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Cooperative functions of Chk1 and Chk2 reduce tumour susceptibility *in vivo*

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Although the linkage of Chk1 and Chk2 to important cancer signalling suggests that these kinases have functions as tumour suppressors, neither *Chk1*$^{+/-}$ nor *Chk2*$^{-/-}$ mice show a predisposition to cancer under unperturbed conditions. We show here that *Chk1*$^{+/-}$*Chk2*$^{-/-}$ and *Chk1*$^{+/-}$ *Chk2*$^{+/-}$ mice have a progressive cancer-prone phenotype. Deletion of a single *Chk1* allele compromises G2/M checkpoint function that is not further affected by Chk2 depletion, whereas Chk1 and Chk2 cooperatively affect G1/S and intra-S phase checkpoints. Either or both of the kinases are required for DNA repair depending on the type of DNA damage. Mouse embryonic fibroblasts from the double-mutant mice showed a higher level of p53 with spontaneous DNA damage under unperturbed conditions, but failed to phosphorylate p53 at S23 and further induce p53 expression upon additional DNA damage. Neither Chk1 nor Chk2 is apparently essential for p53- or Rb-dependent oncogene-induced senescence. Our results suggest that the double Chk mutation leads to a high level of spontaneous DNA damage, but fails to eliminate cells with damaged DNA, which may ultimately increase cancer susceptibility independently of senescence.

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Introduction

Aberrant regulation of DNA-damage response in multicellular organisms is thought to lead to genomic instability and cancer development (Bartkova *et al*, 2005; Gorgoulis *et al*, 2005). Signals initiated by DNA-damage sensors are rapidly transduced to ATM/ATR kinases that in turn phosphorylate a large number of substrates, including checkpoint kinases (Chks), Chk1 and Chk2. In cell culture-based systems, Chk1 and Chk2 behave similarly and appear to regulate activities involving the Cdc25 family of phosphatases (Sanchez *et al*, 1997; Matsuoka *et al*, 1998; Kaneko *et al*, 1999; Tominaga *et al*, 1999), p53 (Shieh *et al*, 2000) and DNA-repair factors (Lee *et al*, 2000; Sorensen *et al*, 2005). In knockout mice, however, the respective phenotypes are very different (Hirao *et al*, 2000; Liu *et al*, 2000; Takai *et al*, 2000, 2002), suggesting that these kinases regulate distinct pathways *in vivo*. In the light of the relationship between these checkpoint kinases and important cancer signalling pathways (Bartkova *et al*, 2005; Gorgoulis *et al*, 2005) and the genetic alterations of *Chk1* and *Chk2* observed in sporadic (Bertoni *et al*, 1999) and familial tumours (Bell *et al*, 1999; Bartek and Lukas, 2003), the lack of an overt tumour-prone phenotype in *Chk1* and *Chk2* knockout mice was somewhat unanticipated (Hirao *et al*, 2000; Liu *et al*, 2000; Takai *et al*, 2000, 2002). Interestingly, tumour incidence was increased in *Chk1*$^{+/-}$, *WNT-1* transgenic mice (Liu *et al*, 2000), *Chk2*$^{-/-}$*Brca1*$^{-/-}$ (McPherson *et al*, 2004), *Chk2*$^{-/-}$ *Brca1*$^{\Delta 11/\Delta 11}$ (Cao *et al*, 2006), *Chk2*$^{-/-}$*NBS1*$^{\Delta B/\Delta B}$ and *Chk2*$^{-/-}$ *Mre11*$^{ATLD1/ATLD1}$ mice (Stracker *et al*, 2008). This may be explained by the redundancy found in biochemical studies, which show that both kinases can phosphorylate the same sites on the same substrates, at least *in vitro*, and that either Chk1 or Chk2 is sufficient to mediate anti-tumour signalling. Alternatively, Chk1 and Chk2 may function in non-redundant DNA-damage response and either response is sufficient to prevent tumour formation *in vivo*.

In this respect, Lam *et al* (2004) showed that Chk1 haploinsufficiency could potentially have a tumour suppressor function. Inactivation of one *Chk1* allele showed inappropriate entry into S phase, accumulation of spontaneous DNA damage during DNA replication and a failure to restrain mitotic entry in the presence of a deregulated S phase. Nevertheless, inactivation of one *Chk1* allele *per se* did not lead to cancer predisposition. Thus, apparent cancer-related phenotypes in *Chk1*$^{+/-}$ cells might be suppressed in *in vivo* tumourigenesis through other DNA-damage responses. To address this critical question, a more exhaustive tumourigenesis study performed in double-mutant mice would be

necessary. This study clearly shows that Chk1 and Chk2 are bona fide and cooperatively haplo-insufficient tumour suppressors *in vivo* that regulate cell cycle checkpoints and apoptosis, but not premature senescence. Mice with the combined loss of two anti-tumour barriers are not able to eliminate cells with a high level of DNA damage and this may be sufficient for the predisposition to spontaneous tumourigenesis.

Results

Tumourigenesis in Chk1/Chk2 double-mutant mice

We generated *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice to conduct a systematic evaluation of the checkpoint kinase function *in vivo* because at least one *Chk1* allele is essential for survival and proliferation of both embryonic (Liu *et al*, 2000; Takai *et al*, 2000) and somatic cells (Shimada *et al*, 2008). Exhaustive characterization of mice bearing single or combined germline *Chk1* and *Chk2* deletions revealed that a significantly higher percentage of mice developed aggressive malignant lymphomas, sarcomas or lung adenomas in *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice (Figure 1A and B; HR = 19.2, 95% CI = 2.5–147.4, $P = 0.004$). Unexpectedly, *Chk1*$^{+/-}$*Chk2*$^{+/-}$ mice also showed a predisposition to cancer, bearing lymphomas, sarcomas or carcinomas (Figure 1C–H) (HR = 9.3, 95% CI = 1.2–74.3, $P = 0.035$), although tumour production occurred later than in *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice. Examination of *Chk1*$^{+/-}$*Chk2*$^{+/+}$ and *Chk1*$^{+/+}$*Chk2*$^{-/-}$ mice revealed no apparent cancer-prone phenotypes during this experimental period (Figure 1A and data not shown). We examined the lineage of lymphomas observed in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice by immunostaining using antibodies to PAX5 as a B-cell marker and CD3 as a T-cell marker. Those lymphomas were positive for PAX5 and negative for CD3, indicating that they were from the B-cell lineage. The typical staining of those lymphomas is shown in Figure 1I.

We then examined whether Chk1 and Chk2 function as typical or haplo-insufficient tumour suppressors. Quantitative real-time PCR using cDNAs from tumour sections revealed that Chk1 was still expressed in tumours of *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice and Chk2 was also expressed in those of *Chk1*$^{+/-}$*Chk2*$^{+/-}$ mice (Figure 1J). These results suggest that both Chk1 and Chk2 are haplo-insufficient tumour suppressors. Aside from their striking cancer susceptibility, *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice were indistinguishable from their wild-type siblings. *Chk1*$^{-/-}$*Chk2*$^{-/-}$ mice, however, died at an early embryonic stage (Supplementary Table SI), indicating that Chk2 depletion failed to rescue embryonic lethality in *Chk1*$^{-/-}$ mice and suggesting that in mice a single *Chk1* allele is sufficient for normal embryonic development or post-natal life.

Aberrant cell cycle checkpoints in Chk1/Chk2 double-mutant cells

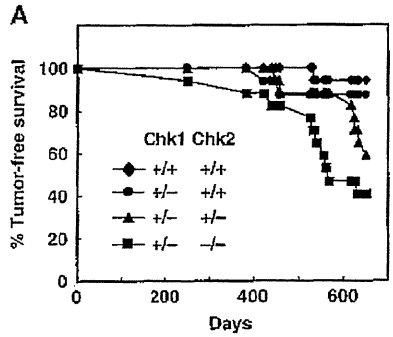
In order to characterize the tumourigenicity observed in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice, we generated primary mouse embryonic fibroblasts (MEFs) from littermates obtained from double-heterozygote breeders. Our strategy to assess the immediate G1/S phase checkpoints after exposure to ionizing radiation (IR) involved staggered CIdU/IdU labelling. The labelling strategy is shown in Figure 2A (left panel). In this assay, single-labelled IdU cells are ones that entered S phase after IR, so this allowed us to evaluate initiation of the G1/S checkpoint without any effect from the intra-S phase checkpoint. Although irradiation reduced the proportion of IdU single-positive cells to 40% in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ cells, those of *Chk1*$^{+/-}$*Chk2*$^{+/+}$ and *Chk1*$^{+/-}$*Chk2*$^{+/-}$ cells were only reduced to 67% ($P<0.05$) and 70% ($P<0.05$), respectively (Figure 2A). No reduction in S phase entry was observed in *Chk1*$^{+/+}$*Chk2*$^{-/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells. Thus, a synergistic effect on this checkpoint was observed by combined deletion of a single *Chk1* allele in *Chk2*$^{-/-}$ cells, indicating that initiation of IR-induced G1/S arrest is independently regulated by Chk1 and Chk2.

Given that initiation of the G1/S checkpoint is regulated by p53 and Cdc25A (Bartek and Lukas, 2001), we examined changes in the expression of p53 and Cdc25A after IR. The level of p53 was increased as early as 1 h and then decreased at 2 h after IR in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ and *Chk1*$^{+/+}$*Chk2*$^{+/-}$ cells (Figure 2B). This increase in p53 protein level was reduced in *Chk1*$^{+/+}$*Chk2*$^{-/-}$ and *Chk1*$^{+/-}$*Chk2*$^{+/-}$ cells and was not observed at all in *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells. Surprisingly, the level of p53 was significantly higher in the absence of DNA damage in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells. Consistent with these effects on p53 level, induction of p21 expression was not detectable in *Chk1*$^{+/+}$*Chk2*$^{-/-}$ or *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells. In contrast to p53, the level of Cdc25A was very low in the absence of DNA damage in *Chk2*$^{-/-}$ cells and the abrupt reduction of Cdc25A after DNA damage was significantly impaired by the single deletion of one *Chk1* allele. These results suggest that Chk1 and Chk2 cooperatively regulate the initiation of the G1/S checkpoint through regulation of Cdc25A protein level. Our results also show that Chk2 is required to maintain a proper level of Cdc25A in the absence of DNA damage, but not after DNA damage, although the molecular mechanism remains to be clarified.

P53 protein level is regulated at multiple levels, transcriptionally, translationally and post-translationally (Appella and Anderson, 2001). One of these controls is Chk1- and Chk2-dependent phosphorylation of p53 at S23 in mice (corresponding to S20 in human) (Shieh *et al*, 2000) and subsequent p53 stabilization by prevention of its interaction with the ubiquitin ligase Mdm2 (Chehab *et al*, 1999). The level of p53 phosphorylation at S23 was increased as early as 1 h and then decreased at 4 h after IR in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ and *Chk1*$^{+/+}$*Chk2*$^{+/-}$ cells, whereas the increase was slightly less in *Chk1*$^{+/+}$*Chk2*$^{-/-}$ and *Chk1*$^{+/-}$*Chk2*$^{+/+}$ cells and was only a little in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells (Figure 2C). These results suggest that Chk1 and Chk2 independently phosphorylate p53 at S23 after DNA damage and cooperatively regulate its stability.

