pre-replication complexes consisting of CDC6, CDT1, and the MCM2-7 helicase complex (Bochman and Schwacha, 2009; Remus and Diffley, 2009). Initiation of DNA replication is then triggered sequentially by the action of at least two sets of protein kinase activities: the CDKs and DBF4-CDC7 (DDK) (Labib, 2010; Sclafani and Holzen, 2007). CDK2 is considered the most important CDK in regulation of DNA replication. However, CDK1 clearly also plays a role, as CDK1 can compensate for loss of CDK2 in regulation of DNA replication in CDK2 knockout mice (Aleem et al., 2005). Furthermore, CDK1 appears to regulate replication of hepatocytes, which contain higher CDK1 levels in S phase than many other cell types (Garnier et al., 2009).

The targets of CDK and DDK activity have received much attention, and the MCM helicase has been shown to be phosphorylated by the kinases, however, the functional role is not yet fully elucidated (Labib, 2010). Two major processes controlled by CDK activity are the loading of CDC45 and AND-1/CTF4 at origins (Zhu et al., 2007). In addition novel CDK targets are emerging with a role in DNA replication, such as TopBP1, Treslin and GEMC1. Once phosphorylated by CDK2, TopBP1 collaborates with the recently discovered Treslin to load CDC45 (Kumagai et al., 2010). Similarly, another CDK target, GEMC1, was recently found to be essential for replication initiation. It also associates with TopBP1 and CDC45 and is required for CDC45 and GINS loading (Balestrini et al., 2010).

The CDC45 protein is a key factor required for initiation, and it associates with the MCM helicase at origins of replication and is dependent on the presence of the GINS complex (Go-Ichi-Ni-San) (Kubota et al., 2003; Moyer et al., 2006; Takayama et al., 2003). MCM unwinding of the DNA duplex generates regions of single-stranded DNA (ssDNA) and this is closely coupled with replication. However, the isolated MCM complex appears rather inactive as a DNA helicase and its activation is likely to involve posttranslational modifications and association with other factors, such as CDC45 and GINS. Further binding of MCM10 leads to the recruitment of AND-1/CTF4 to support origin unwinding and binding of the primase DNA polymerase (Pol) α to initiate replication (Zhu et al., 2007). The first RNA primer is synthesized by the primase activity of Pol α and elongated by its DNA polymerase activity. The RNA–DNA hybrid is recognized by replication factor C (RFC), which loads PCNA, the replicative sliding clamp that mediates the polymerase switch from Pol α to the processive polymerases Pol δ and ε, allowing continuous DNA synthesis (Nasheuer et al., 2002; Takeda and Dutta, 2005).

3.2 CDK-dependent control of origin firing through S phase

Thousands of origins in the genome are fired at distinct times through the S-phase. Usually, euchromatin, with active gene transcription, is replicated early, whereas heterochromatin is replicated late. As many more origins are licensed than are ever used in a normal S-phase most origins are replicated passively (Woodward et al., 2006). Besides being required for firing of origins, CDK activity seems to be required for activation of individual replication clusters/factories as well as driving progress through the replication-timing program (Gillespie and Blow, 2010; Goren and Cedar, 2003; Hiratani et al., 2008; Thomson et al., 2010). These mechanisms are, however, poorly understood. In case of replication fork stalling or replication stress, local dormant origins will fire to compensate for the lack of replication. Under conditions of exogenous DNA damage, checkpoint pathways block the

