Elledge 2003). TopBP1 is another protein that acts in response to DNA damage. TopBP1, a mediator protein containing eight BRCT phospho-recognition motifs, binds and activates ATR/ATRIP complexes in a manner distinct from its role in the initiation of DNA replication (Kumagai et al. 2006). TopBP1 binds to the constitutively phosphorylated C-terminal tail of Rad9 on the 9–1–1 complex at damaged sites, and this binding occurs via its first pair of BRCT repeats. Therefore, although the precise physical mechanisms by which the interactions between TopBP1 and ATR–ATRIP elicit increased ATR activity remain to be determined, TopBP1 appears to be implicated in early events in signaling following recruitment of ATR–ATRIP to sites of DNA damage and replication stress. Involvement of ATM and ATR in the mammalian origin firing program was also proposed from the observations that UV irradiation induced inhibitory effects on initiation of SV40 DNA replication as well as replication of cellular chromosomes in intact cells (Miao et al. 2003). The essential role of ATM and ATR in the origin firing program is further supported by the following observations: (1) ATR is activated during unperturbed S phase in the absence of DNA damage via replication intermediates containing RPA-bound single-stranded DNA (ssDNA) and this occurs in a manner dependent on the Rad9–Rad1–Hus1 (9–1–1) complex (Niida et al. 2007; Sorensen et al. 2004). Furthermore, the activation of ATR slows down the rate of DNA replication by blocking origin firing. (2) Inhibition of ATM and ATR by caffeine or neutralizing antibodies speeds up the rate of DNA replication (Marheineke and Hyrien 2004; Shechter et al. 2004). The effect of caffeine in promoting increased origin firing appears partly the result of feedback from ssDNA–RPA intermediates since partial depletion of RPA from Xenopus egg extracts prevents an increase in the number of origin firings. (3) Cells derived from an AT patient were observed to replicate more quickly than wild-type cells (Painter and Young 1976). Intriguingly, DNA replication checkpoint or S phase DNA damage checkpoint systems were reported to be involved in the origin firing program in vertebrate cells (Willis and Rhind 2009). Therefore, hyperactivation of ATM and ATR by stalled DNA replication or DNA damage during S phase is likely to suppress further origin activation.

Checkpoint inhibition of origin firing is by necessity a global response to DNA damage or stalled replication forks. Given that low doses of IR inhibit origin clusters that are not necessarily directly impacted by DNA damage and that episomal DNA synthesis is prevented even when only nuclear DNA is damaged by IR (Cleaver et al. 1990; Lamb et al. 1989), checkpoint components must act at origins distant from sites of DNA damage to prevent origin firing. These results clearly indicate that ATM and/or ATR play an essential role in the origin firing program under unperturbed and perturbed conditions and that origin regulation is a global response in which ATM and/or ATR act in trans in response to DNA lesions that may be far from the origins being regulated.

Chk1 but not Chk2 is a regulator of the origin firing program functioning downstream of PIKK kinases

With downstream targets, both ATM and ATR are capable of specifically phosphorylating serine or threonine residues in SQ/TQ sequences (Matsuoka et al. 2007), sharing common downstream substrates such as p53 and BRCA1 although they primarily respond to different stimuli (Harper and Elledge 2007). Recently, large-scale proteome analysis revealed that ATM phosphorylates hundreds of substrates that function in cell cycle regulation, DNA repair, checkpoints, and apoptosis (Matsuoka et al. 2007). Of these, the checkpoint kinases Chk1 and Chk2 are probable candidates as trans-acting regulators of origin firing. Chk1 and Chk2 were first identified in fission yeast as essential for cell cycle arrest prior to mitosis in response to DNA damage or DNA replication blockage, respectively. These kinases were also identified in vertebrate cells based on their homology with fission yeast orthologues (Niida and Nakanishi 2006). Chk1 is phosphorylated at Ser317 and Ser345 by ATR in response to DNA damage or DNA replication stress. This phosphorylation is blocked in cells lacking the kinase ATR and markedly inhibited in cells with a reduced amount of Rad17 or lacking Hus1. Chk1 is a constitutively active enzyme and ATR/ATM-dependent phosphorylation appears not to regulate its kinase activity but rather its subcellular localization. For example, in undamaged cells, a significant proportion of Chk1 is chromatin-associated and ATR/ATM-dependent (Niida et al. 2007; Smits et al. 2006). Chk1 phosphorylation following DNA damage results

in its rapid dissociation from chromatin, allowing phosphorylation of targets in the nuclear-soluble fraction.

One of the major downstream targets of Chk1 is a family of Cdc25 phosphatases that catalyze dephosphorylation of the inhibitory cdk1 and cdk2 at T14 and Y15, thereby initiating their kinase activity. Studies in yeasts, *Xenopus*, and mammals have demonstrated that phosphorylation of these Cdc25 proteins by Chk1 creates binding sites for 14-3-3 proteins and downregulates their phosphatase activities. In addition to 14-3-3 binding, in the presence of DNA damage during S phase progression, activated ATR–Chk1 phosphorylates Cdc25A at Ser124 and Thr507, leading to recruitment of 14-3-3 protein as well as at Ser76 triggering its ubiquitination and degradation (Mailand et al. 2000). Downregulated Cdc25A suppresses cdk2 and cdk1 activation that blocks the loading onto chromatin of Cdc45 (Zou and Stillman 1998), a protein required for the initiation of DNA replication through incorporation of DNA polymerase alpha into pre-replication complexes.

Analyses using mice deficient in Chk1 revealed its essential role in early embryonic development (Takai et al. 2000). Use of Chk1-deficient ES cells showed that Chk1 is required for cell cycle arrest before mitosis in response to DNA damage or a DNA replication block (Niida et al. 2005). In addition to its involvement at checkpoints, Chk1, similar to ATR, plays a role at every point in the cell cycle and loss of Chk1 results in the premature onset of mitosis through the dephosphorylation of cdk1 at Tyr15. Premature mitosis leads to the activation of caspases 3 and 9 triggered by cytoplasmic release of cytochrome c and the subsequent mitotic catastrophe. In contrast to Chk1-deficient embryonic cells, Chk1-depleted or Chk1-inhibited somatic mammalian cells or chicken cells exhibit gross abnormality in S phase (Maya-Mendoza et al. 2007; Petermann and Caldecott 2006; Petermann et al. 2006), reflecting increased origin activation, a slow rate of fork progression, and a loss of temporal continuity within the replication program. The abnormal S phase program likely results in an increased number of chromosome breakages that appear as gH2AX foci. The importance of Chk1-dependent S phase regulation is emphasized by the phenotype of Chk1-heterozygous cells from transgenic mice. Such cells provide three clear phenotypes of haplo-insufficiency associated with tumorigenicity in vivo including inappropriate S phase entry, accumulation of DNA damage and failure to restrain mitotic entry (Lam et al. 2004).

Chk1 also plays an important role in transcriptional regulation of the genes involved in S phase progression (Shimada et al. 2008). Chromatin-bound Chk1 phosphorylates histone H3-threonine 11 (H3-T11). Phosphorylation of H3-T11 significantly enhances the binding affinity between GCN5 histone acetyltransferase and histone tails. Thus, changes in the phosphorylation status of H3-T11 in response to DNA damage likely influence GCN5 recruitment at promoters and consequently, transcription of GCN5-dependent genes. GCN5 is utilized as an auxiliary acetyltransferase for E2Fs which regulate the expression of the many genes involved in DNA replication. In addition, GCN5 is recruited to the promoters of many cell cycle genes and GCN5-deficient cells have been shown to exhibit a significant decrease in their growth, which is associated with a reduction in the expression of many cell cycle regulatory genes. Therefore, Chk1-dependent repression of GCN5-dependent gene expression serves as an alternative checkpoint mechanism to promote cell cycle delay or arrest, in addition to the regulation of inhibitory Y15 phosphorylation of cdk1 and cdk2. Consistent with this, $Chk1^{-/-}$ somatic cells permanently arrest the cell cycle at middle S phase, preventing analysis of the S phase program in $Chk1^{-/-}$ cells. Using the Cre/loxP system, we carefully synchronized $Chk1^{del/-}$ MEFs at S phase onset and analyzed their spatiotemporal replication site patterns during early to middle S phase (Katsuno et al. 2009). Double-labeling of cells with IdU and CIdU revealed that Chk1 depletion in mammals resulted in aberrant origin firing as observed in avian cells (Fig. 1). Molecular combing of single DNA molecules demonstrated that Chk1 depletion resulted in a clear reduction in origin spacing (~40% of control cells) as well as in the rate of fork elongation throughout the labeling period. However, since slowing the replication speed very rapidly triggers the recruitment of latent origins (Courbet et al. 2008), it is not clear at this stage whether Chk1 regulates either or both activation of origins or fork velocity. Molecular combing also revealed that loss of Chk1 frequently stalls and collapses active forks.

Intriguingly, ChIP analysis of early S phase cells using FACS-based cell sorting clearly demonstrated that the relative amounts of nascent DNA around

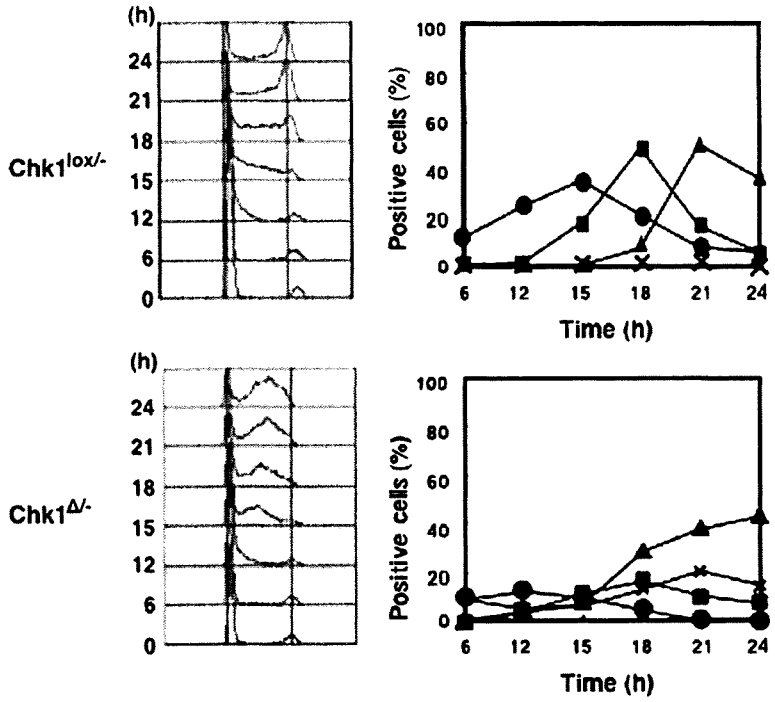

Fig. 1 Chk1 depletion results in an aberrant S phase program. $Chk1^{lox/-}$ MEFs and $Chk1^{del/-}$ MEFs were synchronized at quiescence by serum starvation and then released into G1 phase by the addition of 15% serum. Cells were harvested at the indicated times and their cell cycle distribution was analyzed by FACS (*left panels*). Replication sites were pulse-labeled for 10 min with BrdU and analyzed by fluorescence microscopy. Typical replication site patterns at the indicated times were classified according to a previous study. The number of replication sites in a typical pattern was counted and the positive cells relative to the total number of cells is expressed as a percentage (*right panels*). *Filled circles* represent the early pattern, *filled squares* the middle pattern, *filled triangles* the late pattern, and *x*, the mixed pattern

early replication sites in $Chk1^{del/-}$ cells were almost the same as those in control cells, whereas those of late replication in $Chk1^{del/-}$ cells were significantly higher than those in control cells. Thus, loss of Chk1 is likely to induce abnormal firing of late origins at early S phase.

Unlike Chk1, Chk2 does not appear to be involved in the S phase program, and is dispensable for normal cell survival, cell proliferation, and prenatal development (Takai et al. 2002). Interestingly, heterozygous germline mutations in the human Chk2 gene have been found in a subset of patients with Li–Fraumeni syndrome, a familial type of cancer, without any p53 mutation, suggesting its role as a bona fide tumor suppressor. Chk2 is mainly activated by phosphorylation of its Thr68 in an ATM-dependent manner in response to DSBs. Although biochemical analyses revealed that activated Chk2 is capable of phosphorylating Cdc25A at Ser123, Cdc25C at Ser216, BRCA1 at Ser988, and p53 at several sites, including Ser20, examination of Chk2-deficient mice and their cells showed that this enzyme functions mainly in p53-dependent apoptosis and DNA repair, but not in cell cycle arrest in response to DNA damage. Chk2-deficient mice are resistant to IR as a result of the preservation of splenic lymphocytes, thymocytes, and neurons of the developing brain whose apoptosis is known to be p53-dependent. However, ATM–Chk2 signaling at the intra-S phase checkpoint in response to IR is considered to be important since phosphorylation of Cdc25A by Chk2 was reported to trigger its ubiquitination and degradation in vitro (Falck et al. 2001). However, Chk2 fails to phosphorylate Cdc25A at Ser76 whose phosphorylation is mainly mediated by Chk1 (Jin et al. 2008), which leads to the formation of a phospho-degron that is recognized by the ubiquitin-ligase, b-TRCP. Consistent with this, use of Chk2-deficient cells showed that Chk2 is not essential for the intra-S phase checkpoint response. In addition, Chk2-deficient MEFs do not show any S phase program

abnormalities (Nakanishi et al. unpublished results). Thus, the role of Chk2 in the S phase program remains questionable.