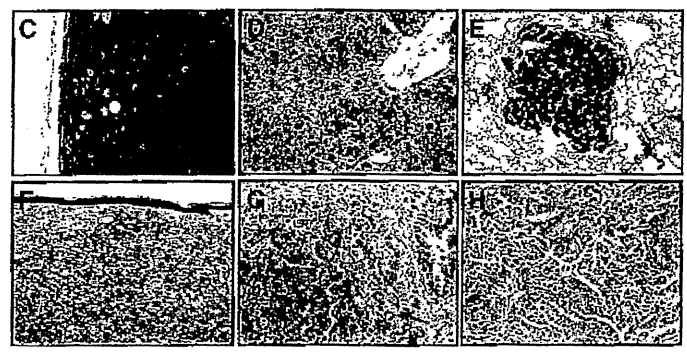
With respect to the intra-S phase checkpoints, we found that IR reduced incorporation of radio-labelled thymidine to 39% in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ cells as compared with untreated cells (Figure 2D). This reduction was significantly impaired in *Chk1*$^{+/+}$*Chk2*$^{-/-}$ cells (51%, $P<0.05$), *Chk1*$^{+/-}$*Chk2*$^{+/+}$ cells (49%, $P<0.05$) and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells (65%, $P<0.01$). Therefore, these results show that the intra-S phase checkpoint in response to double-stranded breaks (DSBs) was regulated by both Chk1 and Chk2.

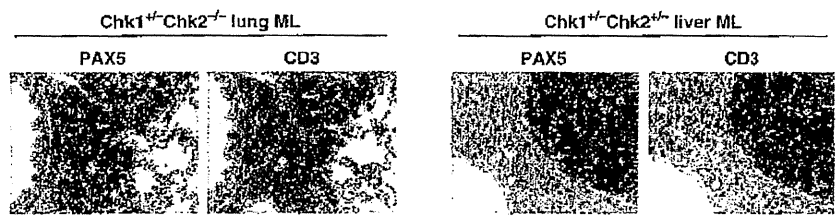
We next examined IR-induced G2 checkpoint function. In contrast to the G1 checkpoint, loss of a single *Chk1* allele resulted in an impaired IR-induced G2 arrest at both low



B

Genotypes	Sex/age (week)	Histology
$Chk1^{+/-}Chk2^{-/-}$	M/36	Malignant lymphoma
	F/54	Malignant lymphoma
	M/62	Malignant lymphoma, lung adenoma
	F/73	Malignant lymphoma
	F/75	Malignant lymphoma
	M/75	Malignant lymphoma
	F/77	Malignant lymphoma
	F/79	Subcutaneous sarcoma
	M/81	Malignant lymphoma, lung adenoma
	F/89	Malignant lymphoma
$Chk1^{+/-}Chk2^{+/-}$	M/63	Pancreas adenocarcinoma
	F/65	Malignant lymphoma
	F/88	Malignant lymphoma
	F/88	Lower abdominal sarcoma
	M/89	Lung adenocarcinoma
	F/90	Malignant lymphoma
	F/93	Malignant lymphoma





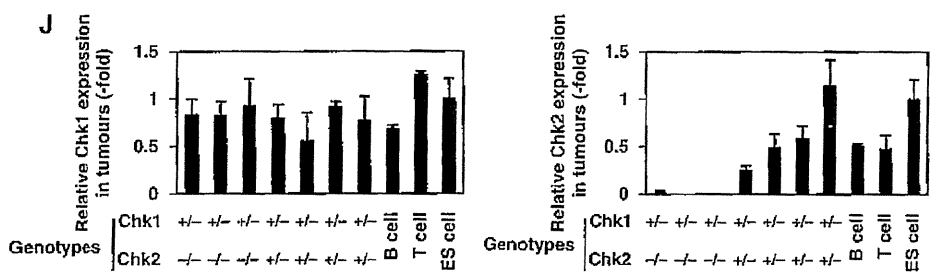


Figure 1 Development of spontaneous tumours in mice bearing germline deletions of *Chk1* and *Chk2*. (**A**) Kaplan–Meier analysis of tumour-free survival of wild type (diamonds, $n=17$), $Chk1^{+/-}Chk2^{+/+}$ (circles, $n=15$), $Chk1^{+/-}Chk2^{+/-}$ (triangles, $n=17$) and $Chk1^{+/-}Chk2^{-/-}$ (squares, $n=17$) mice. The animals were monitored for up to 22 months or until they succumbed to cancer. All tumour cases were identified based on the results of pathological analysis. The statistical significance of the survival curves was assessed using the log-rank test. (**B**) Table summarizing the cancer types observed in $Chk1^{+/-}Chk2^{-/-}$ and $Chk1^{+/-}Chk2^{+/-}$ mice, together with the sexes and ages of the mice; 59% of $Chk1^{+/-}Chk2^{-/-}$, 41% of $Chk1^{+/-}Chk2^{+/-}$, 13% of $Chk1^{+/-}Chk2^{+/+}$ and 6% of $Chk1^{+/+}Chk2^{+/+}$ mice succumbed to cancer during this experimental period. (**C–H**) Images of haematoxylin and eosin histology of six representative tumours found in $Chk1^{+/-}Chk2^{+/-}$ and $Chk1^{+/-}Chk2^{-/-}$ mice. A malignant lymphoma in a lymph node of a $Chk1^{+/-}Chk2^{-/-}$ mouse, ×200 (**C**); a malignant lymphoma in the liver of a $Chk1^{+/-}Chk2^{-/-}$ mouse, ×200 (**D**); a lung adenoma in a $Chk1^{+/-}Chk2^{-/-}$ mouse, ×100 (**E**); a subcutaneous sarcoma in $Chk1^{+/-}Chk2^{-/-}$ mice, ×100 (**F**); a pancreatic adenocarcinoma, ×200 (**G**) and a lung adenocarcinoma, ×200 (**H**). (**I**) Representative immunohistochemical staining of a malignant lymphoma invading the lung of a $Chk1^{+/-}Chk2^{-/-}$ mouse (left) and liver of a $Chk1^{+/-}Chk2^{+/-}$ mouse (right) with PAX5 used as a B-cell marker and CD3 as a T-cell marker, ×100. PAX5 was positive in the nucleus of invading atypical small round cells and CD3 was negative in the membrane of these cells. (**J**) Expression of Chk1 (left panel) and Chk2 (right panel) transcripts in tumours from $Chk1^{+/-}Chk2^{+/-}$ and $Chk1^{+/-}Chk2^{-/-}$ mice. Expression levels of Chk1 and Chk2 transcripts were measured by real-time PCR using RNAs prepared from paraffin-embedded tumour tissues and RNAs from B cells, T cells and ES cells as controls. Normalization was performed relative to the level of GAPDH transcripts and the data are presented as relative expressions to those in ES cells.

(1 Gy) and high (3 Gy) doses of irradiation (Figure 2E), whereas null depletion of *Chk2* had no effect in this regard. Consistent with our observations, recent reports have shown that Chk2 is dispensable for the degradation of Cdc25A and initiation of G2/M arrest after DNA damage in human (Jallepalli *et al*, 2003; Jin *et al*, 2008), mouse (Takai *et al*,