activation of origins that normally fire in late S phase, which constitutes the basis for the S phase checkpoint (Karnani and Dutta, 2011; Santocanale and Diffley, 1998; Woodward et al., 2006).
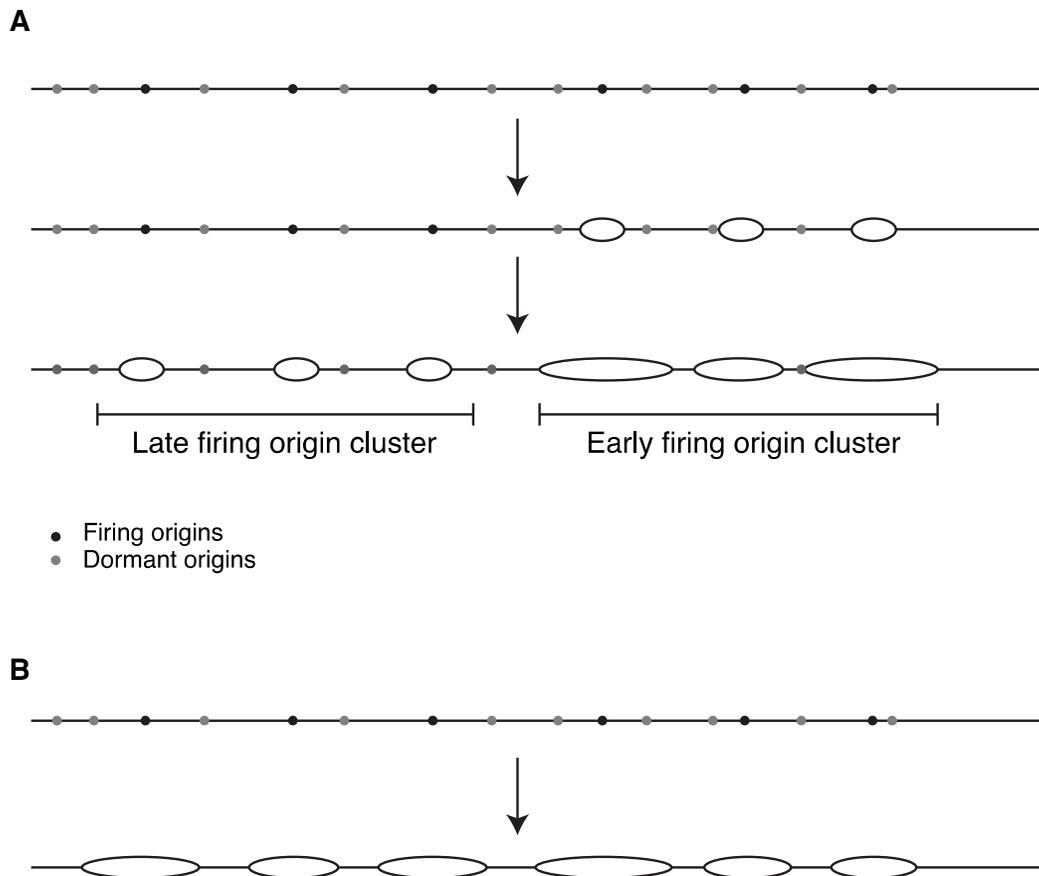

Fig. 3. (A) Many more origins than used are licensed in G1 phase. In S phase a replication program is initiated, where origins are organized into replication clusters that can be divided into early and late firing origins. (B) Under circumstances where CDK activity is deregulated and unusually high the late origins will fire inappropriately.

4. ATR, CHK1 and WEE1 are required for genomic integrity during S phase

4.1 ATR/CHK1 regulate genome integrity during unperturbed S phase

Even though CHK1's involvement in the ATR-CHK1-CDC25A regulation of CDKs to enforce a DNA damage checkpoint has been the major focus, recent discoveries are revealing a critical role of the tight regulation of CDK activity to secure DNA replication. Recent data uncovered that CHK1-mediated control of CDK activity is critical to maintain coordinated duplication of the genome and preventing a catastrophic outcome of the sensitive replication process. When DNA replication is out of control, the genome is destabilized and accumulates a massive amount of DNA double strand breaks (Syljuåsen et al., 2005). These results are consistent with a study in mice where conditional CHK1 heterozygosity caused accumulation of DNA damage during DNA replication (Lam et al., 2004). The occurrence of this DNA damage is still elusive but is clearly replication dependent and directly related to replication forks.

4.2 WEE1 emerges as a regulator of genome integrity in S phase
Until recently, WEE1 was thought to mainly regulate the entry into mitosis. However, WEE1 was recently identified as another central regulator of genome integrity in S phase (Beck et al., 2010). WEE1 depletion rapidly induced DNA damage in S phase in newly replicated DNA, which is accompanied by a marked increase in ssDNA (Beck et al., 2010). This DNA damage is dependent on CDK1 and CDK2 as well as the replication proteins MCM2 and CDT1. This is remarkably similar to the phenotype observed after ablation of CHK1, however, DNA damage after CHK1 inhibition is highly dependent on CDC25A (Beck et al., 2010). It is apparent that the mitotic kinase WEE1 and CHK1 jointly maintain balanced cellular control of CDK activity during normal DNA replication.