The observation that Chk1 is phosphorylated during unperturbed S phase, which regulates the activity and stability of Cdc25 and results in the inactivation of Cdks through increased phosphorylation of their Y15 residues, leads us to speculate that Chk1 regulates the origin firing program through its involvement in certain cyclin–Cdk activities.

Cyclin A–Cdk1 complex regulates the origin firing program, but not replication fork stability in mammalian cells

In yeast, the same Cdk catalytic subunit triggers both DNA replication and mitosis, and this versatility is achieved in part by binding different cyclins that confer distinct substrate specificities. In contrast, multiple Cdk subunits in metazoans pair with preferred cyclin partners to execute specific functions in cell cycle progression. For example, Cdk2 binds with cyclins E and A to promote S phase, and Cdk1 binds with cyclins A and B to complete S phase and trigger mitosis. It remains to be determined whether the Chk1–Cdc25 axis regulates a particular set of cyclin–Cdk complexes or multiple cyclin–Cdk complexes to complete the S phase program. Clb5-dependent Cdk activity was found to be indispensable for activation of late origins in budding yeast, showing that Clb5-deficient cells failed to activate these origins and subsequently showed an extended S phase (Donaldson et al. 1998). Interestingly, this late origin firing defect is suppressed although entry into S phase and is significantly delayed in Clb5/Clb6 double-mutant cells, suggesting that other B-type cyclins promote firing of both early and late replication origins. These observations regarding budding yeast strongly suggest that specific trans-regulators of late origin activation also exist in other eukaryotes.

We found that in normal MEFs, cyclin A2–Cdk1 activity first appeared at middle S phase and increased thereafter, whereas cyclin A2–Cdk2 activity was detected at early S phase (Katsuno et al. 2009). These results are consistent with those of a recent study using centrifugal cell elutriation which showed that cyclin A assembles with Cdk1 only after complex formation with Cdk2 reaches a plateau during late S and G2 phases (Merrick et al. 2008). Importantly, cyclin A2–Cdk1 activity was detected earlier and was enhanced in Chk1$^{del/-}$ MEFs. In contrast, cyclin A2–Cdk2 activity was not obviously affected by Chk1 depletion. Early and enhanced activation of cyclin A2–Cdk1 in Chk1$^{del/-}$ MEFs appeared to be mediated by unregulated Cdc25 since the amount of Cdc25A was highly elevated and the subsequent fast mobility band of Cdk1 (active; Y15 dephosphorylation) was dominant in these cells.

Although hyperactivation of cyclin A2–Cdk1 and cyclin A2–Cdk2 in Chk1$^{del/-}$ MEFs suggests that cyclin A2–Cdk1 is a candidate trans-regulator of the origin firing program, it appears difficult to evaluate its specific role with regard to the cyclin A2–Cdk1 complex because cyclin A2 could form a complex with Cdk1 and Cdk2. Furthermore, Cdk1 is also capable of forming a complex with cyclin B and cyclin A. In order to examine the specific role of each cyclin–Cdk complex in the origin firing program, we generated cyclin A2–Cdk1, cyclin A2–Cdk2, and cyclin B1–Cdk1 fusion constructs. The properties of an active cyclin–Cdk fusion construct were successfully analyzed in the case of cyclin D1–Cdk2 (Chytil et al. 2004). Since cyclin–Cdk activities are regulated mainly by the phosphorylation of Y15, we generated a constitutively active mutant (CdkAF) in which residues at inhibitory phosphorylation sites were replaced with alanine and phenylalanine. Therefore, this mutant would not be affected by the Chk1–Cdc25 axis. Given that the enzymatic kinetic values of these fusion proteins were the same as those of the corresponding cyclin–Cdk complexes when histone H1 and lamin B were used as substrates (Table 1), the cyclin–Cdk fusion constructs were very useful for examining the specific roles of their corresponding complexes in vivo.

Expression of cyclin A2–Cdk1AF and cyclin A2–Cdk2AF fusion proteins at the endogenous level did not appear to affect overall S phase progression whereas that of cyclin B1–Cdk1AF induced severe S phase arrest with gH2AX foci in HeLa cells. Double-labeling with IdU and CIdU in cells expressing cyclin A2–Cdk1AF allowed for the detection of late replication sites during early S phase (Fig. 2). Expression of cyclin A2–Cdk1AF fusion protein reduced origin spacing. These effects were not obvious in cells expressing cyclin A2–Cdk2AF fusion protein. Interestingly, expression of cyclin A2–Cdk2AF did not cause a significant induction of abnormal replication

Table 1 Kinetic values for histone H1 and lamin B of cyclin–Cdk complexes and their fusion proteins

Enzyme	Histone H1 (mM)	Lamin B (mM)
Cyclin A2–Cdk2		
Complex	2.23	2.52
Fusion	2.06	2.09
Cyclin A2–Cdk1		
Complex	2.31	2.74
Fusion	2.25	3.25
Cyclin B1–Cdk1		
Complex	2.22	2.25
Fusion	2.18	1.82

The purified cyclin–Cdk complexes or fusion proteins were incubated with the indicated substrates at varying concentrations. The phosphorylated substrates were separated by SDS-PAGE and their incorporation was determined by radiography with a BAS-2000 imager

structures, unlike Chk1 depletion. These results clearly indicate that cyclin A2–Cdk1AF is a trans-regulator of late origin firing, but is not likely to be involved in fork stabilization during S phase. Other downstream effectors of Chk1 might stabilize replication forks under both unperturbed and perturbed conditions.

Cdk1 is required for proper timing of origin firing

Recently, several reports have demonstrated the essential role of Cdk1 in S phase progression. In check DT40 cells, Cdk1 activity is essential for DNA replication initiation when Cdk2 is depleted (Hochegger et al. 2007). Cdk1 was found to have a similar role in DNA replication observed in *Xenopus* eggs (Krasinska et al. 2008). Elimination of Cdk1, Cdk2, or their partner cyclins alters replication origin spacing, mainly by decreasing the frequency of activation of origin clusters. We found that this is also the case with mammalian cells. FT210 cells harbor a temperature-sensitive Cdk1 gene product and exhibit a 2 h longer S phase at a nonpermissive temperature when compared with the parental FM3A cells which possess a normal Cdk1 gene. Notably, late origin firing was severely impaired in FT210 cells at a nonpermissive temperature but not at a permissive temperature (Fig. 3). Loss of Cdk1 resulted in an increase in origin spacing but did not cause induction of an abnormal replication structure as it did in cells expressing cyclin A2–Cdk1AF fusion protein.

Why would Cdk1 have a specific role in late origin firing? Cdk2 did not appear to complement Cdk1 function as a trans-regulator of these events. It should be noted that Cdk1 preferentially bound to late origins although both Cdk1 and Cdk2 bound to early origins. In yeast and *Xenopus* systems it was reported that cyclins and Cdk1 specifically interact with the origin recognition complex (Romanowski et al. 2000; Wuarin et al. 2002). A recent analysis of the temporal replication profile revealed the physiological impact of the temporal regulation of origin firing although half of the origins in this human study were formed a pan-S phase pattern with equivalent replication in all quarters of S phase (Jeon et al. 2005). These analyses also revealed the cis-factors that influenced replica-

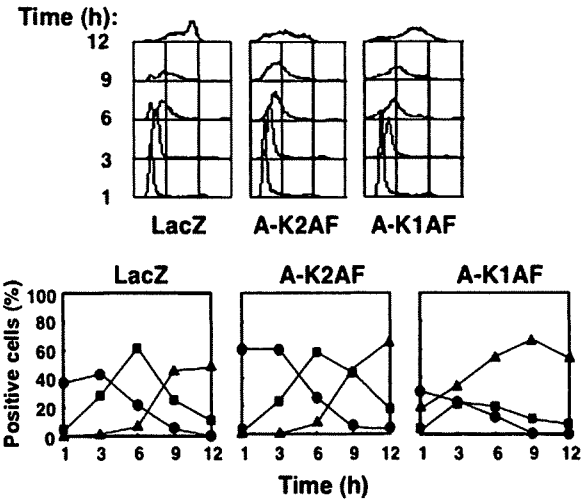

Fig. 2 Ectopic expression of cyclin A2–Cdk1AF, but not cyclin A2–Cdk2AF resulted in an aberrant temporal regulation of origin firing. HeLa cells were infected with adenoviruses expressing either cyclin A2–Cdk2AF, cyclin A2–Cdk1AF, or LacZ (control) 24 h before mimosine wash-out (time 0) and treated with 0.6 mM mimosine 16 h before wash-out to synchronize cells at the G1/S boundary. Mimosine was washed out of the cell medium (time 0), then cells were harvested at the indicated times, and their cell cycle profiles were analyzed by FACScan (*upper panels*). Replication sites were pulse-labeled for 10 min with BrdU and analyzed by fluorescence microscopy. Typical replication site patterns are presented as early, middle, and late. The number of replication sites per pattern was counted and the number of positive cells relative to the total number of cells is expressed as a percentage (*bottom panel*). *Filled circles* represent the early pattern, *filled squares* the middle pattern, and *filled triangles* the late pattern

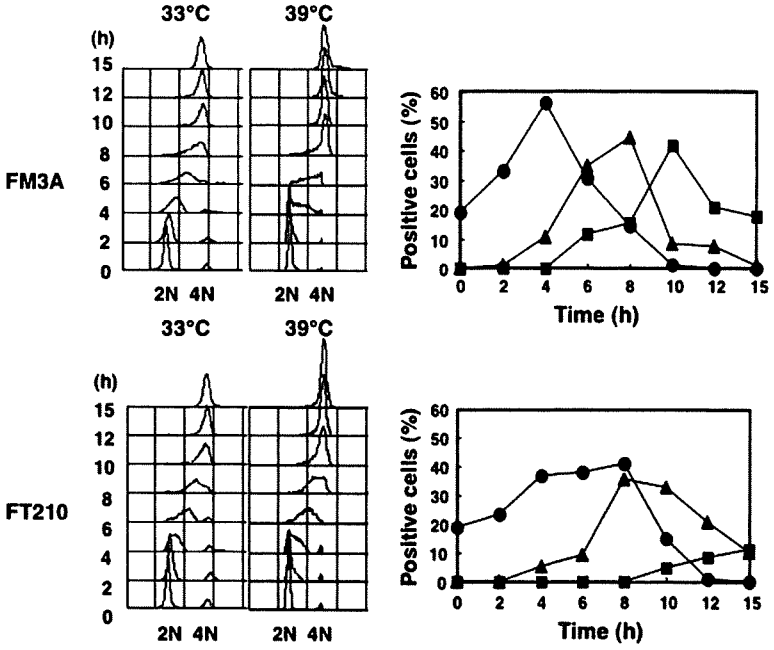

Fig. 3 Essential role of Cdk1 in late origin firing. FT210 (mouse temperature-sensitive Cdk1 mutant cells) and the parental FM3A cells were synchronized at M phase by nocodazole treatment and then released into G1 phase at either a permissive (33°C) or non-permissive (39°C) temperature. Cells were then harvested 6 h after release (time 0) and at various times thereafter. Their cell cycle profiles and replication sites were analyzed as described in the Fig. 2 legend

tion timing. In general, transcribed regions and regions rich in GC or alu are replicated early in the S phase. In contrast, gene-poor regions, heterochromatin, and regions with high concentrations of LINE repeats tend to replicate late. Taken together, some cis-factors including the chromatin status and gene density might affect the binding of Cdks to origins, leading to their preferential binding to late origins.