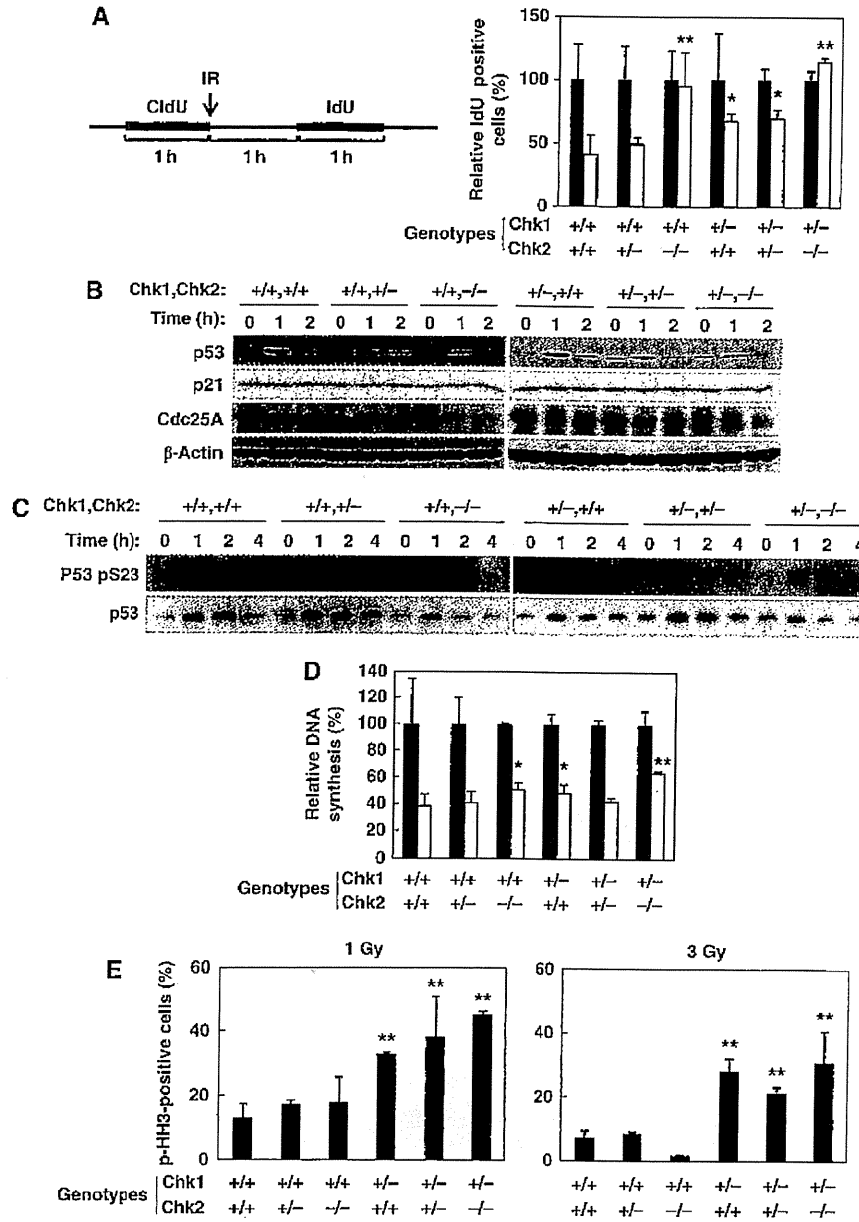


Figure 2 Chk1 and Chk2 regulate non-redundant DNA-damage checkpoints. (**A**) Synergistic regulation of G1/S checkpoint by Chk1 and Chk2. Diagram of our strategy to assess the G1/S checkpoint after IR irradiation. Primary MEFs with the indicated genotypes are labelled with CldU for 1 h and then IR irradiated (white bars) or mock irradiated (black bars). After washing out the CldU, the cells were incubated in fresh medium for 1 h and then incubated with IdU for 1 h. In order to record the number of cells newly entering S phase after IR irradiation, single IdU-positive cells were counted (at least 300 cells) and the results were represented as a percentage of the total cells. Data are means ± s.d. of at least three independent experiments. Statistical significance compared with $Chk1^{+/+}Chk2^{+/+}$ MEFs was assessed by Student's *t*-test (*$P<0.05$, **$P<0.01$). (**B**) Accumulation of p53 and reduction of Cdc25A upon IR treatment. Primary MEFs with the indicated genotypes were irradiated with IR (4 Gy) and harvested at 0, 1 and 2 h after irradiation. Cell lysates were subjected to immunoblotting using anti-p53 (upper panels), anti-p21 (2nd panels), anti-Cdc25A (3rd panels) and anti-β-actin (bottom panels) antibodies. (**C**) Phosphorylation of p53 at S23 after IR treatment. Primary MEFs with the indicated genotypes were irradiated with IR (4 Gy) and harvested at 0, 1, 2 and 4 h after irradiation. Cell lysates were immunoprecipiated with anti-p53 antibodies and the precipitates were subjected to immunoblotting using an anti-phospho-p53 at S23 antibody (upper panels) and an anti-p53 antibody (lower panels). (**D**) Radio-resistant DNA synthesis was examined as described in Materials and methods. The rate of DNA synthesis was determined by the radioactivity of [^{3}H] divided by that of [^{14}C]. The relative DNA synthesis is represented as a percentage of DNA synthesis relative to that observed in cells without DNA damage. Data are means ± s.d. of at least three independent experiments. Statistical significance compared with $Chk1^{+/+}Chk2^{+/+}$ MEFs was assessed by Student's *t*-test (*$P<0.05$, **$P<0.01$). (**E**) Defective G2/M checkpoint in mice lacking a single Chk1 allele. Primary MEFs from mice with the indicated genotypes were treated with two distinct doses of IR (1 Gy: left panel and 3 Gy: right panel). The mitotic index was determined as the percentage of mitotic cells (pH3 Ser10 positive) relative to the total cells at 0.5 h after irradiation. The mitotic index was then calculated as a percentage relative to the non-irradiated cells. Data are means ± s.d. of at least three independent experiments. Statistical significance compared with $Chk1^{+/+}Chk2^{+/+}$ MEFs was assessed by Student's *t*-test (**$P<0.01$).

2002) and Drosophila cells (Varmark *et al*, 2010). Thus, our results suggest that IR-induced G2 arrest is mainly regulated by Chk1, although maintenance of G2 arrest might be affected by Chk2 depletion as reported earlier (Hirao *et al*, 2000; Yu *et al*, 2001).

Aberrant DNA-damage-induced apoptosis and DNA-repair activity in Chk1/Chk2 double-mutant cells

Chk2 has been reported to regulate DNA-damage-induced apoptosis (Hirao *et al*, 2002; Takai *et al*, 2002). Therefore, it is possible that Chk1 and Chk2 may cooperatively regulate p53-dependent apoptosis. Thymocytes from Chk-depleted mice were irradiated and their survival was assessed. Depletion of two *Chk2* alleles, but not a single *Chk1* allele, resulted in impaired induction of thymocyte apoptosis after IR treatment at a high dose (4 Gy) (Figure 3A, right panel). Intriguingly, deletion of a single Chk2 allele also resulted in a partial impairment of apoptosis at a low dose (1 Gy) of IR (Figure 3A, left panel), suggesting that Chk2 is haplo-insufficient for induction of apoptosis.

We then sought to determine whether impaired induction of apoptosis in Chk2-depleted thymocytes was due to reduced induction of p53 protein upon DNA damage. Induction of p53 was observed as early as 1 h and peaked at 2 h after IR in $Chk1^{+/+}Chk2^{+/+}$ thymocytes (Figure 3B). This induction was severely impaired in $Chk1^{+/+}Chk2^{-/-}$ and $Chk1^{+/-}Chk2^{-/-}$ thymocytes. It should be noted that, unlike MEFs, thymocytes from $Chk1^{+/-}Chk2^{+/-}$ and $Chk1^{+/-}Chk2^{-/-}$ mice did not contain a higher level of p53 under unperturbed conditions when compared with $Chk1^{+/+}Chk2^{+/+}$ mice. These results suggest that Chk2 regulates IR-induced apoptosis in thymocytes at least in part through induction of p53 expression.

Chk1 and Chk2 are reported to be involved in DNA repair through phosphorylation of Rad51 (Sorensen *et al*, 2005) and BRCA1 (Lee *et al*, 2000), respectively. Therefore, the cooperative effect of Chk1 and Chk2 in suppressing tumourigenesis may be explained by their ability to ensure proper DNA repair. To address this question, we analysed DNA repair in double Chk1- and Chk2-depleted primary MEFs using an

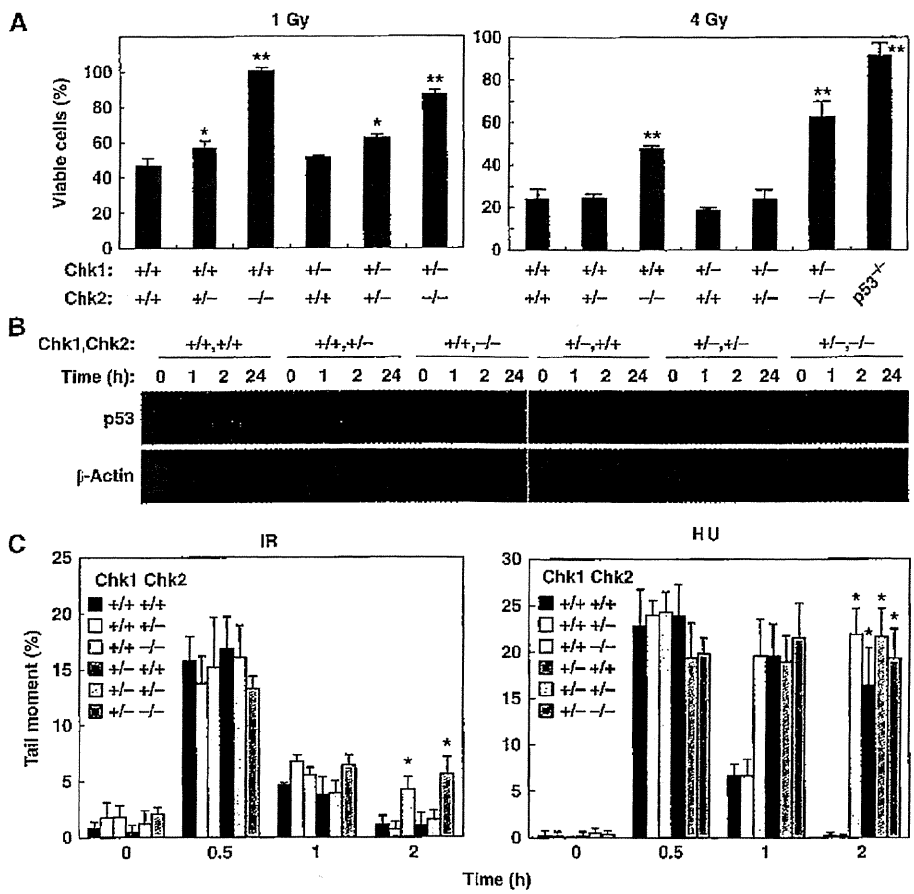


Figure 3 Chk1 and Chk2 have a non-redundant function in DNA-damage-induced apoptosis and DNA repair. (**A**) Chk2 is haplo-insufficient for IR-induced apoptosis of thymocytes. Thymocytes from mice with the indicated genotypes were exposed to 1 Gy (left panel) or 4 Gy (right panel) irradiation. Sub G1 population was determined by FACS and viable cells were calculated as a percentage of non-sub G1 cells relative to the total cell number. Data are means ± s.d. of at least three independent experiments. Statistical significance compared with $Chk1^{+/+}Chk2^{+/+}$ MEFs was assessed by Student's *t*-test (*$P<0.05$, **$P<0.01$) (**B**) Accumulation of p53 upon IR treatment in thymocytes. Thymocytes from mice with the indicated genotypes were irradiated with IR (4 Gy) and harvested at 0, 1, 2 and 24 h after irradiation. Cells were subjected to immunoblotting using anti-p53 and anti-β-actin antibodies. (**C**) Chk1 and Chk2 regulate the efficiency of DNA repair. Primary MEFs of the indicated genotypes were treated with IR (left panel) or hydroxyurea (HU: right panel) and subjected to an alkaline-comet assay at the indicated times. Tail moments were determined using TriTek Comet Score Freeware. Data are means ± s.d. of counting at least 50 cells per sample in three independent experiments. Statistical significance compared with $Chk1^{+/+}Chk2^{+/+}$ MEFs was assessed by Student's *t*-test (*$P<0.005$).

alkaline-comet assay. Deletion of two *Chk2* alleles resulted in a significant aberration of IR-induced DNA repair, although loss of a single *Chk1* allele had no effect (Figure 3C). In contrast, loss of a single *Chk1* allele, as well as deletion of two *Chk2* alleles, caused aberrant DNA repair when DNA damage was induced by hydroxyurea. This indicated that DNA damage-induced activation of DNA repair requires Chk1 or Chk2, but which is required depends on the type of DNA damage.

Spontaneous DNA damage in proliferating Chk1$^{+/-}$ Chk2$^{+/-}$ and Chk1$^{+/-}$Chk2$^{-/-}$ MEFs under unperturbed condition

The increase in the level of p53 observed in proliferative *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs under unperturbed conditions (Figure 2B) raises the possibility that Chk1 and Chk2 directly regulate factors that determine p53 stabilization or prevent accumulation of spontaneous DNA damage that eventually increases p53 protein levels. In *Chk1*$^{+/+}$*Chk2*$^{+/+}$ MEFs, IR induced a biphasic increase in p53, showing a rapid induction of expression within 4 h and a slow induction at 24 h (Figures 2B and 4A). A similar IR-induced biphasic accumulation of p53 was also reported in normal human embryonic cells (Ghosh *et al*, 2000). As seen in Figure 2B, an increased level of p53 was detected in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs under unperturbed conditions (time 0). The level of p21 was also higher in these cells under unperturbed conditions. Surprisingly, the level of Mdm2 was very low in *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs, although the level of p53, a transcriptional activator of Mdm2, was high during this experimental period, suggesting that Chk1 and Chk2 may cooperatively stabilize Mdm2 through an unknown mechanism. In contrast to apparent impairment of the rapid accumulation of p53 observed in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs, a slow induction was observed in all other MEFs tested. Recently, it was reported that Che-1, an RNA polymerase II-binding protein, was phosphorylated by ATM/ATR and Chk1 after DNA damage (Bruno *et al*, 2006). The phosphorylation of Che-1 resulted in its accumulation and recruitment to the *p53* promoter to activate *p53* gene transcription. We examined the induction of Che-1 as well as changes in p53 transcript level after DNA damage. However, in contrast to the previous report (Bruno *et al*, 2006), the accumulation of Che-1 was only impaired upon Chk2 deletion (*Chk1*$^{+/+}$*Chk2*$^{-/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs) when compared with what is observed in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ MEFs (Figure 4A). Deletion of a single *Chk1* allele did appear to slightly affect accumulation of Che-1, consistent with the previous observation that Chk1 as well as Chk2 could phosphorylate Che-1 *in vitro*. P53 transcription was increased as early as 4 h after DNA damage and then decreased in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ and *Chk1*$^{+/-}$*Chk2*$^{+/+}$ MEFs, whereas it did not vary in MEFs in which either 1 or 2 copies of *Chk2* were deleted (Figure 4B). These results show that a single *Chk2* allele is not sufficient for recruitment of Che-1 to the p53 promoter and, therefore, Chk2 is haplo-insufficient for Che-1-dependent transcriptional activation of p53. However, this transcriptional activation was apparently not involved in the slow induction of p53 expression after DNA damage.