Inhibition of ATR/CHK1   Inhibition of WEE1

Cdc25A accumulation

High CDK activity

Unscheduled replication
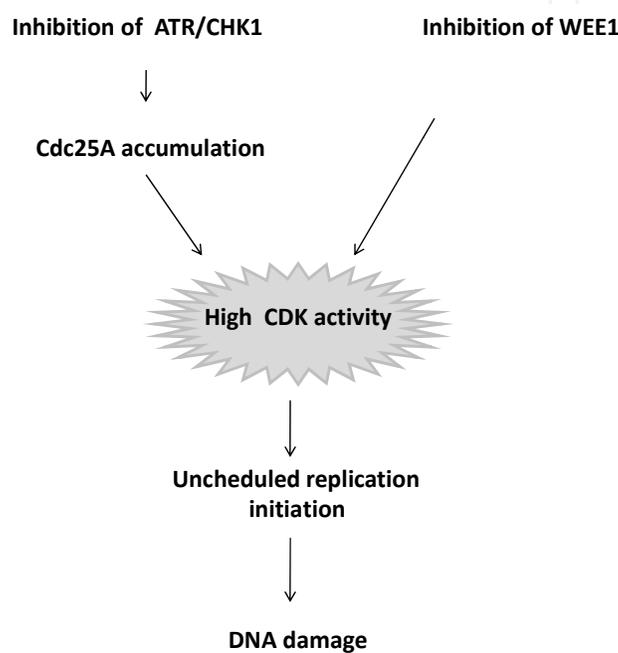initiation

DNA damage

Fig. 4. Model describing induction of DNA damage in S phase following inhibition of the ATR, CHK1 or WEE1 kinases. Inhibition of ATR or CHK1 leads to accumulation of CDC25A and thereby increases CDK activity, while inhibition of WEE1 increases CDK activity directly due to loss of WEE1-mediated inhibitory phosphorylation on CDK. The high CDK activity causes unscheduled firing of replication origins, which subsequently leads to induction of DNA breaks.

4.3 Loss of genome integrity is associated with unscheduled replication of late origins
CHK1 has recently emerged as an important component in the maintenance of genomic integrity because it blocks the appearance of aberrant replication-associated lesions (Syljuåsen et al., 2005). When CHK1 activity is lost CDC25A is stabilized and accumulates. The accompanying hyper-activation of CDK activity results in a loss of control of the replication program. This leads to an increased loading of replication factor CDC45 onto chromatin, as well as a dramatic replication initiation (Syljuåsen et al., 2005). This is accompanied by a subsequent increase in ssDNA at replication forks and association of RPA indicating replication stress (Syljuåsen et al., 2005). In addition, slower replication fork speeds are observed when CHK1 is inhibited (Petermann et al., 2010). This is likely not directly due to the

elevated CDK activity operating at existing forks. The fork slowing may be the consequence of high numbers of initiated replication forks, which can have a major impact on functional replication, for example by titrating out factors that are rate-limiting for replication elongation. Similarly to CHK1, WEE1 depletion also causes increased ssDNA and RPA foci formation indicative of replication stress (Beck et al., 2010), and WEE1 inhibition can also induce a marked increase in origin firing (our unpublished observations). Based on our previous data and the common regulation of CDK activity, we hypothesize that the major cellular defect following depletion of ATR/CHK or WEE1 is unscheduled replication of late origins.

How unscheduled replication leads to induction of DNA breaks is not well understood. DNA breaks can arise in several ways during replication (Lambert and Carr, 2005). The replication process in itself creates sensitive DNA structures since replication forks contain unwound, single-stranded DNA. The template strands on each arm of replication forks are no longer base-paired to their original complementary strands, and they are instead base-paired to newly synthesized DNA. Consequently, single-strand lesions within unwound DNA at replication forks cause double-strand breaks when the replication fork reaches such lesions. Furthermore, stalled replication forks with exposed ssDNA stretches are recombinogenic. Homologous recombination is known to salvage stalled forks but is also thought to generate structures that are resolved by endonuclease Mus81/Eme1 thereby leading to double-strand breaks (Hanada et al., 2007). Nucleases may target the replication forks in a deregulated and excessive manner when CDK activity is deregulated. Based on these observations, it is apparent that the replicating DNA molecules have conformations that facilitate the progression from replication stress to DNA breaks and loss of genome integrity.