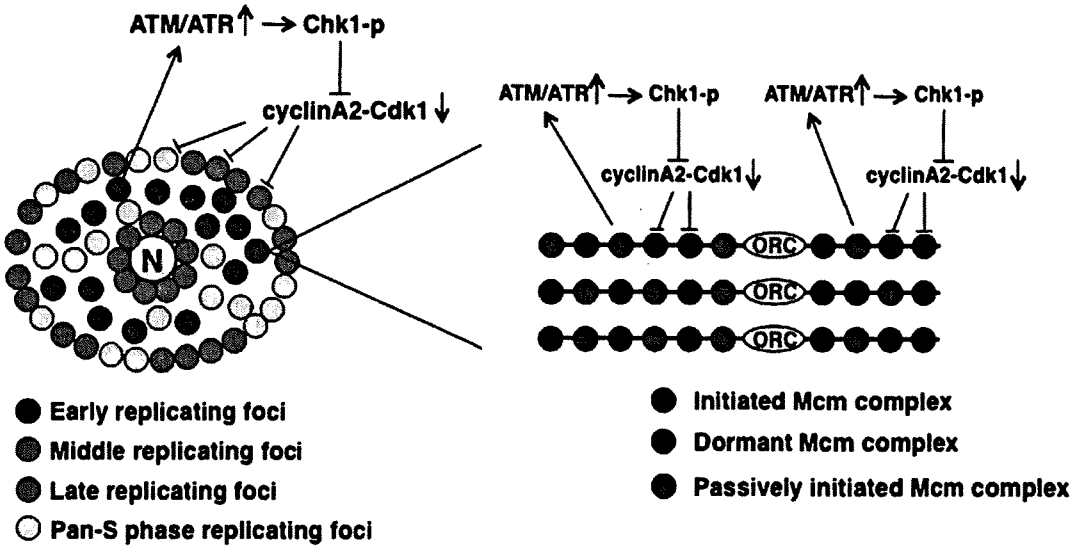

Fig. 4 Proposed model of origin firing program at two distinct levels regulated by the ATM/ATR–Chk1–cyclin A2–Cdk1 axis in mammals. The replication machinery utilizes an established path for sub-chromosomal foci during the temporal progression of S phase. The scheme depicts a cell nucleus harboring a nucleolus (*N*) and DNA organized into sub-chromosomal foci. The spatial organization of the foci at specific replication times is as follows: *red* early, *green* middle, *blue* late, and *yellow* pan S. Cyclin A2-Cdk1 appears to regulate two distinct stages in the origin firing program; one is the sequential activation of replicon clusters characterized as visible replication foci (*left panel*), and the other is the selection of one Mcm2-7 complex around the ORC within a single replication factory (*right panel*)

Conclusions and perspectives

The key factors that regulate late origin firing are summarized in Fig. 4. Although there are some important differences between model systems, we propose that cyclin A2–Cdk1 has an essential role in late origin firing as a trans-regulator. The ATM/ATR–Chk1–Cdc25 axis regulates cyclin A2–Cdk1 activity in unperturbed S phase, ensuring the ordered firing of origins. This axis is very effective for slowing S phase progression in the presence of DNA damage or DNA replication blockage since ATM/ATR can recognize a wide variety of chromosomal abnormalities. Under unperturbed conditions, activated ATM/ATR phosphorylates Chk1 and subsequently suppressed activation of cyclin A2–Cdk1 which is required for late origin firing. Thus, checkpoint mechanisms likely target trans-regulators of the S phase program.

Although trans-factors of the S phase program would be expected to be conserved among eukaryotes since the DNA replication checkpoint underlies one of the basic mechanisms that prevent genomic instability during DNA replication, mechanisms for the regulation of origin firing in eukaryotes appear to be evolutionally diverse. Differential early or late firing of the various replication origins during S phase accounts for much of the temporal programming of replication in budding yeast. The temporal program is imposed by cis-acting chromosomal sequences which are distinct from the origins themselves. However, while cis-acting origin of the replication sequence is clearly defined in prokaryotes, it is ambiguous in eukaryotes. For example, a recent whole-genome analysis using HU revealed an S phase checkpoint in budding yeast that suppresses many origins characterized as late (Feng et al. 2006; Raveendranathan et al. 2006). However, only a few origins fire late during S phase, and the Rad3-dependent S phase checkpoint has little effect on which origins are fired in fission yeast (Hayashi et al. 2007; Heichinger et al. 2006). Thus, the physiological significance of temporal regulation of origin firing in fission yeast remains somewhat unclear.

The physiological importance of the S phase program in mammals is an ongoing topic of interest, as is whether coordinated S phase programs prevent genomic instability during mammalian S phase. Very recently, an interesting observation has been made that the mutation rate, as reflected in recent evolutionary divergence and human nucleotide diversity, is markedly increased in late replicating regions of the human genome (Stamatoyannopoulos et al. 2009). Given that all classes of substitutions are affected by replication timing, an increased mutation rate appears to result from replication time-dependent DNA damage. These observations are also consistent with the finding that the SNP density is increased near later replicating genes. Therefore, these findings clearly suggest that in mammals, the origin firing program plays an important role in maintaining genome stability. Further studies are required to determine whether abnormalities in S phase programs lead to genetic instability diseases such as cancer or premature aging.

References

Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421(6922):499–506

Chytil A, Waltner-Law M, West R, Friedman D, Aakre M, Barker D, Law B (2004) Construction of a cyclin D1-Cdk2 fusion protein to model the biological functions of cyclin D1-Cdk2 complexes. J Biol Chem 279(46):47688–47698

Cleaver JE, Rose R, Mitchell DL (1990) Replication of chromosomal and episomal DNA in X-ray-damaged human cells: a cis- or trans-acting mechanism? Radiat Res 124(3):294–299

Courbet S, Gay S, Arnoult N, Wronka G, Anglana M, Brison O, Debatisse M (2008) Replication fork movement sets chromatin loop size and origin choice in mammalian cells. Nature 455(7212):557–560

DePamphilis ML (2003) Eukaryotic DNA replication origins: reconciling disparate data. Cell 114(3):274–275

Donaldson AD, Raghuraman MK, Friedman KL, Cross FR, Brewer BJ, Fangman WL (1998) CLB5-dependent activation of late replication origins in S. cerevisiae. Mol Cell 2 (2):173–182

Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410 (6830):842–847

Feng W, Collingwood D, Boeck ME, Fox LA, Alvino GM, Fangman WL, Raghuraman MK, Brewer BJ (2006) Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. Nat Cell Biol 8(2):148–155

Ge XQ, Jackson DA, Blow JJ (2007) Dormant origins licensed by excess Mcm2–7 are required for human cells to survive replicative stress. Genes Dev 21(24):3331–3341

Gilbert DM (2004) In search of the holy replicator. Nat Rev Mol Cell Biol 5(10):848–855

Goodarzi AA, Jonnalagadda JC, Douglas P, Young D, Ye R, Moorhead GB, Lees-Miller SP, Khanna KK (2004)

Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. EMBO J 23(22): 4451–4461

Harper JW, Elledge SJ (2007) The DNA damage response: ten years after. Mol Cell 28(5):739–745

Hayashi M, Katou Y, Itoh T, Tazumi A, Yamada Y, Takahashi T, Nakagawa T, Shirahige K, Masukata H (2007) Genome-wide localization of pre-RC sites and identification of replication origins in fission yeast. EMBO J 26(5):1327–1339

Heichinger C, Penkett CJ, Bahler J, Nurse P (2006) Genome-wide characterization of fission yeast DNA replication origins. EMBO J 25(21):5171–5179

Hochegger H, Dejsuphong D, Sonoda E, Saberi A, Rajendra E, Kirk J, Hunt T, Takeda S (2007) An essential role for Cdk1 in S phase control is revealed via chemical genetics in vertebrate cells. J Cell Biol 178(2):257–268

Huberman JA (1998) DNA replication. Choosing a place to begin. Science 281(5379):929–930

Ibarra A, Schwob E, Mendez J (2008) Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. Proc Natl Acad Sci U S A 105(26):8956–8961

Jackson DA (1995) Nuclear organization: uniting replication foci, chromatin domains and chromosome structure. Bioessays 17(7):587–591

Jeon Y, Bekiranov S, Karnani N, Kapranov P, Ghosh S, MacAlpine D, Lee C, Hwang DS, Gingeras TR, Dutta A (2005) Temporal profile of replication of human chromosomes. Proc Natl Acad Sci U S A 102(18):6419–6424

Jin J, Ang XL, Ye X, Livingstone M, Harper JW (2008) Differential roles for checkpoint kinases in DNA damage-dependent degradation of the Cdc25A protein phosphatase. J Biol Chem 283(28):19322–19328

Katsuno Y, Suzuki A, Sugimura K, Okumura K, Zineldeen DH, Shimada M, Niida H, Mizuno T, Hanaoka F, Nakanishi M (2009) Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc Natl Acad Sci U S A 106 (9):3184–3189

Krasinska L, Besnard E, Cot E, Dohet C, Mechali M, Lemaitre JM, Fisher D (2008) Cdk1 and Cdk2 activity levels determine the efficiency of replication origin firing in Xenopus. EMBO J 27(5):758–769

Kumagai A, Lee J, Yoo HY, Dunphy WG (2006) TopBP1 activates the ATR-ATRIP complex. Cell 124(5):943–955

Lam MH, Liu Q, Elledge SJ, Rosen JM (2004) Chk1 is haploinsufficient for multiple functions critical to tumor suppression. Cancer Cell 6(1):45–59

Lamb JR, Petit-Frere C, Broughton BC, Lehmann AR, Green MH (1989) Inhibition of DNA replication by ionizing radiation is mediated by a trans-acting factor. Int J Radiat Biol 56(2):125–130

Machida YJ, Hamlin JL, Dutta A (2005) Right place, right time, and only once: replication initiation in metazoans. Cell 123(1):13–24

Mailand N, Falck J, Lukas C, Syljuasen RG, Welcker M, Bartek J, Lukas J (2000) Rapid destruction of human Cdc25A in response to DNA damage. Science 288 (5470):1425–1429

Marheineke K, Hyrien O (2004) Control of replication origin density and firing time in Xenopus egg extracts: role of a caffeine-sensitive, ATR-dependent checkpoint. J Biol Chem 279(27):28071–28081

Masumoto H, Muramatsu S, Kamimura Y, Araki H (2002) S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature 415(6872):651–655

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316 (5828):1160–1166

Maya-Mendoza A, Petermann E, Gillespie DA, Caldecott KW, Jackson DA (2007) Chk1 regulates the density of active replication origins during the vertebrate S phase. EMBO J 26(11):2719–2731

Merrick KA, Larochelle S, Zhang C, Allen JJ, Shokat KM, Fisher RP (2008) Distinct activation pathways confer cyclin-binding specificity on cdk1 and cdk2 in human cells. Mol Cell 32(5):662–672

Miao H, Seiler JA, Burhans WC (2003) Regulation of cellular and SV40 virus origins of replication by Chk1-dependent intrinsic and UVC radiation-induced checkpoints. J Biol Chem 278(6):4295–4304

Niida H, Nakanishi M (2006) DNA damage checkpoints in mammals. Mutagenesis 21(1):3–9

Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M (2005) Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. J Biol Chem 280(47):39246–39252

Niida H, Katsuno Y, Banerjee B, Hande MP, Nakanishi M (2007) Specific role of Chk1 phosphorylations in cell survival and checkpoint activation. Mol Cell Biol 27 (7):2572–2581

Noguchi E, Shanahan P, Noguchi C, Russell P (2002) CDK phosphorylation of Drc1 regulates DNA replication in fission yeast. Curr Biol 12(7):599–605

O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA (2003) A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet 33(4):497–501

Painter RB, Young BR (1976) Formation of nascent DNA molecules during inhibition of replicon initiation in mammalian cells. Biochim Biophys Acta 418(2):146–153

Petermann E, Caldecott KW (2006) Evidence that the ATR/Chk1 pathway maintains normal replication fork progression during unperturbed S phase. Cell Cycle 5(19):2203–2209

Petermann E, Maya-Mendoza A, Zachos G, Gillespie DA, Jackson DA, Caldecott KW (2006) Chk1 requirement for high global rates of replication fork progression during normal vertebrate S phase. Mol Cell Biol 26(8):3319–3326

Raveendranathan M, Chattopadhyay S, Bolon YT, Haworth J, Clarke DJ, Bielinsky AK (2006) Genome-wide replication profiles of S-phase checkpoint mutants reveal fragile sites in yeast. EMBO J 25(15):3627–3639

Romanowski P, Marr J, Madine MA, Rowles A, Blow JJ, Gautier J, Laskey RA (2000) Interaction of Xenopus Cdc2 x cyclin A1 with the origin recognition complex. J Biol Chem 275(6):4239–4243

Shechter D, Costanzo V, Gautier J (2004) ATR and ATM regulate the timing of DNA replication origin firing. Nat Cell Biol 6(7):648–655

Shiloh Y (1997) Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. Annu Rev Genet 31:635–662

Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, Nakanishi M (2008) Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. Cell 132(2):221–232

Smits VA, Reaper PM, Jackson SP (2006) Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr Biol 16(2):150–159

Sorensen CS, Syljuasen RG, Lukas J, Bartek J (2004) ATR, Claspin and the Rad9-Rad1-Hus1 complex regulate Chk1 and Cdc25A in the absence of DNA damage. Cell Cycle 3 (7):941–945

Stamatoyannopoulos JA, Adzhubei I, Thurman RE, Kryukov GV, Mirkin SM, Sunyaev SR (2009) Human mutation rate associated with DNA replication timing. Nat Genet 41 (4):393–395

Stinchcomb DT, Struhl K, Davis RW (1979) Isolation and characterisation of a yeast chromosomal replicator. Nature 282(5734):39–43

Sun Y, Jiang X, Chen S, Fernandes N, Price BD (2005) A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci U S A 102 (37):13182–13187

Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K, Nakanishi M (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. Genes Dev 14(12):1439–1447

Takai H, Naka K, Okada Y, Watanabe M, Harada N, Saito S, Anderson CW, Appella E, Nakanishi M, Suzuki H, Nagashima K, Sawa H, Ikeda K, Motoyama N (2002) Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. EMBO J 21(19):5195–5205

Willis N, Rhind N (2009) Regulation of DNA replication by the S-phase DNA damage checkpoint. Cell Div 4:13

Woodward AM, Gohler T, Luciani MG, Oehlmann M, Ge X, Gartner A, Jackson DA, Blow JJ (2006) Excess Mcm2–7 license dormant origins of replication that can be used under conditions of replicative stress. J Cell Biol 173(5):673–683

Wuarin J, Buck V, Nurse P, Millar JB (2002) Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication. Cell 111(3):419–431

Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 300(5625):1542–1548

Zou L, Stillman B (1998) Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science 280(5363):593–596

Journal of Dermatological Science 56 (2009) 76–81

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.intl.elsevierhealth.com/journals/jods

Invited review article

DNA damage responses in skin biology—Implications in tumor prevention and aging acceleration

Makoto Nakanishi *, Hiroyuki Niida, Hiroshi Murakami, Midori Shimada

Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

ARTICLE INFO

Article history:
Received 22 June 2009
Received in revised form 4 September 2009
Accepted 8 September 2009

Keywords:
DNA damage
Senescence
Checkpoint
Chk1
Transcription

ABSTRACT

UV irradiation is the main etiological cause of most types of skin cancers and can accelerate skin photoaging. UV irradiation results in several types of DNA damage in eukaryotic cells, such as DNA single strand breaks, DNA interstrand cross-links, and nucleotide base modifications. In response to such DNA damages, mammalian cells exert DNA damage responses including cell cycle checkpoints, well-developed DNA repair, apoptosis and premature senescence to prevent genomic instability. Cell cycle checkpoints are important surveillance systems to maintain genomic integrity. Once checkpoint systems sense the abnormal chromosomal DNA structures, they execute cell cycle arrest through inhibiting the activity of cell cycle regulators and coordinate it with the DNA repair process. Checkpoint responses also execute cellular senescence when cells sense unrepairble and extensive chromosomal abnormalities. Senescent cells are no longer able to divide despite remaining viable for long periods of time, metabolically active, but functionally impaired. Accumulation of senescent cells in skin results in harmful consequences such as skin aging. Therefore, skin photoaging is thought to be a phenotypic hallmark responsible for one of the major mechanisms against skin carcinogenesis. In this review, changes in chromatin modification in response to UV and the molecular mechanisms accelerating aging phenotypes are discussed.