Given that a significant number of heterozygous lobuloalveolar mammary epithelial cells contained spontaneous DNA damage in *Chk1* conditional heterozygotes (Lam *et al*, 2004), we speculate that this was also the case in primary MEFs from *Chk1* heterozygotes. To examine this possibility, MEFs from *Chk1/Chk2* double-mutant mice were stained with phospho-H2AX specific for Ser139 (γH2AX) without the addition of any exogenous DNA-damaging agent. A significant number of γH2AX-positive cells were readily detected in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs, but not in MEFs from other genotypes (Figure 4C). Signals for γH2AX in *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs were much stronger than those in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ MEFs. However, some of the γH2AX signals in *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs as well as most of the signals in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ MEFs showed typical discrete foci, presumably because of the level of spontaneous DNA damage. These results suggest that Chk1 and Chk2 cooperatively prevent the accumulation of cells with spontaneous DNA damage.

Accumulation of DNA damage can easily result in genome instability. We thus examined metaphase chromosome spreads from *Chk1/Chk2* double-mutated primary MEFs and CD19-positive B cells. Aberrations including chromosomal breaks and many fusions appeared in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs (Table I). Although the level of the chromosomal aberrations observed in the activated B cells was relatively low when compared with MEFs, presumably because of the short *in vitro* culture period (72 h) of B cells, higher levels of chromosomal aberrations were detected in B cells from *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice (Table II). These results confirm that *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice are more susceptible to B-cell lymphomas and show that Chk1 and Chk2 synergistically prevent susceptibility to global chromosomal rearrangement.

Chk1 and Chk2 do not appear to be involved in the induction of premature senescence

Senescence offers a major protective mechanism against tumour development and is triggered by DNA damage responses (Lowe *et al*, 2004). In several studies using human diploid cells, activation of Chk1 and Chk2 was proposed to be important for oncogene-induced and replicative senescence (d'Adda di Fagagna *et al*, 2003; Bartkova *et al*, 2006; Di Micco *et al*, 2006; Mallette *et al*, 2007). Therefore, we hypothesized that the cooperative function of Chk1 and Chk2 in suppression of tumourigenesis might be a means to ensure induction of senescence upon exposure of cells to genotoxic stress. To test this hypothesis, we first examined stress-induced senescence in culture, known as 'culture shock', in which mammalian cells are exposed to an unrelenting onslaught of mitogenic signals (Sherr and DePinho, 2000). Wild type and other types of Chk1/Chk2-depleted primary MEFs tested were cultured in serum-containing medium and passaged every 3 days after a 3T3 subculture schedule. All types of MEFs had similar doubling times for the first 10 generations. Their growth rate then slowed and eventually reached a non-dividing state even at a subconfluent density (Figure 5A). These cells then recovered their growth capability and became immortal at around 40 days with a doubling time similar to that of the initial culture. Unexpectedly, ectopic expression of oncogenic Ras clearly induced both growth arrest (Figure 5B) and a senescent phenotype as assessed by senescence-associated β-gal staining (Figure 5C) in all types of double Chk1- and Chk2-depleted primary MEFs.

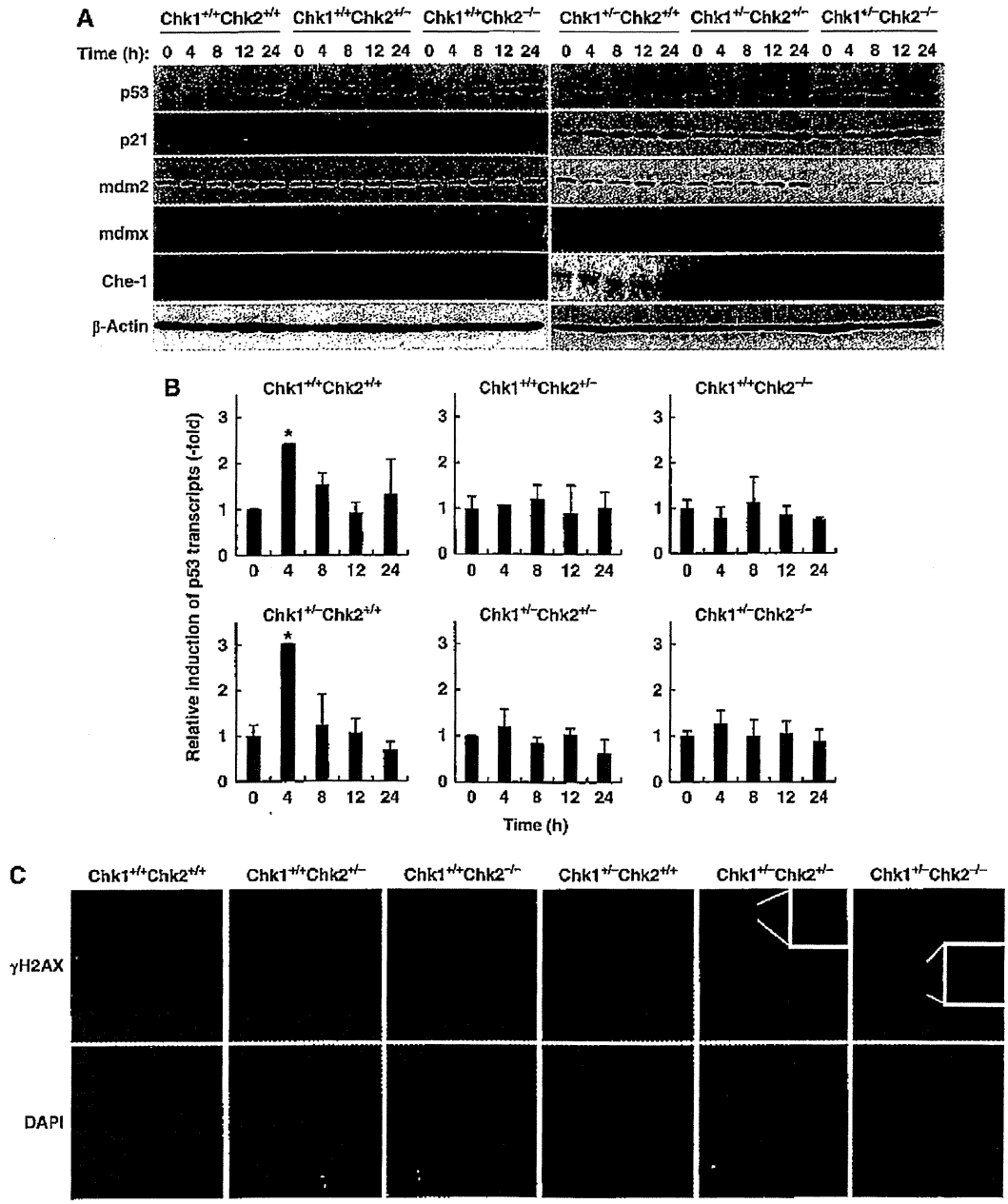


Figure 4 Spontaneous DNA damage and induction of p53 under unperturbed conditions in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs. (**A**) Cell lysates from the indicated MEFs were irradiated with IR (4 Gy). Cells were harvested at the indicated times after IR and cell lysates were subjected to immunoblotting with anti-p53 (top), anti-p21 (second), anti-mdm2 (third), anti-mdmX (fourth), anti-Che-1 (fifth) and anti-β-actin (bottom) antibodies. (**B**) Changes in the p53 transcripts after IR irradiation. Primary MEFs with the indicated genotypes were irradiated with IR (4 Gy) and harvested at the indicated times after irradiation. Total RNA was then extracted and the expression levels of p53 transcript were measured by quantitative real-time PCR. The results were normalized to the level of GAPDH transcripts used as an internal control and data are presented as means ± s.d. of at least three independent experiments. Statistical significance compared with cells without IR irradiation (time 0) was assessed by Student's *t*-test (*$P < 0.01$). (**C**) Spontaneous DNA damage under unperturbed conditions in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs. Asynchronized primary MEFs with the indicated genotypes without any genotoxic stress were fixed and subjected to immunofluorescence staining with anti-phospho-H2AX (γH2AX) antibodies (upper panel). Cells were counterstained with DAPI to detect nuclei (lower panel), ×200. Magnified images of cells with γH2AX foci are shown in the white boxes (*Chk1*$^{+/-}$*CHk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells).

Given that homozygous deletion of *Chk1* resulted in growth arrest at S phase (Shimada and Nakanishi, 2008) and presented a senescence-like morphology, and knockdown of Chk1 by its siRNA drastically increased the number of SA-β-gal-positive cells (Shimada *et al*, paper in preparation), our results indicated that both Chk1 and Chk2 are apparently dispensable for oncogene-induced senescence.

As the induction of senescent phenotypes is regulated by both p16-Rb and p53-dependent mechanisms (Courtois-Cox

Table I Chromosomal abnormalities in primary MEFs from Chk1/Chk2 double-mutant mice[a]

Samples	Metaphases analysed	Chromosomes/metaphase	Total aberrant cells (%)
Chk1$^{+/+}$*Chk2*$^{+/+}$	58	40.30 ± 0.60	2 (3.45%)
Chk1$^{+/+}$*Chk2*$^{+/-}$	62	40.37 ± 1.09	4 (6.45%)
Chk1$^{+/+}$*Chk2*$^{-/-}$	58	42.54 ± 1.38	9 (15.52%)
Chk1$^{+/-}$*Chk2*$^{+/+}$	59	39.96 ± 0.66	5 (8.47%)
Chk1$^{+/-}$*Chk2*$^{+/-}$	58	40.66 ± 0.61	11 (18.97%)
Chk1$^{+/-}$*Chk2*$^{-/-}$	59	44.12 ± 1.56	12 (20.34%)

[a]Chromosomes were stained with 4′, 6′-diamidino-2-phenylindole (DAPI) and observed under a Zeiss axioplan imaging 2 microscope. The abnormal chromosomes including fusions and fragments were counted in metaphase chromosome spreads of MEFs with the indicated genotypes.