4.4 ATR, CHK1 and WEE1 are required to prevent breakage at the replication fork

The occurrence of high level CDK-mediated DNA damage was initially surprising since this is not observed in yeast in a similar manner. The lesions in mammalian cells have been linked directly to replication forks and are dependent on replication factors like CDC45 and CDT1 (Beck et al., 2010; Syljuåsen et al., 2005). However, the generation of the double strand breaks is not understood. In addition to the over-initiation of origins, additional possible explanations also exist that more directly explain the occurrence of double strand breaks. CHK1 may negatively regulate endonucleases, such as MUS81/EME1. MUS81 generates breaks at stalled forks to support their repair by homologous recombination repair, which also re-establishes the replication fork (Hanada et al., 2007). If CHK1 negatively regulates such endonucleases during normal DNA replication, loss of CHK1 will lead to activation of the endonucleases and potentially excessive induction of DNA breaks. Given that WEE1 inhibition leads to a similar phenotype, it would be expected that it is the deregulated CDK activity that is the causative effect in activating such a DNA processing activity.

ATR and CHK1 are also known to directly support fork stability (Bartek et al., 2004; Lambert and Carr, 2005). Another possibility would thus be that lack of ATR/CHK1-mediated support of stalled forks will lead to fork collapse and DNA breaks. It is however not clear if WEE1 has a similar role, in addition, the role of CHK1 effects appears largely through the CDC25A-CDK pathway (Beck et al., 2010). A major and direct contribution of fork stability issues to the phenotype is therefore not very likely. Unbalanced or depleted nucleotide pools could also cause fork stalling and eventual collapse similar to that observed in cells treated with hydroxyurea (Katou et al., 2003), an inhibitor of nucleotide metabolism.

However, this would likely not occur with the rapid kinetics observed after CHK1 and WEE1 depletion, where inhibitors induce DNA damage within 2 hours of treatment. Hence, it appears more likely that CDK targets with enzymatic activity, i.e. a nuclease exhibiting aberrant activity, could cause the DNA damage. It remains to be determined to what extent such activities control genome integrity as well as the nature of the deregulated enzymes.

5. The disease links of ATR, CHK1, and WEE1 and their potential as targets for treatment of cancer

5.1 Cancer-associated ATR/CHK1 mutations

An important issue is whether genomic instability arising from replicative problems caused by ATR, CHK1 and WEE1 disruption may contribute to the development of human disease such as cancer. Heterozygous ATR and CHK1 mutations have been reported in a subset of endometrial, colon, and stomach cancers (Bertoni et al., 1999; Kim et al., 2007; Lewis et al., 2005; Menoyo et al., 2001; Vassileva et al., 2002; Zighelboim et al., 2009), and CHK1 mutations were also found in malignant melanoma (Kumar et al., 2005). Supporting that such mutations are likely of functional importance, is expression of truncating mutations of ATR in human cell lines abrogated CHK1 phosphorylation and topotecan-induced S phase arrest (Lewis et al., 2005). On the other hand, ATR and CHK1 mutations were not found in other types of cancer such as hereditary breast and ovarian cancers (Heikkinen et al., 2005; Marsh et al., 2007; Solyom et al., 2010) or in families with the cancer prone Li-Fraumeni syndrome (Vahteristo et al., 2001). It is important to note that in addition to inactivating mutations, suppression of ATR/CHK1 signaling in human cancer might potentially occur in many other different ways, and in vivo assessment of ATR and CHK1 kinase activities would be required in order to exclude that defects in the function of these kinases occur during tumor progression. Interestingly, a recent report suggested that as opposed to mutations, CHK1 deletions may contribute to breast cancer progression (Mu et al., 2011). Further evidence that heterozygous mutations of ATR and CHK1 might contribute to tumor progression stems from studies of ATR and CHK1 heterozogosity in mice. In one report ATR heterozygous (+/-) mice showed a modest increase in late tumor development (Brown and Baltimore, 2000) although increased tumorigenesis was not observed in other cases (Murga et al., 2009; Ruzankina et al., 2007). However, ATR heterozygozity caused a significant increase in tumorigenesis on a mismatch repair-deficient (Mlh1 -/-) background (Fang et al., 2004). CHK1 heterozygous (+/-) mice were prone to tumorigenesis on a WNT-1 transgenic background (Liu et al., 2000) and CHK1 heterozygosity induced in mouse mammary glands using a Cre/loxP system caused induction of mammary tumors in a p53 heterozygous background (Fishler et al., 2010).