Contents

1. Introduction

In the life of a cell, DNA damage poses a great threat to genome stability, leading to loss or amplification of chromosomal activity, which may result in carcinogenesis or tissue aging. To prevent genomic instability upon such DNA damage, eukaryotic cells are equipped with coordinated systems to contend with DNA damage, so-called DNA damage responses. Numerous key players have been identified in terms of checkpoint sensor proteins, transducer kinases and effectors (Fig. 1), but their coordination, interconnectedness, and mechanisms by which they execute important anti-tumor protective responses have only recently become evident. Mammalian cells possess at least three distinct anti-tumor barriers including cell cycle checkpoints, apoptosis, and premature senescence (Fig. 2). Although molecular mechanisms by which checkpoints and apoptosis are regulated are relatively well characterized, those regulating premature senescence are largely unknown.

Several hypotheses have been proposed to explain the mechanisms underlying induction of senescence. For example,

* Corresponding author. Tel.: +81 52 853 8144; fax: +81 52 842 3955.
E-mail address: mkt-naka@med.nagoya-cu.ac.jp (M. Nakanishi).

0923-1811/$36.00 © 2009 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jdermsci.2009.09.001

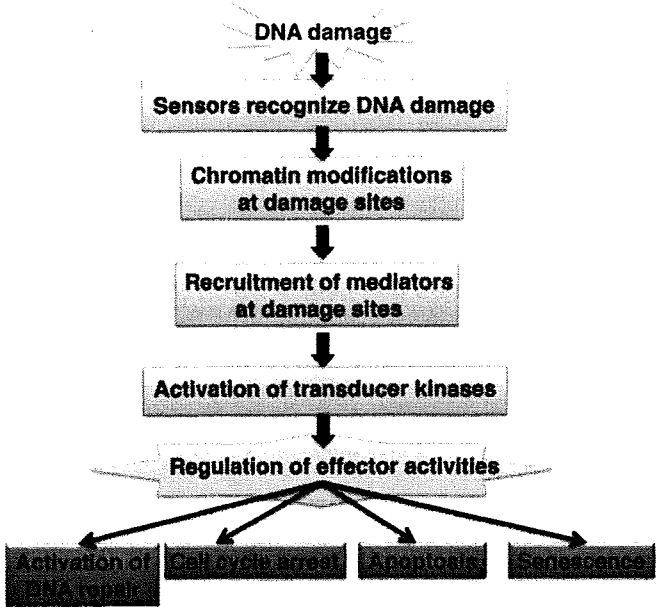

Fig. 1. Conceptual organization of the signal transduction of checkpoint responses. DNA damages are recognized by sensor proteins and induce chromatin modifications at damage sites that can function as a platform for recruitment of mediator proteins and DNA repair proteins. These signals are transmitted to transducers (mainly kinases) and the transducer molecules regulate effectors, thereby arresting the cell cycle, inducing apoptosis or senescence, and activating DNA repair mechanisms.

telomeres, the end of eukaryotic chromosomes, become progressively shorter with every round of cell division [1], presumably due to telomere end problem during DNA replication and the lack of telomerase activity. Critical telomere shortening thus results in telomere dysfunction and ultimately activates DNA damage responses that contribute to cellular senescence. However, the molecular basis by which DNA damage induces senescent phenotypes is largely unexplored. In this review, we will focus on the current knowledge of molecular cascades of DNA damage responses from chromatin modifications to cellular phenotypes.

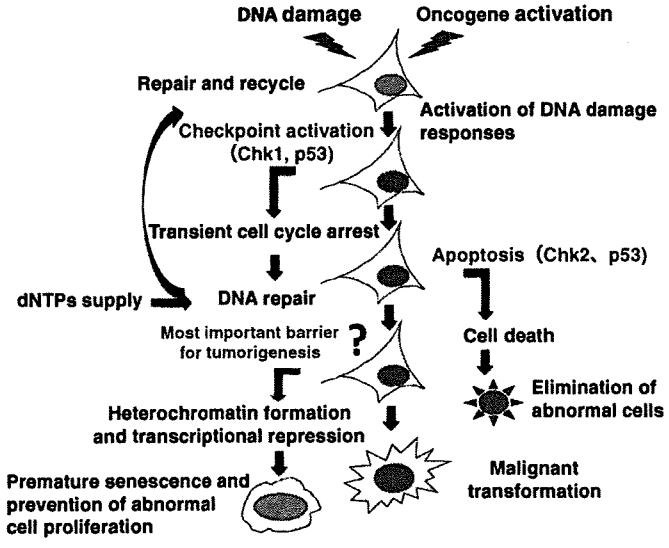

Fig. 2. Schematic model of mammalian anti-tumor barriers. Mammalian cells possess at least three anti-tumor barriers. Once cellular DNA is damaged or oncogenes are activated, DNA damage responses are activated. If DNA damage is relatively weak and repairable, phosphorylated Chk1 results in transient cell cycle arrest during which damaged DNA is efficiently repaired. If DNA damage is severe, cells with abnormal DNA structures are eliminated by Chk2 and p53-dependent apoptosis. However, certain cell types in mammals are quite resistant to apoptosis. In such cases, DNA damage responses also induce premature senescence through permanent transcriptional repression and heterochromatin formation of cell cycle regulatory genes, such as cyclin B, Cdk1.

2. Changes in chromatin modification at DNA damage sites induced by UV

Eukaryotic genomic DNA is tightly packaged with histone and non-histone proteins into highly condensed chromatin structures at the nucleus, which prevent mechanistic breaks of DNA strand and accidental access of proteins capable of binding to DNA. Hence, efficient repair of DNA damage should require modification and remodeling to render chromatin structure more accessible to DNA repair enzyme and checkpoint signaling proteins. Nucleosomes constitute the fundamental unit of chromatin. This supramolecular assembly includes a nucleosomal core and a variable DNA linker. The nucleosomal core particle comprises about 146 bp of DNA wrapped around an octameric complex containing two copies each of histones H2A, H2B, H3, and H4. Core histones are mainly globular molecules, however, they also show an unstructured N-terminal tail that contains a large number of residues that can be modified in various ways including acetylation, phosphorylation, and methylation. Alteration of the chromatin structure can be achieved by covalent modification of histone tails or by altering histone composition. Most well-characterized histone modification at DNA damage sites is phosphorylation of H2AX at Ser139 by ATM, ATR, and DNA-PK [2–4]. This phosphorylation (γ-H2AX) covers a region that extends up to megabases away from DNA double-strand break site [5] and can be a platform for protein complexes required for transmit the signals to downstream mediator kinases [6]. In yeast, a platform of γ-H2AX surrounding the damage sites is also required for the recruitment of chromosomal modification and remodeling factors, NuA4 histone acetyltransferase and INO80-SWR1 remodeling complexes that likely unravel the packed chromatin, allowing repair enzymes to access the DNA [7–10]. Similar to yeast NuA4, the Tip60-HAT complex is also required for DNA damage repair in mammals [11]. Tip60 phosphorylates histone H4 at multiple sites and these phosphorylations are required for the recruitment of DNA repair proteins [12]. Tip60-dependent histone acetylation is also essential for exchange of γ-H2AX with unphosphorylated H2AX in mammals for termination of damage signaling [13].

In addition to acetylation, histone methylation has also played an important role in recruitment of protein complexes required for damage signaling. For example, methylation of histone H3 at K79 by Dot1L is indispensable for recruitment of 53BP1 at damage sites [14]. Collectively, histone modifications and remodelings result in changes in the higher order chromatin structure and recruitment of protein complexes required for DNA repair and transmission of signaling to transducer kinases.

p53, a known tumor suppressor protein, has also been reported to function in DNA damage-induced chromatin modifications [15]. Decades ago, it has been shown that UV irradiation make chromatin more accessible [16] and histone acetylation facilitates nucleotide excision repair (NER) [17,18]. UV-induced chromatin relaxation in living cells spread out throughout the entire nucleus in a manner dependent on p53. Intriguingly, the blockage of the elongating RNA polymerase II also resulted in a similar p53-dependent chromatin relaxation. Therefore, transcription could sense the presence of UV-induced DNA damages and thus induces the stalling of the RNA polymerase that results in activation of transcription-coupled repair (TCR) directly or global genome repair (GGR) indirectly by increasing the global chromatin accessibility with p53 as effector.

3. Recognition of UV-induced abnormal chromatin by sensor proteins

Abnormal chromosomal structures induced by UV should effectively be recognized by so-called sensor proteins. For example, studies in yeasts and mammals have demonstrated that Rad9, Rad1, Hus1, and Rad17 are essential factors that activate checkpoint signaling in response to various types of DNA damage induced by UV [19]. Rad9, Rad1, and Hus1 form a heterotrimeric complex (the 9-1-1 complex) whose structure resembles a PCNA-like sliding clamp [20]. Rad17 forms an RFC-related complex with four small RFC subunits, Rfc2, Rfc3, Rfc4, and Rfc5 that acts as a clamp-loading complex [21]. The Rad17/Rfc2-5 complex is recruited to ssDNA where it is loaded in an RPA-dependent manner [22]. The presence of a dsDNA–ssDNA junction, as might be found at a stalled replication fork, activates this complex to load a second complex, the PCNA-related 9-1-1 clamp (Rad9-Rad1-Hus1). The chromatin-bound 9-1-1 complex then facilitates phosphorylation mediated by ATR and ATM. Indeed, the 9-1-1 complex, Rad17/Rfc2-5 complex, and PCNA colocalize in foci formed upon DNA damage [23–25].

Recently, several possible connections between the 9-1-1 complex and the DNA repair pathways have been suggested. The 9-1-1 complex directly interacts with DNA polymerase beta (Pol β) and stimulates its activity [26]. Pol β stimulation results in an increase in its affinity for the primer-template. This notion might raise the possibility that the 9-1-1 complex might attract Pol β to DNA damage sites, thus connecting directly checkpoints and DNA repair. The 9-1-1 complex also interacts and activates factors required for DNA repair, such as Lig I endonuclease [27,28], Fen I nuclease [29,30], and Mut Y DNA glycosylase homologue [31,32]. Taken together, these results suggest that the 9-1-1 complex could act as a recruiting platform for different factors involved in DNA repair.

4. Activation of transducer kinases

In mammals, once sensor complexes recognize DNA damage induced by UV, ATR kinase, which is extremely large proteins that phosphorylate a great number of substrates and shares a significant structural homology with ATM, is rapidly activated. An *ATM* mutation results in a devastating syndrome called ataxia telangiectasia that causes immunodeficiency, genome instability, clinical radio-sensitivity and a predisposition to cancer [33]. ATR was discovered from its sequence similarity to ATM and Rad3 [34], and was shown to play an essential role in DNA damage and DNA replication checkpoint activation [35,36]. Mutations in ATR gene have been reported in a subset of patients with Seckel syndrome [37], which is a human autosomal recessive disorder causing severe intrauterine growth retardation, proportionate dwarfism, microcephaly, with skeletal and brain abnormalities, and cancer predisposition.