Table II Chromosomal abnormalities in primary B cells from Chk1/Chk2 double-mutant mice[a]

Samples	Metaphases analysed	Chromosomes/metaphase	Total aberrant cells (%)
Chk1$^{+/+}$*Chk2*$^{+/+}$	65	39.97 ± 0.17	1 (1.54%)
Chk1$^{+/+}$*Chk2*$^{+/-}$	52	39.88 ± 0.61	1 (1.92%)
Chk1$^{+/+}$*Chk2*$^{-/-}$	55	39.53 ± 1.36	3 (5.45%)
Chk1$^{+/-}$*Chk2*$^{+/+}$	36	37.80 ± 3.38	2 (5.56%)
Chk1$^{+/-}$*Chk2*$^{+/-}$	37	39.02 ± 2.11	3 (8.11%)
Chk1$^{+/-}$*Chk2*$^{-/-}$	33	39.39 ± 1.03	3 (9.09%)

[a]Primary B cells from *Chk1/Chk2* double-mutant mice were isolated using magnetic bead-conjugated antibodies against CD19 and AutoMACS, and stimulated by lipopolysaccharide as described in 'Materials and methods'. Chromosomes were stained with 4′, 6′-diamidino-2-phenylindole (DAPI) and observed under a Zeiss axioplan imaging 2 microscope. The abnormal chromosomes including fusions and fragments were counted in metaphase chromosome spreads of B cells with the indicated genotypes.

et al, 2008), we wondered whether senescent phenotypes in Chk-depleted MEFs were induced by either or both pathways. Expression of oncogenic Ras resulted in the clear induction of p16 protein in all types of MEFs (Figure 5D). As described above, the level of p53 was significantly higher in control-transfected *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs than in MEFs of other genotypes. Intriguingly, the levels of p53 and p21 in control-transfected *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs were almost identical to those observed in Ras-transfected senescent cells. These results suggest that senescent phenotypes among *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs might be induced by activation of p16-Rb pathway. To further clarify this point, we introduced papillomavirus E7, which binds to and inactivates Rb (Chellappan *et al*, 1992), into *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ primary MEFs in the presence or absence of oncogenic Ras. Surprisingly, E7 failed to prevent growth arrest and a senescence-like cell morphology in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ primary MEFs expressing oncogenic Ras as well as in *Chk1*$^{+/+}$*Chk2*$^{+/+}$ MEFs cells (Figure 6A and B). Co-introduction of papillomavirus E6, which targets p53 for degradation (Scheffner *et al*, 1990), with E7 completely reversed growth arrest and morphological changes in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ primary MEFs expressing oncogenic Ras. These results indicate that senescence is induced in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ cells at least in part by oncogene-induced activation of the p53 pathway even in the absence of p16-Rb pathway. Although the actual contribution of the p16-Rb pathway to senescence induction in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ MEFs is unknown, it should be noted that abrogation of a p53-Arf pathway (Serrano *et al*, 1997; Palmero *et al*, 1998), but not loss of p16 (Sharpless *et al*, 2001), is sufficient to prevent oncogenic Ras-induced senescence in primary MEFs. Taken together, our results indicate that cancer predisposition of *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice is not due to impaired induction of senescence in response to genotoxic stress.

Discussion

Our results clearly indicate that Chk1 and Chk2 act cooperatively to prevent tumourigenesis by regulating partly redundant, but mainly non-redundant responses to DNA damage or genotoxic stress, including cell cycle arrest, apoptosis and DNA repair. As a result, combined loss of Chk1 and Chk2 causes the accumulation of cells with spontaneous DNA damage under unperturbed conditions leading to genomic instability and then tumour development. Accumulation of cells with DNA damage under unperturbed conditions appears to result from increased DNA damage during S phase because of reduced Chk1 activity and failure to eliminate cells with DNA damage by loss of Chk2 function. This idea is strongly supported by the fact that Chk1 depletion causes severe DNA damage during S phase (Niida *et al*, 2005; Syljuasen *et al*, 2005) and that Chk2-deficient MEFs are highly resistant to DNA-damage-induced apoptosis (Figure 3A) (Hirao *et al*, 2002; Takai *et al*, 2002). In addition, increased expression of p53 in Chk1/Chk2 double-depleted cells under unperturbed conditions was observed only in proliferative MEFs and not in G0-arrested T cells (Figures 2B, 3B and 4A), further supporting the idea that DNA damage in these cells occurred during S phase. Recently, Zaugg *et al* (2007) reported that Chk1 and Chk2 functioned in mostly non-redundant DNA-damage response and that the loss of Chk1 activated Chk2 in thymocytes, suggesting the existence of physiological cross-talk between Chk1 and Chk2. Taken together, these results indicate that high tumour susceptibility

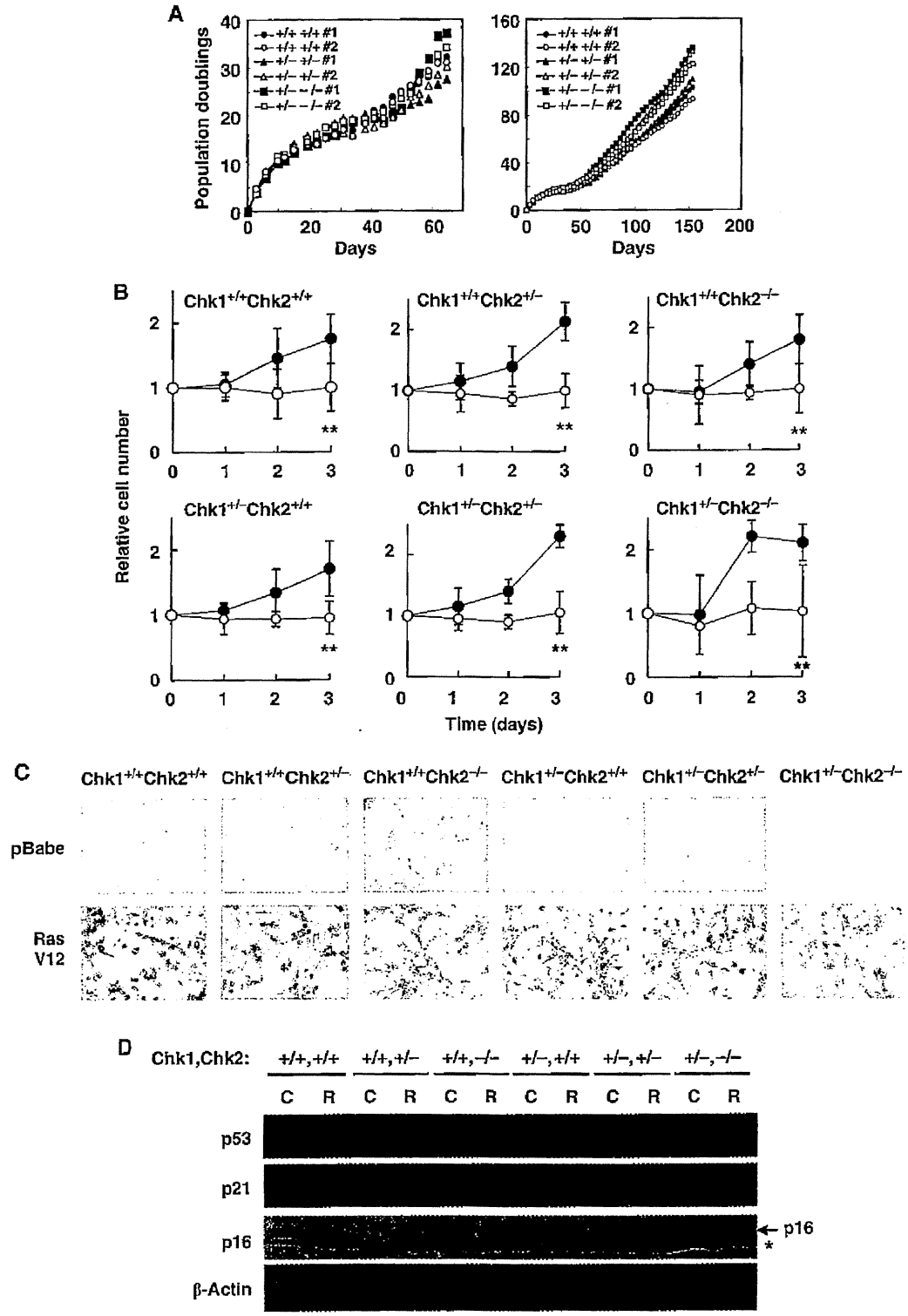


Figure 5 Chk1 and Chk2 are apparently dispensable for culture-induced (culture shock) and oncogene-induced senescence. (**A**) Growth rates of short-term (left panel) and long-term (right panel) cultures of primary MEFs of the indicated genotypes. (**B**) Representative growth curves corresponding to primary MEFs with the indicated genotypes infected with control (closed circles) or H-RasV12-expressing (open circles) retroviruses. Statistical significance of relative numbers of cells expressing RasV12 at day 3 compared with those expressing control vector was assessed by Student's *t*-test (**$P<0.01$). (**C**) Photographs of cells stained for SA-β-gal activity 3 days after infection. (**D**) Induction of p53, p21 and p16 proteins in cells of the indicated genotypes infected with either control (C) or H-RasV12 (R). An asterisk represents non-specific bands.

in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice is likely due to the combined impairment of non-redundant DNA-damage response mediated by both Chk1 and Chk2.

Although mutations in *CHEK2* (the gene encoding Chk2) do not account for the cancer-predisposing Li–Fraumeni syndrome as originally thought, rare germline mutations

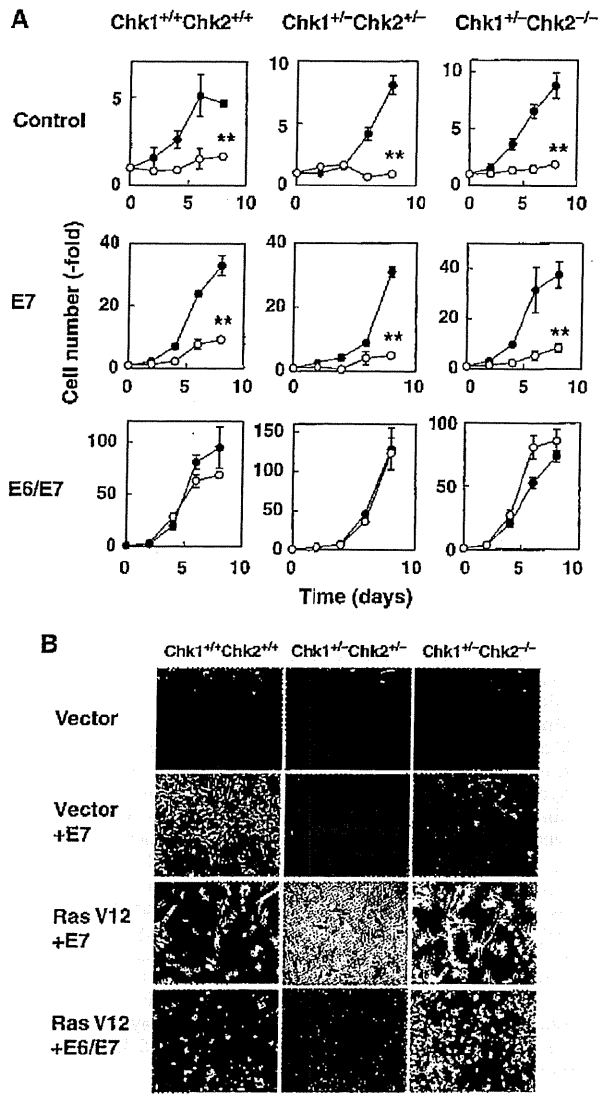


Figure 6 Chk1 and Chk2 are apparently dispensable for p53- and Rb-dependent senescence pathways. (**A**) Growth curve of MEFs with or without RasV12-expressing mock (control), E7 (E7) or both E6 and E7 (E6/E7). The cells were infected with empty (open circles) or Ras-expressing retrovirus (filled circles). Cell numbers were counted at the indicated times after selection. Data are means ± s.d. of triplicate experiments. Statistical significance of relative numbers of cells expressing RasV12 at day 8 compared with those expressing control vector was assessed by Student's *t*-test (**$P<0.01$). (**B**) Morphology of cells obtained at 8 days after selection. The MEFs expressing mock (top panel), E7 (second and third panels) and E6/E7 (bottom panel) were infected with empty (top and second panels) or Ras-expressing retrovirus (third and bottom panels).

have been detected with high incidence in a number of familial cancers and rare somatic mutations have been reported in some tumours, suggesting that *CHEK2* is indeed a cancer susceptibility gene (Meijers-Heijboer *et al*, 2002; Vahteristo *et al*, 2002; Antoni *et al*, 2007). However, we and others have shown that complete loss of both Chk2 alleles is not sufficient to increase tumour incidence in mice (Hirao *et al*, 2002; Takai *et al*, 2002). In this regard, it should be noted that most of the *CHEK2* abnormalities in human tumours are mutations (not complete gene deletion) that generate truncated or mutated forms of the Chk2 protein (Antoni *et al*, 2007). Thus, these modified forms may impair other tumour suppressive pathways in a dominant-negative manner. Alternatively, it may take a much longer time to generate tumours by loss of Chk2 function, so that they are not observed during the short life span of the mice.