The roles of ATR and CHK1 in control of DNA replication likely contribute to their tumor suppression function. Supporting that the extent of downregulation by hypomorphic mutations is sufficient to cause harmful DNA lesions associated with deregulated replication, conditional CHK1 heterozygosity in mice caused spontaneous DNA damage in S phase (Lam et al., 2004). Also, a mouse model for the human Seckel syndrome based on a mutation in the ATR gene revealed high incidence of cells showing pan-nuclear staining of gamma-H2AX in cultured MEFs *in vitro* as well as in embryos *in vivo* (Murga et al., 2009). The strong gamma-H2AX staining occurred only in Cyclin A positive cells and was attributed to increased replication stress occurring as a consequence of reduced ATR function. However, no tumors were found in these mice even in the absence of p53, which

may suggest that the induced replication stress in these mice had reached a level of severity that rather caused cell death (Murga et al., 2009). Another mouse model hypomorphic for ATR also displayed increased DNA damage as assayed by gamma-H2AX levels, which could likely be due to replicative problems, although analysis of cell cycle was not included (Ragland et al., 2009). Altogether, it seems plausible that replication associated DNA damage due to insufficient CHK1 or ATR levels in S phase caused by hypomorphic mutations in these genes could contribute to promote genomic instability and tumor progression. Analogous, it was proposed that oncogene-induced DNA damage due to increased CDK activity and replication stress promotes tumor progression at early stages (Bartkova et al., 2010; Bartkova et al., 2005; Gorgoulis et al., 2005; Halazonetis et al., 2008).

In addition to its role in cancer, ATR hypomorphic mutations have also been associated with the Seckel syndrome, which is characterized by severe microcephaly, dwarfism and dysmorphic facial features (O'Driscoll et al., 2003). In addition, a deletion in ATR was found in one patient with the Blepharophimosis-ptosis-epicanthus inversus syndrome showing microcephaly and growth retardation (O'Driscoll et al., 2007). It remains to be investigated whether replication associated DNA damage are involved in the development of these syndromes.

5.2 Cancer-associated downregulation of WEE1

Mutations of WEE1 have not yet been reported in human cancer. However, several reports suggest that WEE1 function may be sometimes compromised due to other types of cancer-associated alterations. Expression of microRNA-155 (miR-155) is elevated in several human cancers and was recently shown to cause downregulation of WEE1 (Tili et al., 2011). Downregulation of the WEE1 protein was also observed in pituitary adenomas, and this was also associated with miRNA expression (Butz et al., 2010). Moreover, prostate epithelium, which is prone to prostate cancer development, also expressed very low levels of WEE1 (Kiviharju-af Hallstrom et al., 2007). Based on our studies (Beck et al., 2010), low levels of WEE1 during human tumorigenesis would likely lead to deregulated replication with subsequent spontaneous DNA damage in S phase. We propose that prevention of such damage might contribute to the tumor suppressor function of WEE1 in some cases.