ATR constitutively forms a heterodimer with ATRIP that binds to UV-damaged DNA or to RPA-coated single-stranded DNA [38]. In addition to ATRIP, in response to DNA damage, TopBP1, a mediator protein containing eight BRCT phospho-recognition motifs, binds and activates ATR/ATRIP complexes in a manner distinct from the role of TopBP1 in initiation of DNA replication [39,40]. TopBP1 binds the constitutively phosphorylated C-terminal tail of Rad9 on the 9-1-1 complex, at damage sites and this binding occurs via its first pair of BRCT repeats on TopBP1. Therefore, although the precise physical mechanisms by which the interactions between TopBP1 and ATR–ATRIP elicit increased ATR activity remain to be determined, TopBP1 appears to be implicated in early events in signaling following recruitment of ATR–ATRIP to sites of DNA damage and replication stress. For the downstream targets, ATR is capable of specifically phosphorylating serine or threonine residues in SQ/TQ sequences as with ATM, sharing common downstream substrates such as p53 [41–44] and BRCA1 [45,46] although they primarily respond to different stimuli [47].

5. Regulation of checkpoint kinase, Chk1

The checkpoint kinase Chk1 is first identified in fission yeast as essential for cell cycle arrest before mitosis in response to DNA damage. This kinase also identified in vertebrate cells based on its homology with fission yeast Chk1. Chk1 is phosphorylated at Ser317 and Ser345 by ATR in response to DNA damage or DNA replication stress. This phosphorylation is blocked in cells lacking the kinase ATR [48] and markedly inhibited in cells with a reduced amount of Rad17 [49] or those lacking Hus1 [50]. Chk1 is a constitutively active enzyme and the ATR/ATM-dependent phosphorylation appears not to regulate its kinase activity but rather its subcellular localization [51,52]. For example, following ATR/ATM-dependent phosphorylation, Chk1 is targeted to centrosomes [52,53], where cyclin B1-cdk1 is first activated at the onset of mitosis [54]. In undamaged cells, a significant proportion of Chk1 is chromatin associated and ATR/ATM-dependent Chk1 phosphorylation following DNA damage results in rapid Chk1 dissociation from chromatin.

One of the major downstream targets of Chk1 is a family of Cdc25 phosphatases [55,56]. Cdc25 phosphatases catalyze dephosphorylation of the inhibitory phosphorylation of cdk1 and cdk2 at T14 and Y15 [57], and thus activate their kinase activity. Studies in yeasts, Xenopus, and mammals have demonstrated that phosphorylation of these Cdc25 proteins by Chk1 creates binding sites for 14-3-3 proteins and downregulates their phosphatase activities [55]. In addition to 14-3-3 binding, in the presence of DNA damage during S phase progression, activated ATR-Chk1 phosphorylates Cdc25A triggering its ubiquitination and degradation [58]. The downregulated Cdc25A suppresses cdk2 and cdk1 activation that blocks the loading of Cdc45 [59,60], a protein required for the initiation of DNA replication through recruitment of DNA polymerase alpha into pre-replication complexes, onto chromatin.

Analyses using mice deficient in Chk1 revealed its essential role in early embryonic development [48,61]. Chk1-deficient ES cells demonstrated that Chk1 is prerequisite for cell cycle arrest before mitosis in response to DNA damage induced by UV or DNA replication block as described above. In addition to its involvement in checkpoints, Chk1, like ATR [35], plays a role throughout the cell cycle and notably loss of Chk1 results in the premature onset of mitosis through the dephosphorylation of cdk1 at Tyr15. Premature mitosis, in turn, leads to the activation of caspases 3 and 9 triggered by cytoplasmic release of cytochrome c and the subsequent mitotic catastrophes [62].

Furthermore, Chk1 plays an important role in transcriptional regulation. Chromatin-bound Chk1 phosphorylates histone H3-threonine 11 (H3-T11) [63]. Phosphorylation of H3-T11 significantly enhances the binding affinity between GCN5 histone acetyltransferase and histone tails (Fig. 3). Thus, changes in the phosphorylation status of H3-T11 in response to DNA damage likely influence GCN5 recruitment at promoters and thus transcription of GCN5-dependent genes. GCN5 acetylates mainly histone H3-K9 and is utilized as an accessory acetyltransferase for E2Fs [64], which regulate the expression of the many genes involved in DNA replication [65]. In addition, GCN5 is recruited to the promoters of many cell cycle genes [66] and GCN5-deficient cells have been shown to exhibit a significant decrease in their growth capability which is associated with a reduction in the expression of many cell cycle regulatory genes [67]. Therefore, Chk1-dependent repression of GCN5-dependent gene expression serves as an alternative checkpoint mechanism to promote cell

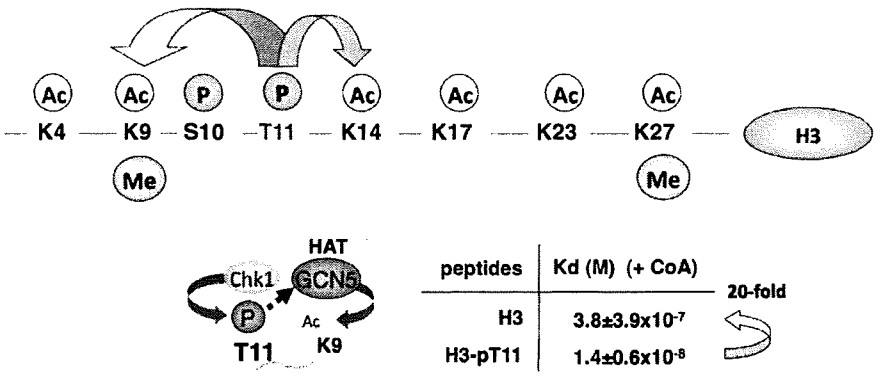

Fig. 3. Histone H3-T11 phosphorylation enhances interaction between GCN5 histone acetyltransferase and histone H3. Biacore analysis using purified HAT domain of GCN5 and histone H3 amino-terminal peptides revealed that phosphorylation of T11 drastically enhanced GCN5 bindings to histone H3 amino-terminal peptides with 20-fold higher affinity. GCN5 mainly acetylates histone H3-K9.

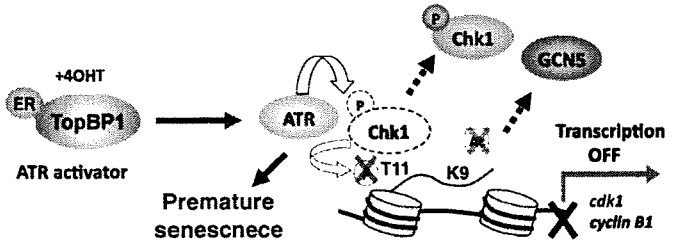

Fig. 4. Expression of TopBP1 fragment, which activates endogenous ATR, effectively induces senescent phenotypes. Activated ATR phosphorylates Chk1 at Ser317 and Ser345. Chk1 phosphorylation triggers its dissociation from chromatin. Chk1 dissociation from chromatin results in dephosphorylation of H3-T11, which in turn dissociates GCN5 from chromatin and subsequently suppresses transcriptions of cyclin B1 and Cdk1 genes.

cycle delay or arrest, beside the regulation of inhibitory Y15 phosphorylation of cdk1 and cdk2.

6. Implication of ATR-Chk1 pathway in skin carcinogenesis and photoaging

UV-induced DNA damage or activation of diverse oncogenes in mammals induces the phenotypic hallmarks of cellular senescence, a state of permanent growth arrest unable to respond any physiological growth stimuli with preserved metabolic activity, with a large and flattened morphology. Emerging results reveals that the phenomenon of cellular senescence represents a physiological tumor suppressive mechanism with a potential correlation to tissue aging [68,69], showing a significant increase in the number of senescent cells in tissues from old-aged persons. Consistent with this notion, markers of activated DNA damage checkpoints in human clinical specimens from early stages of colorectal and urinary bladder tumors correlated well with markers of heterochromatinization characteristic for cellular senescence. The recent reports using cell culture models clearly demonstrated that ATR-dependent DNA damage signaling is required for the downstream events that lead to the establishment of cellular senescence. Experimental activation of the ATR-Chk1-dependent DNA damage responses, for example thorough ectopic activation of ATR by a TopBP1 fragment, resulted in typical senescent phenotypes into several types of cultured cells [70] (Fig. 4). Surprisingly, ATR-depletion induced most of senescent phenotypes into MEFs and leads to the rapid onset of a broad range of age-related phenotypes in adult mice including skin degeneration, alopecia, and premature hair graying [71] although it remains to be elusive whether accumulation of senescent cells results in age-related phenotypes in skins. The skin abnormalities in ATR-deficient mice have been previously noted in some Seckel patients [72–75]. A recent mouse model of ATR-Seckel which possesses a hypomorphic Seckel mutation in ATR gene also shows accelerated skin aging such as decreased density of hair follicles and thinner epidermis [76]. In addition, both depletion and ectopic activation of Chk1 also induced most of premature senescent phenotypes (Shimada et al. unpublished results). Therefore, a proper signal level of ATR-Chk1 pathway under unperturbed condition may be critically required for prevention of cells to enter into a state of permanent cell cycle arrest such as senescence and maintenance of tissue homeostasis. Taken together, skin photoaging appears to be phenotypical results from activation of tumor protecting barrier against UV-induced DNA damage.

7. Conclusions

The coordinated activation of DNA damage responses such as cell cycle checkpoints, DNA repair, and premature senescence are essential for the maintenance of genome integrity and tumor suppression. Given that mutations or decreased expression of the genes implicated in DNA damage responses are detected in the most of cancers, proper DNA damage signaling is essential for preventing cancer. However, enforced and prolonged activation of DNA damage responses induce premature senescent phenotypes into normal cells that ultimately accelerate tissue aging. Thus, decreasing exposure to genotoxic stresses such as UV appears to be of value to prevent both carcinogenesis and tissue aging.

Acknowledgement

We thank Dr. Doaa Zineldeen for critical reading of the manuscript.

References

[1] Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990;345:458–60.
[2] Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Lobrich M, Jeggo PA. ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. Cancer Res 2004;64:2390–6.
[3] Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem 2001;276:42462–7.
[4] Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem 2001;276:47759–62.
[5] Shroff R, Arbel-Eden A, Pilch D, Ira G, Bonner WM, Petrini JH, et al. Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. Curr Biol 2004;14:1703–11.