Chk1$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice developed B-cell tumours, but not T-cell lymphomas. Intriguingly, B-cell lymphomas have been observed in mice bearing a *p53 S23A/S23A* knockin mutation (corresponding to human S20) (MacPherson *et al*, 2004). It is, therefore, possible that tumourigenicity in the double-mutant mice resulted from impaired phosphorylation of p53 at S23 that is redundantly regulated by both Chk1 and Chk2 in response to DNA damage, as indicated from *in vitro* studies (Shieh *et al*, 2000). P53 is activated by numerous types of stress, such as DNA damage, viral infection and metabolic stress, through phosphorylation of S23. CK1 is a p53 S23 kinase activated in response to DNA viral infection (MacLaine *et al*, 2008), AMPK is another p53 S23 kinase activated in response to elevation of AMP/ATP ratio (Maclaine and Hupp, 2009) and DAPK-1 is a third p53 S23 kinase activated in response to inappropriate oncogene activation (Craig *et al*, 2007). However, the kinase responsible for S23 phosphorylation in response to IR has not been identified. Unexpectedly, knockdown of either or both Chk1 and Chk2 in human cells failed to abrogate the damage-dependent induction of p53 expression (Ahn *et al*, 2003), and depletion of both *Chk2* alleles in human colon cancer cells does not compromise p53 phosphorylation at S20 (corresponding to mouse S23) (Jallepalli *et al*, 2003). Although these results cast doubt on the function of Chk1 and Chk2 in the damage-induced phosphorylation of human p53 at S20 (corresponding to mouse S23), our present results clearly show that DNA-damage-induced mouse p53 phosphorylation at S23 was abrogated in primary MEFs from *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice. One potential explanation for this discrepancy is that our experiments were performed with primary knockout MEFs, whereas other studies used human cancer cells and did not analyse the phosphorylation status of p53 at S20 (corresponding to mouse S23) in Chk1/Chk2 double-knockdown cells. Therefore, although the contribution of Chk1 and Chk2 to the phosphorylation of p53 at S23 in response to DNA damage appears to be somewhat different between human and mouse cells, both enzymes are likely to function as damage-induced p53 S23 kinases in a redundant manner.

Chk2 has also been reported to cooperate with other factors involved in DNA-damage response to suppress oncogenic potential. For example, *Brca1*$^{\Delta 11/\Delta 11}$*Chk2*$^{-/-}$ mice showed cancer predisposition (Cao *et al*, 2006). Similarly, *Chk2*$^{-/-}$ mice with a conditional deletion of *Brca1* in the thymus or mammary gland developed tumours in these tissues (McPherson *et al*, 2004). Very recently, *NBS1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ and *Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$ mice have also been reported to develop tumours with latency similar to that observed in *Brca1*$^{\Delta 11/\Delta 11}$*Chk2*$^{-/-}$ mice (Stracker *et al*, 2008). Both the MRN complex and Brca1 have an essential function in sensing DNA double-strand breaks and in transmitting the signal to downstream targets. In contrast, Chk1 and Chk2 are specifically activated depending on the type of DNA damage and thus act in a complementary manner (Bartek and Lukas, 2003). Our results suggest that Chk1-dependent cell cycle arrest might serve as a backup system for DSBs that must be

repaired before completion of DNA replication or eliminated by Chk2-dependent apoptosis to prevent severe genomic instability and transformation of normal cells into cancer cells. In this regard, it should be noted that ATM regulates the recruitment of ATR to DSBs, leading to double-strand break-induced Chk1 phosphorylation (Adams *et al*, 2006; Jazayeri *et al*, 2006).

Surprisingly, neither Chk1 nor Chk2 was apparently involved in mouse senescence pathways, which may explain why tumour development in *Chk1*$^{+/-}$*Chk2*$^{+/-}$ and *Chk1*$^{+/-}$*Chk2*$^{-/-}$ mice had a late onset. These observations are in clear contrast with those observed in human cells, in which both Chk1 and Chk2 are activated after induction of premature or replicative senescence (d'Adda di Fagagna *et al*, 2003; Gire *et al*, 2004; Bartkova *et al*, 2006; Di Micco *et al*, 2006; Mallette *et al*, 2007). In addition, knockdown of Chk2 suppresses oncogene-induced senescence (Di Micco *et al*, 2006), and ectopic co-expression of dominant-negative forms of Chk1 and Chk2 (d'Adda di Fagagna *et al*, 2003) or Chk2 knockdown suppress replicative senescence in human cells (Gire *et al*, 2004). In this regard, mouse cells do not undergo replicative senescence, because they possess far longer telomeres than human cells. Therefore, Chk2 might have a specific function in the induction of replicative senescence that is not observable in mice. We found that complete loss of Chk1 led to a senescence-like permanent growth arrest in MEFs. Intriguingly, constitutive activation of ATR in mouse cells can also induce senescence (Toledo *et al*, 2008). We and others reported that Chk1 localizes at chromatin under unperturbed conditions and its phosphorylation by ATR releases Chk1 from chromatin (Smits *et al*, 2006; Niida *et al*, 2007). Chromatin-bound Chk1 is required for the expression of various cell cycle regulatory genes through phosphorylation of H3-T11 (Shimada *et al*, 2008). Therefore, constitutive activation of ATR mimics loss of Chk1 bound on chromatin. Taken together, these results indicate that permanent loss of Chk1 from chromatin can drive cells into senescence through epigenetic modifications on cell cycle gene promoters. This appears to be consistent with the apparently dispensable function of Chk1 in the induction of premature senescence.

In conclusion, our results suggest that a combination of partial defects in several tumour-protective barriers might engender a more progressive cancer-prone condition than a severe defect in one mechanism. This is in agreement with the association between genetic variations or mutations in *Chk1* and *Chk2* genes and the high cancer risk. Therefore, inhibition of Chk1 or Chk2 as an approach to cancer therapy should be undertaken with careful consideration.

Materials and methods

Immunoblotting

For preparation of whole cell extracts, cells were lysed in IP kinase buffer as previously described. The antibodies used for immunoblotting or immunofluorescence were directed against p53 (NCL-p53-505, Novocasta Laboratory), p21 (sc-6246, SantaCruz), p16 (sc-1207, SantaCruz), Cdc25A (sc-7389, SantaCruz), Che-1 (ab39631, Abcam), Mdm2 (sc-965, SantaCruz), Mdmx (sc-28222, SantaCruz), PAX5 (sc-1974, SantaCruz), CD3 (ab49943, Abcam), β-actin (ab2676-100, Abcam), phosphor-Ser-10-histone H3 (06-570, Upstate) and BrdU (B44, BD for IdU; BI1/75, ICR1 for CldU).

Mice and MEFs

Chk1$^{+/-}$ mice (Takai *et al*, 2000) were crossed with *Chk2*$^{+/-}$ mice (Takai *et al*, 2002) to obtain *Chk1*$^{+/-}$*Chk2*$^{+/-}$ mice. Experimental cohorts were derived from littermates obtained from double-heterozygote breeders. All mice studied had a mixed 129 × C57BL/6 genetic background and were genotyped by PCR. All experiments were performed in compliance with the Nagoya City University Animal Care Committee guidelines. Primary MEFs were derived from E14.5 embryos of double-heterozygote breeders.

Cell cycle, apoptosis analysis and comet assay

The mitotic index was measured as described previously using anti-phosphor-Ser-10-histone H3 (06-570, Upstate) antibodies (Niida *et al*, 2005). For G1/S checkpoint analysis, primary MEFs were labelled with CldU (10 μM) for 1 h, then treated with mock or IR (5 Gy) and additionally labelled with IdU (10 μM) for 1 h after incubation with fresh medium for 1 h (Figure 2A). The cells were then fixed in three parts methanol: one part glacial acetic acid at −20°C for 30 min, after which they were treated with 2 N HCl for 30 min. The cells were then washed with PBS, permeabilized with 0.05% Triton X-100 in PBS and blocked with 5% FBS, 0.2% Triton X-100 and 0.1% BSA in PBS. CldU was detected with a 1/100 dilution of anti-BrdU rat monoclonal antibody (BU1/75: ab6326, Abcam), and IdU was detected with a 1/50 dilution of anti-BrdU mouse antibody (clone B44: 347580, BD Biosciences). Clone B44 recognizes both IdU and CldU, but washing for 30 min with a high salt buffer (100 mM Tris, 0.5 M NaCl, 0.5% Tween, pH 8.0) releases it from CldU. A 1/500 dilution of Alexa 594-conjugated goat anti-rat IgG was used to detect BU1/75, and a 1/500 dilution of Alexa 488-conjugated goat anti-mouse IgG was used to detect B44. The cover slips were then mounted onto slides with Mowiol Mounting Medium for Fluorescence with 4′, 6′-diamidino-2-phenylindole (DAPI. For intra-S phase checkpoint analysis, primary MEFs were cultured with medium containing 10 nCi/ml [^{14}C] thymidine (Amersham) for 24 h, washed with PBS and cultured again for 30 min in medium without [^{14}C] thymidine. The cells were then X-irradiated (10 Gy), cultured for 1 h and then pulse labelled with medium containing 2.5 μCi/ml [^{3}H] thymidine for 30 min. The labelled cells were harvested and fixed in ice-cold 70% ethanol overnight. The fixed cells were applied to membranes on the filtration plate, and the membranes were washed with 70% ethanol and then 95% ethanol (Nalgen Nunc; no. 255984). Radioactivity on the membranes was determined with a liquid scintillation counter. Radio-resistant DNA synthesis was determined by the radioactivity of [^{3}H] divided by that of [^{14}C]. For apoptosis analysis, thymocytes were irradiated and cultured for 24 h. Cells were fixed, stained with propidium iodide and analysed by FACS. An alkaline-comet assay was performed using the OxiSelect™Comet Assay kit according to the manufacturer's instructions (CELL BIOLABS INC). DNA was stained with Vista Green DNA Dye.

Isolation of CD19-positive B cells

Splenocytes were obtained by dissection of *Chk1/Chk2* double-mutant mice and by manual disruption of the organ. The cell suspension was passed through a BD Falcon cell strainer (REF352340), and centrifuged at 300 *g* for 10 min at 4°C and suspended in 500 μl MACS running buffer. CD19-positive B cells were selected from single-cell suspensions of splenocytes by labelling the cells with magnetic bead-conjugated antibodies against CD19 (Miltenyi Biotec, 130-052-201), followed by Auto-MACS magnetic bead sorting according to the manufacturer's instructions. The CD19-positive cells (2×10^6) were stimulated by 25 μg/ml lipopolysaccharide for 72 h and treated with 0.1 ng/ml colcemid for an additional 2 h. The resultant cells were then subjected to karyotype analysis.