5.3 Overexpression of WEE1 and CHK1 in human cancer

On the other hand, WEE1 is overexpressed in human glioblastoma and a subset of breast cancers (Iorns et al., 2009; Mir et al., 2010), and CHK1 mRNA expression was elevated in MYC-amplified neuroblastoma (Cole et al., 2010). The mechanism behind upregulation of CHK1 in MYC-amplified neuroblastoma is not known (Cole et al., 2010). However, the high WEE1 expression in gliomas may be due to low levels of microRNA mir-128 as high WEE1 expression correlated with low expression of mir-128 in gliomas, and forced overexpression of mir-128 in glioma cells resulted in downregulation of WEE1 (Wuchty et al., 2011). High levels of WEE1 and CHK1 would be expected to suppress rather than to promote cell growth, and at first glance it may be difficult to reconcile how high levels of CHK1 or WEE1 would be consistent with a selective pressure during tumorigenesis towards genetic alterations allowing uncontrolled growth. An explanation may be that other alterations in these tumors exist that promote increased replication stress, and if the CDK activity was too high, the replication associated damage would reach a level of severity resulting in cell death. The elevated expression of WEE1 or CHK1 could thus likely be needed for cell

survival following other genetic alterations that have occurred during tumorigenesis. In line with this hypothesis, inhibition of WEE1 led to induction of DNA damage and cell death in tumors expressing high WEE1 levels (Mir et al., 2010). Furthermore, MYC is known to cause replication stress and elevated CHK1 expression was found selectively in MYC-amplified neuroblastoma (Cimprich et al., 1996).

5.4 CHK1-inhibition as a strategy for cancer treatment

ATR, CHK1 and WEE1 have been suggested as targets for cancer treatment. Indeed, several inhibitors of CHK1 are currently in clinical trials (Dai and Grant, 2010; Ma et al., 2010). When used in combination with chemotherapeutic agents or radiation, CHK1-inhibitors can cause selective sensitization of p53 negative cells (Ashwell and Zabludoff, 2008; Dixon and Norbury, 2002; Petersen et al., 2010). It was proposed that p53-negative cancer cells are particularly sensitive to CHK1-inhibitors in combination with DNA damaging agents because they lack the p53-dependent G1 checkpoint and therefore may depend more on the G2 checkpoint for DNA damage repair (Russell et al., 1995). However, CHK1-inhibiton also sensitizes p53 positive cells (Hirose et al., 2001; Tse et al., 2007), and p53-status does not always predict responses to CHK1-inhibition (Petersen et al., 2010; Zenvirt et al., 2010).

In addition to G2 checkpoint abrogation, other effects of CHK1-inhibition likely contribute to cause cell death, including inhibition of homologous recombination repair (Morgan et al., 2010; Sørensen et al., 2005) as well as induction of DNA damage in S phase due to replication problems (Cole et al., 2010; McNeely et al., 2010; Syljuåsen et al., 2005). The cytotoxic effects of CHK1-inhibition associated with increased CDK activity and induction
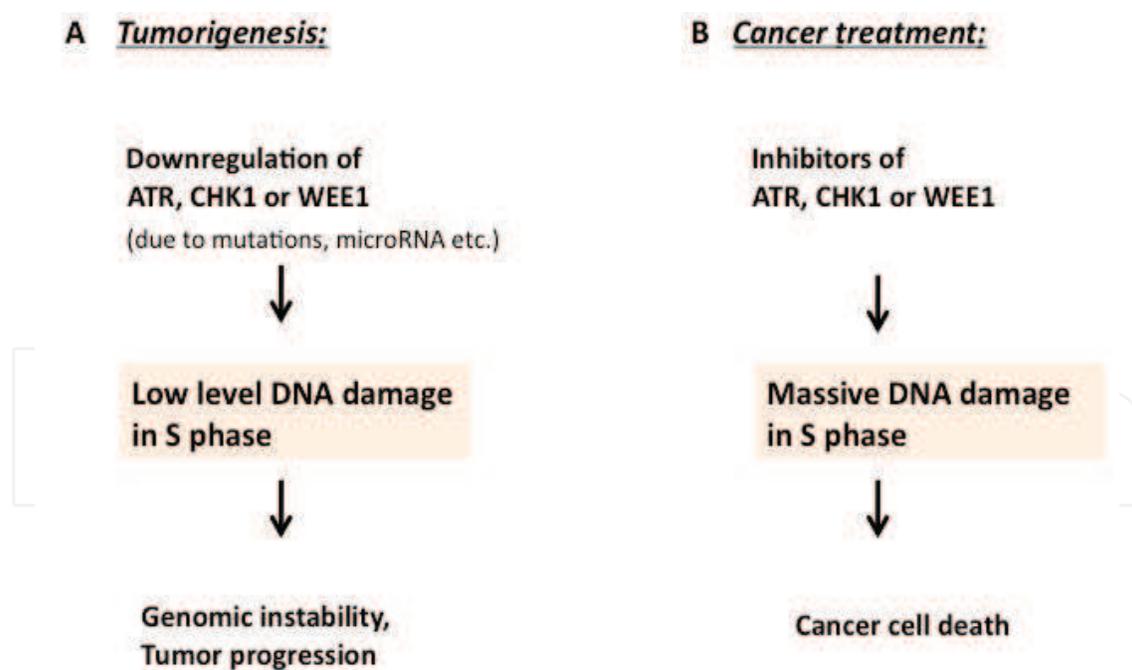