[6] Stucki M, Jackson SP. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA Repair (Amst) 2006;5:534–43.
[7] Downs JA, Allard S, Jobin-Robitaille O, Javaheri A, Auger A, Bouchard N, et al. Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites. Mol Cell 2004;16:979–90.
[8] Morrison AJ, Highland J, Krogan NJ, Arbel-Eden A, Greenblatt JF, Haber JE, et al. INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. Cell 2004;119:767–75.
[9] van Attikum H, Gasser SM. The histone code at DNA breaks: a guide to repair? Nat Rev Mol Cell Biol 2005;6:757–65.
[10] Kobor MS, Venkatasubrahmanyam S, Meneghini MD, Gin JW, Jennings JL, Link AJ, et al. A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol 2004;2:E131.
[11] Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, Horikoshi M, et al. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 2000;102:463–73.
[12] Murr R, Loizou JI, Yang YG, Cuenin C, Li H, Wang ZQ, et al. Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nat Cell Biol 2006;8:91–9.
[13] Ikura T, Tashiro S, Kakino A, Shima H, Jacob N, Amunugama R, et al. DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. Mol Cell Biol 2007;27:7028–40.
[14] Huyen Y, Zgheib O, Ditullio Jr RA, Gorgoulis VG, Zacharatos P, Petty TJ, et al. Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature 2004;432:406–11.
[15] Rubbi CP, Milner J. p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage. EMBO J 2003;22:975–86.
[16] Smerdon MJ, Lieberman MW. Nucleosome rearrangement in human chromatin during UV-induced DNA-repair synthesis. Proc Natl Acad Sci USA 1978;75:4238–41.
[17] Smerdon MJ, Lan SY, Calza RE, Reeves R. Sodium butyrate stimulates DNA repair in UV-irradiated normal and xeroderma pigmentosum human fibroblasts. J Biol Chem 1982;257:13441–7.
[18] Ramanathan B, Smerdon MJ. Enhanced DNA repair synthesis in hyperacetylated nucleosomes. J Biol Chem 1989;264:11026–34.
[19] Melo J, Toczyski D. A unified view of the DNA-damage checkpoint. Curr Opin Cell Biol 2002;14:237–45.
[20] Shiomi Y, Shinozaki A, Nakada D, Sugimoto K, Usukura J, Obuse C, et al. Clamp and clamp loader structures of the human checkpoint protein complexes, Rad9-1-1 and Rad17-RFC. Genes Cells 2002;7:861–8.
[21] Griffith JD, Lindsey-Boltz LA, Sancar A. Structures of the human Rad17-replication factor C and checkpoint Rad 9-1-1 complexes visualized by glycerol spray/low voltage microscopy. J Biol Chem 2002;277:15233–6.
[22] Zou L, Liu D, Elledge SJ. Replication protein A-mediated recruitment and activation of Rad17 complexes. Proc Natl Acad Sci USA 2003;100:13827–32.
[23] Dahm K, Hubscher U. Colocalization of human Rad17 and PCNA in late S phase of the cell cycle upon replication block. Oncogene 2002;21:7710–9.
[24] Meister P, Poidevin M, Francesconi S, Tratner I, Zarzov P, Baldacci G. Nuclear factories for signalling and repairing DNA double strand breaks in living fission yeast. Nucl Acids Res 2003;31:5064–73.
[25] Burtelow MA, Kaufmann SH, Karnitz LM. Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. J Biol Chem 2000;275:26343–8.
[26] Toueille M, El-Andaloussi N, Frouin I, Freire R, Funk D, Shevelev I, et al. The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair. Nucl Acids Res 2004;32:3316–24.
[27] Smirnova E, Toueille M, Markkanen E, Hubscher U. The human checkpoint sensor and alternative DNA clamp Rad9-Rad1-Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery. Biochem J 2005;389:13–7.
[28] Wang W, Lindsey-Boltz LA, Sancar A, Bambara RA. Mechanism of stimulation of human DNA ligase I by the Rad9-rad1-Hus1 checkpoint complex. J Biol Chem 2006;281:20865–72.
[29] Friedrich-Heineken E, Toueille M, Tännler B, Bürki C, Ferrari E, Hottiger MO, et al. The two DNA clamps Rad9/Rad1/Hus1 complex and proliferating cell nuclear antigen differentially regulate flap endonuclease 1 activity. J Mol Biol 2005;353:980–9.
[30] Wang W, Brandt P, Rossi ML, Lindsey-Boltz L, Podust V, Fanning E, et al. The human Rad9-Rad1-Hus1 checkpoint complex stimulates flap endonuclease 1. Proc Natl Acad Sci USA 2004;101:16762–7.
[31] Chang DY, Lu AL. Interaction of checkpoint proteins Hus1/Rad1/Rad9 with DNA base excision repair enzyme MutY homolog in fission yeast, *Schizosaccharomyces pombe*. J Biol Chem 2005;280:408–17.
[32] Shi G, Chang DY, Cheng CC, Guan X, Venclovas C, Lu AL. Physical and functional interactions between MutY glycosylase homologue (MYH) and checkpoint proteins Rad9-Rad1-Hus1. Biochem J 2006;400:53–62.
[33] Shiloh Y. Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. Annu Rev Genet 1997;31:635–62.
[34] Cimprich KA, Shin TB, Keith CT, Schreiber SL. cDNA cloning and gene mapping of a candidate human cell cycle checkpoint protein. Proc Natl Acad Sci USA 1996;93:2850–5.
[35] Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 2000;14:397–402.
[36] Brown EJ, Baltimore D. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes Dev 2003;17:615–28.
[37] O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet 2003;33:497–501.
[38] Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 2003;300:1542–8.
[39] Kumagai A, Lee J, Yoo HY, Dunphy WG. TopBP1 activates the ATR-ATRIP complex. Cell 2006;124:943–55.
[40] Hashimoto Y, Tsujimura T, Sugino A, Takisawa H. The phosphorylated C-terminal domain of *Xenopus* Cut5 directly mediates ATR-dependent activation of Chk1. Genes Cells 2006;11:993–1007.
[41] Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 1998;281:1674–7.
[42] Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998;281:1677–9.
[43] Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, et al. ATM associates with and phosphorylates p53: mapping the region of interaction. Nat Genet 1998;20:398–400.
[44] Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, et al. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev 1999;13:152–7.
[45] Cortez D, Wang Y, Qin J, Elledge SJ. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science 1999;286:1162–6.
[46] Tibbetts RS, Cortez D, Brumbaugh KM, Scully R, Livingston D, Elledge SJ, et al. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev 2000;14:2989–3002.
[47] Harper JW, Elledge SJ. The DNA damage response: ten years after. Mol Cell 2007;28:739–45.
[48] Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev 2000;14:1448–59.
[49] Zou L, Cortez D, Elledge SJ. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev 2002;16:198–208.
[50] Weiss RS, Matsuoka S, Elledge SJ, Leder P. Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway. Curr Biol 2002;12:73–7.
[51] Smits VA, Reaper PM, Jackson SP. Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr Biol 2006;16:150–9.
[52] Niida H, Katsuno Y, Banerjee B, Hande MP, Nakanishi M. Specific role of Chk1 phosphorylations in cell survival and checkpoint activation. Mol Cell Biol 2007;27:2572–81.
[53] Kramer A, Mailand N, Lukas C, Syljuåsen RG, Wilkinson CJ, Nigg EA, et al. Centrosome-associated Chk1 prevents premature activation of cyclin-B-Cdk1 kinase. Nat Cell Biol 2004;6:884–91.
[54] Jackman M, Lindon C, Nigg EA, Pines J. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat Cell Biol 2003;5:143–8.
[55] Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science 1997;277:1501–5.
[56] Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, et al. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 1997;277:1497–501.
[57] Rudolph J. Cdc25 phosphatases: structure, specificity, and mechanism. Biochemistry 2007;46:3595–604.
[58] Mailand N, Falck J, Lukas C, Syljuåsen RG, Welcker M, Bartek J, et al. Rapid destruction of human Cdc25A in response to DNA damage. Science 2000;288:1425–9.
[59] Zou L, Stillman B. Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science 1998;280:593–6.
[60] Mimura S, Takisawa H. Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk. EMBO J 1998;17:5699–707.
[61] Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, et al. Aberrant cell cycle checkpoint function and early embryonic death in Chk1(−/−) mice. Genes Dev 2000;14:1439–47.
[62] Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M. Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. J Biol Chem 2005;280:39246–52.
[63] Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, et al. Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. Cell 2008;132:221–32.
[64] Lang SE, McMahon SB, Cole MD, Hearing P. E2F transcriptional activation requires TRRAP and GCN5 cofactors. J Biol Chem 2001;276:32627–34.
[65] Nevins JR. The Rb/E2F pathway and cancer. Hum Mol Genet 2001;10:699–703.
[66] Caretti G, Salsi V, Vecchi C, Imbriano C, Mantovani R. Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. J Biol Chem 2003;278:30435–40.
[67] Kikuchi H, Takami Y, Nakayama T. GCN5: a supervisor in all-inclusive control of vertebrate cell cycle progression through transcription regulation of various cell cycle-related genes. Gene 2005;347:83–97.

[68] Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, et al. Tumour biology: senescence in premalignant tumours. Nature 2005;436:642.
[69] Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, et al. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature 2005;436:660–5.
[70] Toledo LI, Murga M, Gutierrez-Martinez P, Soria R, Fernandez-Capetillo O. ATR signaling can drive cells into senescence in the absence of DNA breaks. Genes Dev 2008;22:297–302.
[71] Ruzankina Y, Pinzon-Guzman C, Asare A, Ong T, Pontano L, Cotsarelis G, et al. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. Cell Stem Cell 2007;1:113–26.
[72] Arnold SR, Spicer D, Kouseff B, Lacson A, Gilbert-Barness E. Seckel-like syndrome in three siblings. Pediatr Dev Pathol 1999;2:180–7.
[73] Boscherini B, Iannaccone G, La Cauza C, Mancuso G, Girotti F, Finocchi G, et al. Intrauterine growth retardation. A report of two cases with bird-headed appearance, skeletal changes and peripheral GH resistance. Eur J Pediatr 1981;137:237–42.
[74] Butler MG, Hall BD, Maclean RN, Lozzio CB. Do some patients with Seckel syndrome have hematological problems and/or chromosome breakage? Am J Med Genet 1987;27:645–9.
[75] Fathizadeh A, Soltani K, Medenica M, Lorincz AL. Pigmentary changes in Seckel's syndrome. J Am Acad Dermatol 1979;1:52–4.
[76] Murga M, Bunting S, Montana MF, Soria R, Mulero F, Cañamero M, et al. A mouse model of ATR-Seckel shows embryonic replication stress and accelerated aging. Nat Genet 2009;41:891–8.

Makoto Nakanishi, MD, PhD, Professor in Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University. *Education*: Nagoya City University Medical School, awarded the degree of MD; Nagoya City University Medical School, awarded the degree of PhD in Biochemistry. *Research and Professional Experience*: Present: Professor and Chair at Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University.

Biochemical and Biophysical Research Communications 388 (2009) 529–532

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Casein kinase II is required for the spindle assembly checkpoint by regulating Mad2p in fission yeast

Midori Shimada [a], Ayumu Yamamoto [b], Yuko Murakami-Tonami [a,1], Makoto Nakanishi [a], Takashi Yoshida [a], Hirofumi Aiba [c], Hiroshi Murakami [a,*]

[a] Department of Biochemistry and Cell Biology, Graduate School of Medicine, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
[b] Department of Chemistry, Shizuoka University, 836 Ohya, Suruga-ku, Sizuoka 422-8529, Japan
[c] Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan

ARTICLE INFO

Article history:
Received 4 August 2009
Available online 8 August 2009

Keywords:
Cell cycle
Schizosaccharomyces pombe
Mitosis

ABSTRACT

The spindle checkpoint is a surveillance mechanism that ensures the fidelity of chromosome segregation in mitosis. Here we show that fission yeast casein kinase II (CK2) is required for this checkpoint function. In the CK2 mutants mitosis occurs in the presence of a spindle defect, and the spindle checkpoint protein Mad2p fails to localize to unattached kinetochores. The CK2 mutants are sensitive to the microtubule depolymerising drug thiabendazole, which is counteracted by ectopic expression of *mad2*$^+$. The level of Mad2p is low in the CK2 mutants. These results suggest that CK2 has a role in the spindle checkpoint by regulating Mad2p.

Introduction

Cell cycle checkpoints monitor cell cycle progression to ensure the integrity of the genome and the fidelity of sister chromatid separation [1–3]. In most eukaryotes, the spindle assembly checkpoint (SAC) is a surveillance mechanism that delays anaphase onset until all chromosomes are correctly attached in a bipolar fashion to the mitotic spindle [4]. This checkpoint prevents chromosome mis-segregation during mitosis. The main spindle checkpoint proteins, which include Mad1, Mad2, BubR1 (Mad3 in yeast), Bub1, Bub3, and Mps1, have been identified [4] and are conserved in all eukaryotes. Additionally, there are several structural components of the kinetochore and centromere whose functions are required to sustain the checkpoint, such as the Ndc80, Mcm21, Mtw1 complexes and centromere proteins A (CENP-A), C, E, F, I [5,6]. Several other checkpoint components, such as Zw10 and Rod, have been identified in higher eukaryotes but have no yeast orthologues [7]. Recently, it was reported that the conserved human PRP4 protein kinase, which is implicated in the regulation of mRNA splicing, is required for the SAC [8].

The SAC acts by inhibiting the anaphase promoting complex or cyclosome (APC/C), a multi-subunit ubiquitin ligase required to promote degradation of both the anaphase inhibitor securin and B-type cyclins [9,10]. Degradation of securin and cyclin triggers chromosome segregation at the metaphase-anaphase transition and exit from mitosis, respectively. APC/C inhibition during checkpoint activation is mediated by a direct interaction of Mad2p with the Slp1p/Cdc20 protein. In fission yeast, as well as in other organisms, Mad2p localizes to unattached kinetochores upon activation of the SAC [11].

We have been studying the relationships among cell cycle controls induced by DNA replication inhibition, DNA damage, spindle assembly defects and RNA splicing defects [12,13]. We have isolated a casein kinase II (CK2) mutant (*orb5-3c13*) that is defective in arrest induced by RNA splicing defects but not by the replication and DNA damage checkpoints [12]. CK2 is a highly conserved serine-threonine kinase that is typically found in tetrameric complexes consisting of two catalytic (α and/or α′) subunits and two β regulatory subunits [14]. In fission yeast, Orb5p is a catalytic subunit of CK2, whereas Ckb1p is a regulatory subunit [15]. Here, we investigate the function of CK2 in the SAC.

Materials and methods

Yeast strains, media, and genetic methods. Complete medium (YES) and minimal medium (EMM) were prepared and standard methods were used as described [16]. Procedures for gene disruption and COOH-terminal tagging of proteins with the HA, Myc, or GFP epitope were previously described [17]. Transformation of *Schizosaccharomyces pombe* was performed by the lithium method [18]. For microscopic analysis, cells were fixed with 70% ethanol and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described [19]. The *S. pombe* strains used in this study included HM4 [*h*$^-$ *leu1-32*], HM817 [*h*$^+$ *leu1-32 nda3-311*], HM1338

* Corresponding author. Fax: +81 52 842 3955.
E-mail address: hmura@med.nagoya-cu.ac.jp (H. Murakami).
[1] Present address: Department of Biochemistry, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.08.030

[h^+ *leu1-32 ura4-D18 mad2::ura4*$^+$], HM3434 [h^- *leu1-32 orb5-3c13*], HM3584 [h^- *leu1-32 ckb1::kan*], HM3625 [h^- *leu1-32 ura4-D18 ckb1-3HA-kan*], HM4846 [h^- *leu1-32 ura4-D18 nda3-311 mad2::ura4*$^+$], HM4956 [h^- *leu1-32 nda3-311 orb5-3c13*], HM4957 [h^- *leu1-32 nda3-311 ckb1::kan*], HM5172 [h^- *leu1-32 ckb1::kan mad2-GFP-kan*], HM5177 [h^+ *leu1-32 ura4-D18 ckb1::kan mad2::ura4*$^+$], HM5210 [h^- *leu1-32 nda3-311 ckb1-3HA-kan*], HM5213 [h^- *leu1-32 nda3-311 ckb1-GFP-kan*], HM5265 [h^- *leu1-32 orb5-3c13 mad2-GFP-kan*], HM5170 [h^- *leu1-32 nda3-311 mad2-GFP-kan*], and HM5741 [h^- *leu1-32 ura4-D18 nda3-311 ckb1::kan mad2-GFP-kan*], and HM5867 [h^- *leu1-32 ura4-D18 nda3-311 orb5-GFP-kan*].