Retroviral-mediated gene transfer

To prepare retroviral particles, PlatE cells (2×10^6) were plated on a 10 cm culture dish, and then transfected with retroviral vectors using FuGene6 (Roche). For infection, primary MEFs at passage 2 were plated at a density of 2×10^5 cells per 10 cm dish and infected by virus from PlatE cells for 24 h. The infection process was repeated four times at 4–12 h intervals. After selection in the presence of 2 μg/ml puromycin for 4 days, growth curves, immunoblotting and senescence analysis of MEFs was carried out. To determine senescence, MEFs were stained for SA-β-gal activity as described previously (Bartkova *et al*, 2006; Di Micco *et al*, 2006). For retroviral transfection of E6 and E7 vectors, infected MEFs were selected in medium containing 100 μg/ml hygromycin for 4 days.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: HN and KM performed the majority of the experiments and data analysis. MS, KO, KO, KS, HF, AKK, BB, PMH, TM IM, TS, NM, MD and EA provided experimental data. HN, MD, NM and EA helped to write the paper. MN conceived of the project, planned and guided the research, and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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REVIEW

Regulation of yeast forkhead transcription factors and FoxM1 by cyclin-dependent and polo-like kinases

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Members of the forkhead-box (Fox) family of transcription factors are present in many eukaryotes. More than 100 such proteins that share homology in the winged-helix DNA-binding domain have been identified in higher eukaryotes. This family of transcription factors is implicated in the regulation of a variety of cellular processes, including the cell cycle, apoptosis, DNA repair, stress resistance and metabolism. A subfamily of Fox proteins are required to activate expression of the genes encoding B-type cyclins, Cdc25 and Polo-like kinase (Plk) during the mitotic cell cycle and meiosis in organisms from yeast to mammals. These proteins are activators of cyclin-dependent kinase 1 (Cdk1). Cdk1 and Plk phosphorylate Fox and its associated proteins at different sites, resulting in activation or repression of Fox transcriptional activity, depending on the target genes. In addition to their documented transcriptional functions, Fox proteins are involved in the regulation of pre-mRNA processing, at least in yeast. In this review, we will focus on the role of Fox proteins in the fission yeast *Schizosaccharomyces pombe* and budding yeast *Saccharomyces cerevisiae*, in addition to the role of FoxM1 in mammals in the cell cycle and in pre-mRNA processing, as revealed in recent studies.

Introduction

In eukaryotes, cell cycle progression is primarily controlled by cyclin-dependent kinases (Cdks). Polo-like kinases (Plks) also help to regulate the cell cycle, mainly at the M phase. In addition, the transcriptional program supports cell cycle control. In yeast, there are many genes that show cell cycle-dependent expression and this periodic expression is often controlled by a single transcription factor complex. One family of such transcription factors is the forkhead (FKH) box (Fox) proteins.

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In this review, we first describe basic cell cycle regulation during the mitotic and meiotic cell cycle by Cdks in the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae*, as well as in mammals. Next, we review the roles of Fox transcription factors in these two yeasts and FoxM1 in mammals, in cell cycle and development. Because of the great number of Fox transcription factors in mammals, it is almost impossible to review in detail the contribution of all of the Fox genes to development and function. This review therefore focuses on Fox proteins in fission and budding yeasts and FoxM1 in mammals because, relative to other mammalian Fox proteins, the sequence of mammalian FoxM1 is comparatively similar to those of yeast Fox proteins. We also discuss the relation of Fox proteins and associated proteins to Cdks and Plks, and how this is linked to regulation of cell cycle progression.

Regulation of the mitotic cell cycle by Cdk. Cell cycle progression is controlled by the fluctuating activity of Cdks/cyclin complexes,[1,2] which play a central role in the control of the mitotic and meiotic cell cycle in eukaryotes.[2,3] In both fission and budding yeasts, a single Cdk1 (Cdc2 in fission yeast and Cdc28 in budding yeast) that binds to specific cyclins at different stages of the cycle is sufficient to drive the cell cycle.[4] In budding yeast, there are six B-type cyclins: Clb5 and Clb6 trigger DNA replication: Clb3 and Clb4 initiate the formation of mitotic spindles; and Clb1 or Clb2 induce nuclear division. Although there are several cyclins (Cdc13, Cig2, Rem1, etc.) in fission yeast, the single mitotic B-type cyclin Cdc13 is sufficient to drive the cell cycle in this organism.[5] Therefore, the single Cdk1 and the single cyclin are enough to control the cell cycle, at least in fission yeast.

In most multi-cellular organisms, there are many Cdks and cyclins. Human cells harbor 13 Cdks and 25 cyclins, although only a subset of Cdk-cyclin complexes are thought to be directly required to drive the cell cycle.[6] They include three interphase Cdks (Cdk2, Cdk4 and Cdk6), a mitotic Cdk1, and four classes of cyclins (the A-, B-, D- and E-type cyclins). Mitogenic signals induce expression of the D-type cyclins that usually bind to Cdk4 and Cdk6 during G_1. Activation of these Cdk complexes leads to phosphorylation of Rb proteins that are bound to the E2F transcription factor; phosphorylated Rb proteins then release E2F. Free E2F induces expression of E-type cyclins that bind and

activate Cdk2 and Cdk2-cyclin E complexes further phosphorylate Rb proteins leading to their complete inactivation. Cdk2 is subsequently bound by cyclin A to traverse G_2 phase, and at the end of G_2 phase, Cdk1 is activated by cyclin A. After cyclin A degradation during nuclear envelope breakdown, the Cdk1-cyclin B complexes drive the cells through mitosis. In multi-cellular organisms, it is generally believed that the the interphase Cdks are essential for transition of each phase of the cell cycle. However, recent genetic evidence suggests that in these organisms Cdk1 is sufficient to drive the basic cell cycle, similar to the situation in the two above-mentioned yeasts, while the interphase Cdks are only required for proliferation of specialized cells.[7-9]

Given that they play crucial roles within the cell, the activities of Cdks are regulated by a tight network of controlled mechanisms that include activatory/inhibitory phosphorylation and that are influenced by the availability of cyclins and the presence of Cdk inhibitor. Cdks are negatively controlled by phosphorylation at the tip of their glycine-rich loop (or ATP-binding pocket), namely at the residues equivalent to Thr14 and Tyr15 in Cdk1, whereas in order for the Cdk1 catalytic cleft to open, they require phosphorylation of the activation loop, equivalent to Thr161 in Cdk1. Phosphorylation of Tyr15 is catalyzed by the Myt1 and Wee1 kinases, whereas that of Thr14 is conducted by the Wee1 kinase. Cdk-cyclin complexes are activated once the inhibitory phosphate groups are removed via the Cdc25 phosphatase family members.

In addition to Cdk, members of the Plk family exist in a variety of organisms and include budding yeast Cdc5, fission yeast Plo1 and mammalian Plks.[10-13] Members of this family of kinases are key regulators of M phase processes including mitotic entry, spindle pole functions and cytokinesis. Plk contains a Ser/Thr kinase domain in its N-terminal region and two Polo box domains (PBD) in its C-terminal region. The PBD is a binding site for phosphorylated motifs in target proteins. Plk promotes G_2/M transition through phosphorylation of Cdc25 for activation and Wee1 and Myt1 for inhibition, although the details of this regulation are different depending on the species.

Regulation of CDK and the transcriptional program in meiosis. Meiosis is the process by which haploid germ cells are formed from a diploid cell. Meiosis is required for producing genetic diversity within a species by random chromosome segregation and recombination between homologous chromosomes. Pre-meiotic DNA replication occurs followed by a high level of homologous recombination. After recombination, homologous chromosomes separate to opposite poles of the nucleus (meiosis I). Separation of sister chromatids then takes place (meiosis II).

Similar to the situation in the mitotic cycle, in both fission and budding yeasts Cdk1 is also required for pre-meiotic DNA synthesis and for the first and second meiotic divisions, although the degree of the requirement for Cdk activity differs depending on the cell cycle stage.[3,14,15] Cdk1 activity increases after meiotic "Start" and before meiosis I. Between meiosis I and meiosis II the kinase activity transiently decreases, before increasing again during meiosis II. Cdk1 is phosphorylated on Tyr15 after passing meiotic Start and until the onset of the first meiotic division. The regulation of Cdk1 between meiosis I and meiosis II is mediated via the cyclin protein level.

During meiosis in fission and budding yeasts, hundreds of genes are expressed in successive waves of transcription that correlate with major biological events of meiosis that include the pre-meiotic S phase, recombination, meiotic nuclear divisions and sporulation.[16,17] Periodic gene expression is thought to be a mechanism that supports the orderly execution of cell cycle events.[18] In fission yeast the group of middle genes (561 genes) that contain the FLEX motif is expressed during meiotic nuclear divisions and is mainly regulated by the meiosis-specific transcription factor Mei4,[19] (discussed in a later section). In budding yeast, expression of more than 150 middle genes is controlled by Ndt80. Ndt80 is a transcription factor also required for expression of middle and middle/late genes, as well as five of the six *CLB* genes, except *CLB2*.[20] All the *CLB* genes except *CLB2* are induced by ectopic expression of Ndt80 during the mitotic cell cycle. It is likely that the first meiotic division is regulated by *CLB* genes whose expression is dependent on Ndt80,[20] although it has not been done that overexpression of the *CLB* gene drives cells into the first meiotic division in *ndt80* mutant cells. The fact that expression of both *CLB* and middle genes is dependent on Ndt80 ensures that meiotic progression and ascospore formation are properly coordinated. These facts suggest that Mei4 may be a functional, but not structural, homolog of Ndt80.

Fox Transcription Factors in Budding and Fission Yeasts and Mammals

The Fox family of transcription factors is defined by a common DNA-binding domain called the FKH box or winged helix domain (Fig. 1). The FKH domain containing around 100 amino acids shows structural homology with the linker histones, suggesting that this domain may bind nucleasomal DNA.[21-23] Fox proteins are present throughout the animal kingdom as well as in yeast, but not in plants.[24] Despite the sequence similarity in the DNA-binding domain, the remaining region is divergent, allowing for differential functional regulation. In humans, 19 Fox gene subfamilies (FoxA-FoxS) are present in the genome and over 100 proteins have been found in humans (Fig. 1). This family of transcription factors participates in a wide variety of cellular processes including cell cycle regulation, development, stress resistance, apoptosis, immunity, metabolism and aging.[25-27] In addition, mutations of FoxP2 have been found in human disease, with a phenotype related to speech dysfunction.[28] Fox proteins both activate and repress expression of their target genes, usually through the recruitment of co-factors or repressors.[29-31] A forkhead-associated (FHA) domain is often detected in Fox proteins as a phosphopeptide-binding motif that promotes phosphoprotein interaction.