Fig. 5. Possible implications of loss of ATR, CHK1 and WEE1–mediated control of S phase events for malignant progression and cancer treatment. (A) Reduced expression or activity of ATR, CHK1 and WEE1 during tumorigenesis may cause DNA replication failures leading to DNA damage in S phase and subsequent genomic instability and tumor progression. (B) During cancer treatment with inhibitors of ATR, CHK1 or WEE1, massive induction of DNA damage in S phase due to deregulated replication may induce marked cancer cell death.

of DNA damage are expected to occur in S phase cells of cancerous as well as normal tissues, and would thus likely contribute to normal tissue damage following treatment with CHK1-inhibitors. However, the tumors would often contain a higher fraction of cycling cells than the surrounding normal tissue, resulting in tumor selective effects. In addition, tumor cells with elevated replication stress due to other genetic alterations (Bartkova et al., 2010; Halazonetis et al., 2008) may be more dependent on CHK1-mediated control of CDK activity in S phase, which would likely further promote the selective killing of tumor cells compared to normal tissue (Gilad et al., 2010).

5.5 Inhibition of WEE1 and ATR for cancer treatment

Similar to CHK1-inhibitors, inhibitors of WEE1 kinase are also in clinical trials and were reported to selectively sensitize p53-deficient tumor cells to DNA damaging agents (Hirai et al., 2010; Hirai et al., 2009; Leijen et al., 2010; Rajeshkumar et al., 2011). Furthermore, a non-transformed mammary epithelial cell line was less affected by WEE1 silencing compared to breast cancer cell lines, suggesting that WEE1-inhibition would not be toxic to normal cells (Murrow et al., 2010). Inhibition of ATR can also sensitize cancer cells and ATR has also been suggested as a therapeutic target, although small-molecule ATR-inhibitors are not yet available (Wagner and Kaufmann, 2010). Based on the roles of WEE1 and ATR in restraining CDK activity and thereby preventing unscheduled DNA replication, we propose that induction of DNA damage in S phase will contribute to the cytotoxic effects of WEE1 and ATR –inhibitors and potentially affect both tumor and normal cells in a similar manner as discussed above for CHK1-inhibitors.

6. Conclusion

It is essential for living organisms to secure that the genetic material is passed faithfully to daughter cells. Defects compromise genetic integrity and can ultimately lead to cancer and additional diseases. The duplication of the human genome in the S-phase of the cell cycle is therefore highly regulated with a large number of control mechanisms securing correct timing and quality of the process. Recent work has revealed that the checkpoint kinases ATR, CHK1 and WEE1 are constitutively active during normal S phase progression in the absence of exogenous DNA damage, and this function is critical to maintain genome integrity. These checkpoint kinases control genome integrity by restraining CDK activity. Loss of checkpoint kinase –mediated control of CDK activity will cause unscheduled firing of replication origins in S phase and thereby lead to the induction of DNA breaks in a not yet fully understood mechanism. Such replication-associated DNA lesions may contribute to promote loss of genome integrity and cancer progression following heterozygous mutations or other ways of inactivation of ATR, CHK1 or WEE1 during tumorigenesis. Furthermore, replication-associated DNA damage occurring in response to small-molecule inhibitors of ATR, CHK1 and WEE1 should be taken into account when such inhibitors are considered for cancer treatment.

7. Acknowledgements

8. References

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DNA Replication-Current Advances

Edited by Dr Herve Seligmann

The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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