Protein extraction and western blotting. Protein extracts were prepared and western blotting was performed as described [19]. Mouse monoclonal antibodies to HA (1:1000 dilution, Roche), GFP (1:1000 dilution, Roche) and α-tubulin (1:50,000 dilution, Sigma) were used. Immune complexes were detected with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (both at 1:1000 dilution, Amersham) and ECL reagents (Amersham).

Preparation of synchronous cultures. G_2 cells were synchronized using 7–30% lactose gradients as described [12]. The percentage of cells that had passed mitosis was determined microscopically by ascertaining the number of cells that had begun or finished septation; this number was then divided by the total number of cells, and the quotient was multiplied by 100.

Results and discussion

We reasoned that if casein kinase II (CK2) functions as a spindle checkpoint protein in fission yeast, cells deficient in CK2 may be hypersensitive to microtubule-destabilizing drugs such as thiabendazole (TBZ), because of their inability to arrest cell cycle progression in the presence of a defective spindle. The *orb5* mutant (*orb5-3c13*) was viable, but its growth rate was reduced at high temperature (Fig. 1A) [12]. This strain was hypersensitive to TBZ (Fig. 1A). Cells lacking *ckb1* are viable but have a cold-sensitive phenotype [15], and they were also hypersensitive to TBZ.

To determine whether cells harboring defective CK2 proteins are also sensitive to other spindle defects, we tested for synthetic lethality among CK2 and *nda3* mutants, which are defective in the β-tubulin gene (Fig. 1B). At a restrictive temperature *nda3-311* mutants arrest at prometaphase and lack a mitotic spindle due to a cold-sensitive mutation [20]. Both *ckb1* and *nda3* single mutants maintained high viability at 32 °C. In contrast, the *nda3 ckb1* double mutant rapidly lost viability at all temperatures tested. However, the viability of the *nda3 orb5* double mutant was similar to that of the *nda3* mutant. This may be because the *orb5* mutant is temperature-sensitive. These findings suggest that CK2 mutants lose viability when spindles are defective.

Given that we identified CK2 as having a potential role in the SAC in *nda3* cells, we further investigated the phenotype of CK2 mutants. We synchronized mutants in early G_2 phase by lactose gradient centrifugation, shifted the temperature to 18 °C at time 0 and monitored mitotic progression (Fig. 1C). Whereas *wt* cells underwent nuclear division ~120 min after the temperature shift, *nda3 mad2* cells did so later. In contrast to *nda3* cells, *nda3 ckb1* cells underwent nuclear division, although progression through mitosis was somewhat delayed compared with that in *mad2 nda3* cells, probably due to the slow growth of *nda3 ckb1* cells at low temperature. *nda3 orb5* cells also underwent nuclear division, although their progression through mitosis was severely delayed (data not shown). These results suggest that CK2 is required for the SAC.

Mad2p localizes to unattached kinetochores [11] and this localization is believed to be required for activation of the checkpoint.

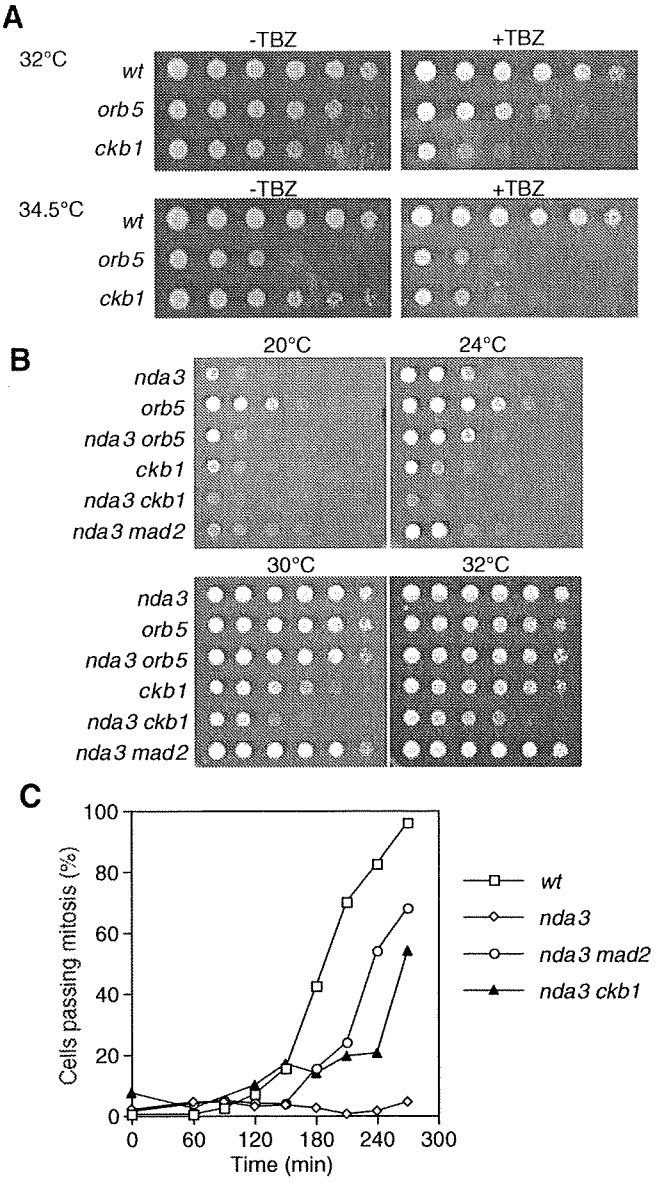

Fig. 1. CK2 is required for the spindle assembly checkpoint. (A, B) Cells of the indicated genotypes (*wt*, HM4; *orb5*, HM3434; *ckb1*, HM3584; *nda3*, HM817; *nda3 orb5*, HM4956; *nda3 ckb1*, HM4957; *nda3 mad2*; and HM4846) were grown exponentially and spotted onto YES plates with or without 0.015 mg/ml TBZ and incubated at the indicated temperatures for 2 days. (C) Cells of the indicated genotypes in (A), (B) were grown to mid-log phase in YES medium at 32 °C, and those in early G2 phase were collected by lactose gradient centrifugation. The cells were cultured in YES medium at 18 °C. Samples were then subjected to DAPI staining for determination of the percentage of cells entering mitosis.

To determine the role of CK2 in Mad2p localization, we used yeast strains in which Mad2p was tagged with the green fluorescent protein (GFP) [13] and monitored Mad2-GFP in cells lacking *ckb1*. The temperature was shifted to 18 °C at time 0 and the distribution of Mad2-GFP was determined. Mad2-GFP was observed in the nuclear periphery and chromatin domain in *nda3* and *nda3 ckb1* cells at 0 h (Fig. 2A and B). Whereas Mad2-GFP accumulated at kinetochores in *nda3* cells at 9 h, this increase was not observed in *nda3 ckb1* cells (Fig. 2A and B). These results suggest that *ckb1*$^+$ is required for the localization of Mad2-GFP to unattached kinetochores.

Since Mad2p does not associate with unattached kinetochores in *ckb1* cells, we next tested whether ectopic expression of *mad2*$^+$ could rescue the sensitivity of *ckb1* cells to TBZ (Fig. 2C). Ectopic

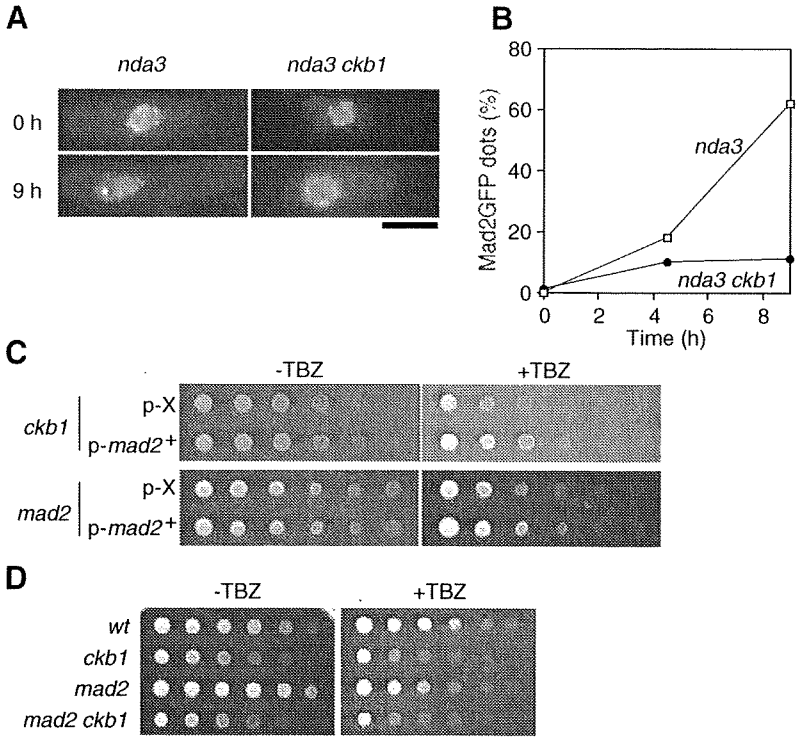

Fig. 2. CK2 is required for the localization of Mad2p at unattached kinetochores. (A, B) Cells (*nda3*, HM5170; *nda3 ckb1*, HM5741) were grown exponentially in EMM2 medium at 34 °C. (A) At time 0, the temperature was shifted to 18 °C. Mad2-GFP was visualized at the indicated times. Bar, 5 μm. (B) The percentage of cells with Mad2-GFP dots was determined. (C) Exponentially growing cells (*ckb1*, HM3584; *mad2*, HM1338) harboring pREP81-*mad2*$^+$ (p-*mad2*$^+$) or an empty vector (p-*x*) were spotted onto EMM plates without (−TBZ) or with 0.015 mg/ml TBZ (+TBZ). Plates were incubated at 32 °C for *ckb1* and at 30 °C for *mad2* for 3 days. (D) Exponentially growing cells (*wt*, HM4; *ckb1*, HM3584; *mad2*, HM1338; *mad2 ckb1*, HM5177) were spotted onto YES plates without (−TBZ) or with 0.015 mg/ml TBZ (+TBZ). Plates were incubated at 30 °C for 2 days.

expression of *mad2*$^+$ indeed restored viability to *ckb1*-deleted cells, as also observed for *mad2*-deleted cells after TBZ treatment. We thus concluded that *mad2*$^+$ functions downstream of *ckb1*$^+$.

To clarify the relative contributions of *ckb1*$^+$ and *mad2*$^+$ to the regulation of the SAC, we constructed a *mad2 ckb1* double mutant and monitored its viability after exposure to TBZ (Fig. 2D). The viability of *mad2 ckb1* cells was similar to that of the *ckb1* mutant, suggesting that *ckb1*$^+$ and *mad2*$^+$ act in the same pathway.

We next examined the effects of TBZ exposure on the abundance of Mad2p in CK2 mutants (Fig. 3A and B). The amount of Mad2p in *wt* cells did not vary after treatment of exponential cultures with TBZ at either 32 °C or 20 °C. In contrast, the amount of Mad2p in *ckb1* cells was low in the presence or absence of TBZ (Fig. 3A). The amount of Mad2p increased after heat shock treatment, whereas this increase was not observed in *orb5* cells (Fig. 3B). These results suggest that CK2 is partially required for the maintenance of Mad2p independent of the SAC activation. It is possible that in *ckb1* cells failure of the localization of Mad2-GFP to unattached kinetochores is due to the low protein level of Mad2p. However, it is unlikely since Mad2-GFP was detected in the nuclear periphery and chromatin domain in *ckb1* cells.

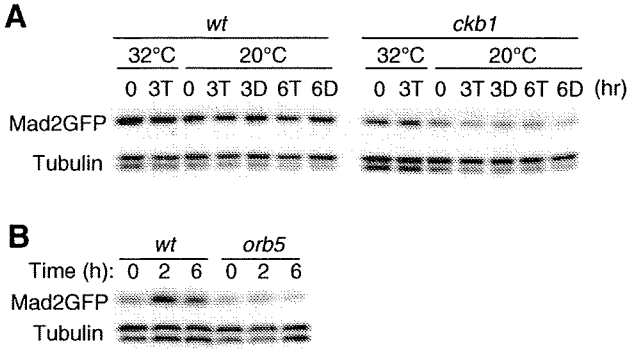

Fig. 3. The Mad2p level is low in CK2 mutants. (A) *wt* (HM5170) and *ckb1* (HM5172) cells were grown to mid-log phase in YES medium at 32 °C (32 °C, 0). TBZ (0.1 mg/ml) was added at time 0 and samples were collected after 3 h (32 °C, 3T). After the cells were grown at 32 °C, the temperature was shifted to 18 °C in YES medium without (D) or with TBZ (T). Samples were collected at the indicated times and subjected to immunoblot analysis using an anti-GFP antibody. (B) *wt* (HM5170) and *orb5* (HM5265) cells were grown to mid-log phase in YES medium at 24 °C. The temperature was shifted to 36 °C at time 0. Samples were analyzed as in (A).

We next tested whether protein levels and localization of Orb5p and Ckb1p change in response to activation of the SAC. The protein level did not vary with TBZ treatment (Supplementary figure 1). However, the levels of both proteins increased in *nda3* mutant cells compared with *wt* cells, but these levels did not change after the temperature shift. This result may reflect the fact that Orb5p and Ckb1p did not vary in response to SAC activation, but due to other defects in *nda3* cells. We found that Orb5p and Ckb1p were constitutively present in the nucleus with and without the activation of the SAC (Supplementary figure 2). In higher eukaryotes, CK2 is associated with centromeres and the mitotic spindle during mitosis [21]. It is likely that a sub-fraction of fission yeast CK2 is associated with the kinetochores, where it might regulate the localization of Mad2p when the SAC is activated.

We have shown that *ckb1* and *orb5* mutants exhibit the defining feature of a metaphase checkpoint defect – the failure to arrest in metaphase in the presence of spindle damage. In addition, these mutants show sensitivity to the microtubule-destabilizing drug TBZ. We have also found that Ckb1p is required for the localization of Mad2p to unattached kinetochores. In all of these respects, *ckb1* and *orb5* mutant phenotypes are qualitatively similar to those described for perturbations of the classical checkpoint components Mad1p and Mad2p. However, in *ckb1* and *orb5* mutants the protein

level of Mad2p is low. These facts suggest that CK2 is required for the SAC both directly and indirectly. It has been shown that human CK2 is involved in mitotic arrest following spindle damage [22]. We propose that CK2 represents additional components or regulators of the metaphase checkpoint that have been conserved among eukaryotes.

Loss of mitotic checkpoint control is a common event in human cancer cells, which is thought to be responsible for their frequently observed chromosome instability, although many cancer-derived samples do not contain mutations in SAC proteins [23]. The molecular nature of the defect underlying the absence of the SAC in most of these cell lines is not known [24]. Recent studies have shown that reduced levels of Mad2 expression can be detected in nasopharyngeal carcinoma, ovarian cancer and breast cancer cell lines and ovarian cancer [25,26]. Complete loss of Mad2p in various results in embryonic lethality owing to chromosome mis-segregation [27]. It is likely that partial loss of the SAC leads to tumor development in cells that are undergoing tumorigenesis. We have shown that the Mad2p level is decreased in CK2 mutants in fission yeast. Since CK2 is highly conserved among eukaryotes, it is possible that tumor cells harboring a SAC defect have CK2 mutations.

In conclusion, we have shown that CK2 plays an essential role on the SAC that has been conserved among eukaryotes. The molecular mechanism how CK2 regulates Mad2p remains to be determined. Future studies should provide invaluable insights into understanding the role of CK2 in the SAC.

Acknowledgments

We thank P. Nurse and T. Toda for yeast strains and K. Okazaki for plasmids and helpful discussion. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H.M. and Y.M-T.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.030.

References

[1] D.W. Cleveland, Y. Mao, K.F. Sullivan, Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling, Cell 112 (2003) 407–421.
[2] S. Hauf, Y. Watanabe, Kinetochore orientation in mitosis and meiosis, Cell 119 (2004) 317–327.
[3] H. Murakami, P. Nurse, DNA replication and damage checkpoints and meiotic cell cycle controls in the fission and budding yeasts, Biochem. J. 349 (2000) 1–12.
[4] A. Musacchio, E.D. Salmon, The spindle-assembly checkpoint in space and time, Nat. Rev. Mol. Cell Biol. 8 (2007) 379–393.
[5] L.J. Vos, J.K. Famulski, G.K. Chan, How to build a centromere: from centromeric and pericentromeric chromatin to kinetochore assembly, Biochem. Cell Biol. 84 (2006) 619–639.
[6] G.K. Chan, S.T. Liu, T.J. Yen, Kinetochore structure and function, Trends Cell Biol. 15 (2005) 589–598.
[7] R. Karess, Rod-Zw10-Zwilch: a key player in the spindle checkpoint, Trends Cell Biol. 15 (2005) 386–392.
[8] E. Montembault, S. Dutertre, C. Prigent, R. Giet, PRP4 is a spindle assembly checkpoint protein required for MPS1, MAD1, and MAD2 localization to the kinetochores, J. Cell Biol. 179 (2007) 601–609.
[9] M. Yanagida, Cell cycle mechanisms of sister chromatid separation; Roles of Cut1/separin and Cut2/securin, Genes Cells 5 (2000) 1–8.
[10] J.M. Peters, The anaphase-promoting complex: proteolysis in mitosis and beyond, Mol. Cell 9 (2002) 931–943.
[11] A.E. Ikui, K. Furuya, M. Yanagida, T. Matsumoto, Control of localization of a spindle checkpoint protein, Mad2, in fission yeast, J. Cell Sci. 115 (2002) 1603–1610.
[12] M. Shimada, C. Namikawa-Yamada, M. Nakanishi, H. Murakami, Regulation of Cdc2p and Cdc13p is required for cell cycle arrest induced by defective RNA splicing in fission yeast, J. Biol. Chem. 280 (2005) 32640–32648.
[13] I. Sugimoto, H. Murakami, Y. Tonami, A. Moriyama, M. Nakanishi, DNA replication checkpoint control mediated by the spindle checkpoint protein Mad2p in fission yeast, J. Biol. Chem. 279 (2004) 47372–47378.
[14] F. Meggio, L.A. Pinna, One-thousand-and-one substrates of protein kinase CK2?, FASEB J 17 (2003) 349–368.
[15] I. Roussou, G. Draetta, The *Schizosaccharomyces pombe* casein kinase II alpha and beta subunits: evolutionary conservation and positive role of the beta subunit, Mol. Cell Biol. 14 (1994) 576–586.
[16] S. Moreno, A. Klar, P. Nurse, Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*, Methods Enzymol. 194 (1991) 795–823.
[17] J. Bahler, J.Q. Wu, M.S. Longtine, N.G. Shah, A. McKenzie 3rd, A.B. Steever, A. Wach, P. Philippsen, J.R. Pringle, Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*, Yeast 14 (1998) 943–951.
[18] K. Okazaki, N. Okazaki, K. Kume, S. Jinno, K. Tanaka, H. Okayama, High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*, Nucleic Acids Res. 18 (1990) 6485–6489.
[19] H. Murakami, P. Nurse, Meiotic DNA replication checkpoint control in fission yeast, Genes Dev. 13 (1999) 2581–2593.
[20] T. Toda, K. Umesono, A. Hirata, M. Yanagida, Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*, J. Mol. Biol. 168 (1983) 251–270.
[21] W. Krek, G. Maridor, E.A. Nigg, Casein kinase II is a predominantly nuclear enzyme, J. Cell Biol. 116 (1992) 43–55.
[22] M. Sayed, S. Pelech, C. Wong, A. Marotta, B. Salh, Protein kinase CK2 is involved in G2 arrest and apoptosis following spindle damage in epithelial cells, Oncogene 20 (2001) 6994–7005.
[23] C.S. Lopes, C.E. Sunkel, The spindle checkpoint: from normal cell division to tumorigenesis, Arch. Med. Res. 34 (2003) 155–165.
[24] T. Takahashi, N. Haruki, S. Nomoto, A. Masuda, S. Saji, H. Osada, T. Takahashi, Identification of frequent impairment of the mitotic checkpoint and molecular analysis of the mitotic checkpoint genes, hsMAD2 and p55CDC, in human lung cancers, Oncogene 18 (1999) 4295–4300.
[25] X. Wang, D.Y. Jin, Y.C. Wong, A.L. Cheung, A.C. Chun, A.K. Lo, Y. Liu, S.W. Tsao, Correlation of defective mitotic checkpoint with aberrantly reduced expression of MAD2 protein in nasopharyngeal carcinoma cells, Carcinogenesis 21 (2000) 2293–2297.
[26] M.J. Percy, K.A. Myrie, C.K. Neeley, J.N. Azim, S.P. Ethier, E.M. Petty, Expression and mutational analyses of the human MAD2L1 gene in breast cancer cells, Genes Chromosomes Cancer 29 (2000) 356–362.
[27] L.S. Michel, V. Liberal, A. Chatterjee, R. Kirchwegger, B. Pasche, W. Gerald, M. Dobles, P.K. Sorger, V.V. Murty, R. Benezra, MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells, Nature 409 (2001) 355–359.

Biochemical and Biophysical Research Communications 380 (2009) 460–466

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ptpcd-1 is a novel cell cycle related phosphatase that regulates centriole duplication and cytokinesis

Doaa H. Zineldeen, Midori Shimada, Hiroyuki Niida, Yuko Katsuno, Makoto Nakanishi *

Department of Cell Biology and Biochemistry, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

ARTICLE INFO

Article history:
Received 6 January 2009
Available online 24 January 2009

Keywords:
Centrosome
Phosphatase
Mitosis
Polo-like kinases
Cytokinesis
Cdc14

ABSTRACT

Proper progression of mitosis requires spatio-temporal regulation of protein phosphorylation by orchestrated activities of kinases and phosphatases. Although many kinases, such as Aurora kinases, polo-like kinases (Plks), and cyclin B-Cdk1 are relatively well characterized in the context of their physiological functions at mitosis and regulation of their enzymatic activities during mitotic progression, phosphatases involved are largely unknown. Here we identified a novel protein tyrosine phosphatase containing domain 1 (Ptpcd 1) as a mitotic phosphatase, which shares sequence homology to Cdc14. Immunofluorescence studies revealed that Ptpcd1 partially colocalized with γ-tubulin, an archetypical centrosomal marker. Overexpression of this phosphatase prevented unscheduled centrosomal amplification in hydroxyurea arrested U2OS cells. Intriguingly, Ptpcd 1-associated and colocalized with polo-like kinase 1(Plk1). Hence, overexpression of Ptpcd1 rescued prometaphase arrest of Plk-1 depleted cells, but resulted in aberrant cytokinesis as did as Plk1 overexpression. These results suggested that Ptpcd1 is involved in centrosomal duplication and cytokinesis.

Faithful transmission of genetic information relies on the coordinated regulatory system of the cell cycle [1]. In higher eukaryotes, mitosis involves many dynamic processes at chromosomes, including condensation and segregation, both of which are mainly regulated by protein phosphorylation and dephosphorylation [2]. Proper segregation of chromosomes requires centrosomal maturation, separation, spindle formation and alignment of chromosomes [2,3]. Centrosome is the major microtubule organizing center in animal cells composed of two centrioles, which are barrel shaped structures with nine triple microtubules and a pericentriolar matrix responsible for nucleating microtubules and organizing mitotic spindles for bipolar separation of sister chromatids [4,5]. The identification of several centrosome-associated protein kinases has proposed the concept that multiple regulatory phosphorylation pathways tightly control centrosome cycle during cell cycle [6–8]. Around G1/S transition, a procentriole forms adjacent to each parental centriole and continues growing during S phase. At the onset of mitosis, the two centrosomes separate and the daughter centriole matures and instructs mitotic spindle formation [4]. In post-mitotic cells, centrosome migrates to the cell surface and one of the centrioles differentiates into a basal body that nucleates microtubules to form a cilium [5].

Polo-like kinases (Plks) regulate a multitude of several mitotic processes, including centrosome duplication, maturation [9], bipolar spindle formation [10], microtubule/kinetochore interactions, and cytokinesis [11,12]. Spatio-temporal coordination of Plks activities is achieved through binding to phosphorylated docking proteins with distinct subcellular localizations, such as centrosomes, kinetochore, and the midzone [12,13]. At early mitosis, Cdk1 creates the phosphorylated docking sites on the substrates [1], whereas Plks create their own docking sites on other partners after inactivation of Cdk1 [1,12,13]. In budding yeast, some parts of mitotic function of Plks appear to be mediated by Cdc14p. Cells lacking Cdc14p are unable to exit from mitosis, with defects in both movement of chromosomes to the spindle poles and elongation of anaphase spindles [14]. Mammalian cells possess two Cdc14 paralogue, Cdc14A and Cdc14B, identified based on their sequence similarity to Cdc14p [15]. Recent studies suggested that Cdc14A and Cdc14B might be involved in distinctive cellular functions; the former functioned in centrosomal separation and cytokinesis [3,16], and the latter in centrosomal duplication and microtubule stabilization [17]. However, Cdc14B deficient cells were viable and lacked apparent defects in chromosome segregation and cytokinesis [18], suggesting that alternative phosphatase(s) might be capable of complementing the mitotic functions of Cdc14B. Here, we identified Ptpcd1 as a possible functional isozyme of mammalian Cdc14B that is genetically linked to Plk1.

Materials and methods

Cloning of Ptpcd1. The complete ORF of Ptpcd1 (corresponding exactly to AW456874; No. NM 207232 and MGI: 2145430) was

* Corresponding author. Fax: +81 52 842 3955.
E-mail address: mkt-naka@med.nagoya-cu.ac.jp (M. Nakanishi).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.01.113