Budding yeast fox proteins. In budding yeast, there are four Fox proteins: Fkh2 (Forkhead homolog 2), Fkh1 (Forkhead homolog 1), Fhl1 (Forkhead-like 1) and Hcm1 (high copy suppressor of calmodulin) (Fig. 2). Fkh2 is required for the expression of a wide variety of genes comprising the *CLB2* cluster.[32-34] The *CLB2* cluster consisting of about 35 genes is co-regulated during the G_2 and M phases.[35] The upstream region of the *CLB2* cluster has a binding site for Fkh2, Ndd1 (nuclear division defective 1) and

Mcm1 (minichromosome maintenance 1)[36,37] (Fig. 3). Mcm1 is a member of the MADS box proteins and is required for transcription, minichromosome maintenance, cell cycle control, mating and stress tolerance.[38] In addition, Mcm1 is required for Fkh2 binding to the *CLB2* promoter, serving as an anchor for the factors that specify the regulatory features of expression of the *CLB2* cluster.[39,40] Ndd1 is a coactivator of Fkh2 and overexpression of *NDD1* increases expression of the *CLB2* cluster genes.[37]

Fkh1 controls cell cycle-regulated gene expression in collaboration with Fkh2.[34,41-43] However, genetic and biochemical data suggest that Fkh1 and Fkh2 have opposing functions. Cells lacking either Fkh1 or Fkh2 show opposing phenotypes in cell cycle progression and of general transcription, including expression of *CLB2* mRNA. However, cells lacking either Fkh1 or Fkh2 fail to show periodic expression of G_2/M genes.[31,33,36,42,44] A monomer of either Fkh1 or Fkh2 can associate with the *CLB2* promoter in vitro, but only Fkh2 binds it in vivo.[31,33,34,36,42,45] This is probably due to the fact that Fkh2, but not Fkh1, binds the promoter region cooperatively with Mcm1.[42] The N-terminal region of the Fkh2 DNA-binding domain, which is absent in Fkh1, mediates direct interaction with Mcm1.[39,43]

FHL1 was originally discovered as a multi-copy suppressor of a *pol3* mutant and was then shown to control cell proliferation through regulation of expression of the genes encoding ribosomal proteins.[46] A coactivator of Fhl1 is Ifh1 (Interacts with forkhead), which was discovered as a multi-copy suppressor of slow growth of *fhl1*-deleted cells.[47] Ifh1 binds to Fhl1 through the FHA domain of Fhl1 and its interaction is required for expression of ribosomal protein genes.[48-50] Following stress, Ifh1 dissociates from Fhl1 and Fhl1 recruits the inhibitory factor Crf1 (Co-repressor with *FHL1*), leading to decreased transcription.[51]

HCM1 was first identified as a multi-copy suppressor of the temperature-sensitive calmodulin mutant that is defective in chromosome segregation.[52] Hcm1 is structurally different from the other Fox proteins in budding yeast in that it lacks an FHA domain (Fig. 2). *HCM1* is periodically transcribed at late G_1 and early S phase. The S-phase-specific expression of genes required for budding and chromosome segregation depends on Hcm1.[53] In addition, Hcm1 is involved in the timely expression of Fkh1, Fkh2 and Ndd1. Consistent with the role of Hcm1 in the S-phase-specific expression of chromosome-segregation genes, *hcm1* mutant cells show frequent chromosome loss and a strong genetic interaction with genes that are required for the spindle assembly checkpoint. The binding site of the Fox proteins is highly conserved; therefore, it is possible that there could be an overlap in binding specificities among the Fox proteins. However, the overlap is minimal between Fkh1/Fkh2 and Hcm1.[53]

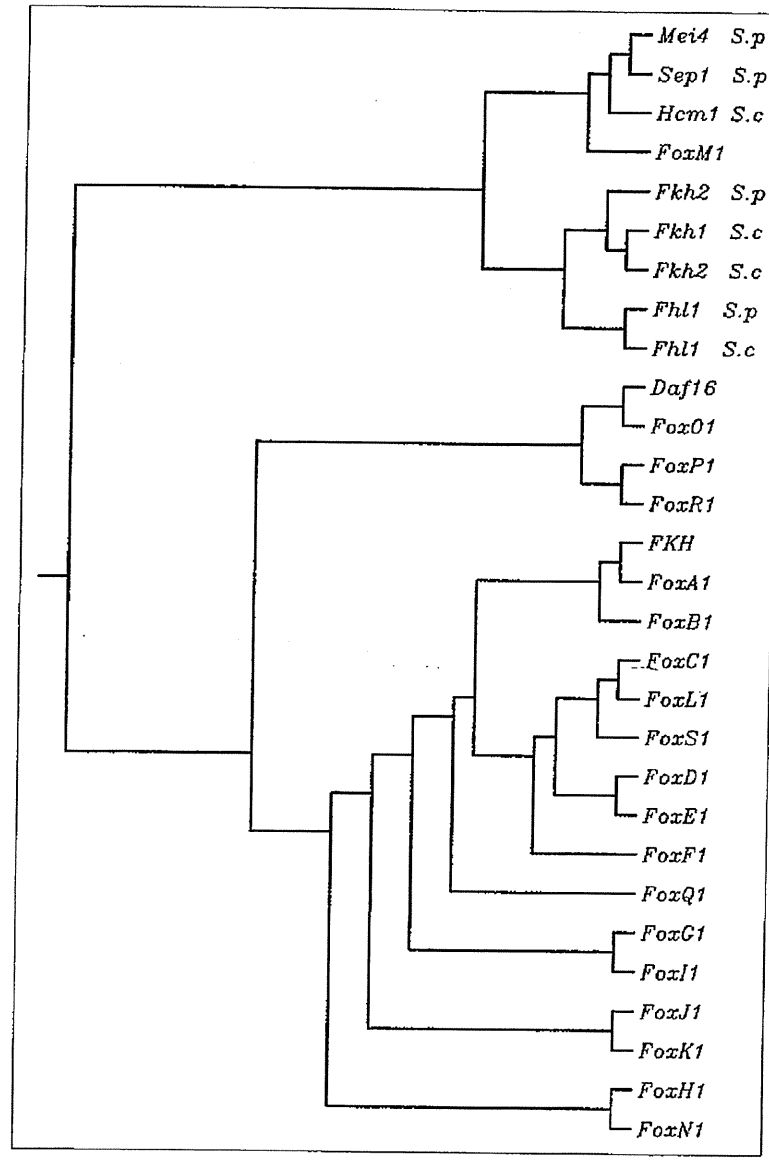

Figure 1. Evolutionary tree showing the yeast Fox proteins, *Caenorhabditis elegans* Daf16, *Drosophila melanogaster* FKH and human Fox proteins. Sequences were aligned using CLUSTALW (www.genome.jp) and an unrooted tree was generated. *S.p., Schizosaccharomyces pombe; S.c., Saccharomyces cerevisiae.*

Fission yeast fox proteins. In fission yeast, there are four Fox proteins: Mei4, Fkh2, Fhl1 and Sep1 (Fig. 2). Mei4 is a transcription factor that is required for the first meiotic nuclear division, recombination and sporulation.[54,55] Mei4 is meiosis-specific, its mRNA and proteins being found only in meiosis.[55-57] As mentioned above, expression of about half of the middle genes required for recombination and nuclear division is regulated by Mei4 during meiosis.[17,19] In *mei4* null cells, the first meiotic division does not occur at all. The versatile roles of Mei4, in combination with the large array of Mei4 putative target genes, make it unlikely that there is one critical target of Mei4 responsible for the first meiotic division. However, activation of Cdc2 by

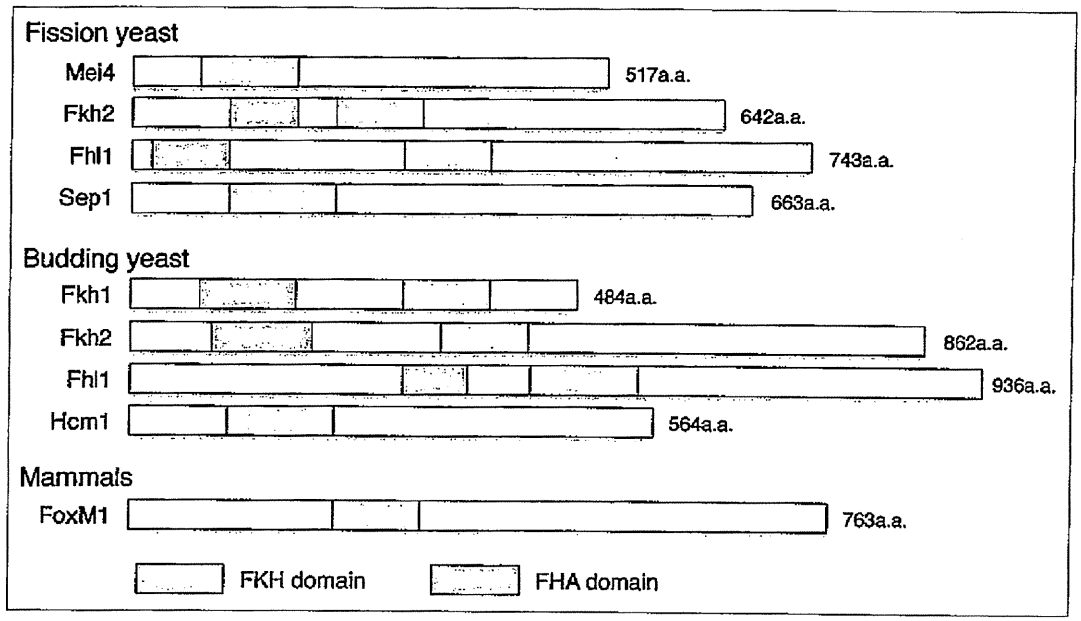

Figure 2. Structural features of Fox proteins in fission and budding yeasts, and in mammals. All proteins contain a highly conserved Forkhead (FKH) domain as a DNA-binding domain. The forkhead-associated (FHA) domain is often found in the Fox family of proteins and serves as a phosphopeptide binding site, enhancing the interaction between phosphoproteins.

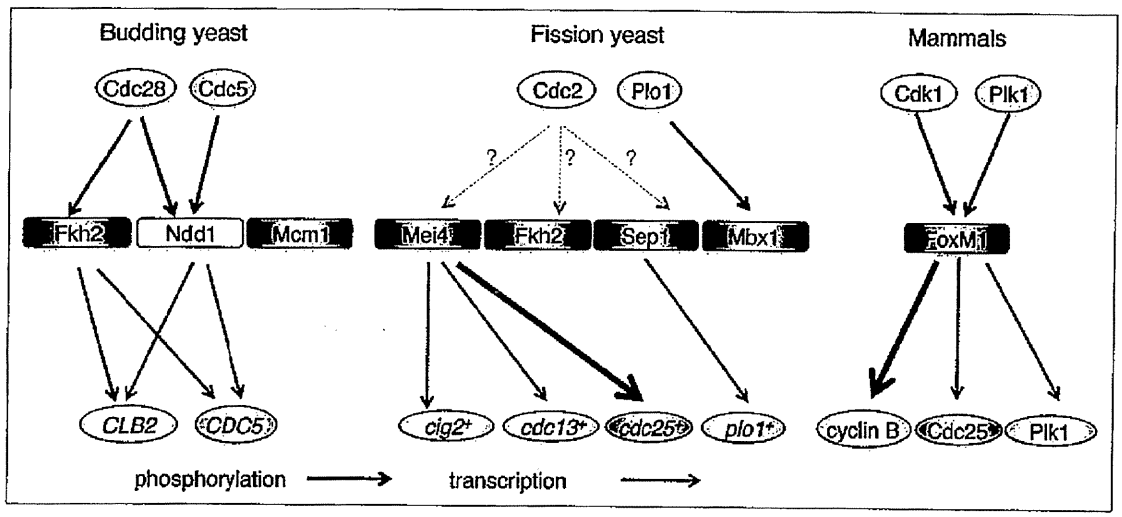

Figure 3. Regulation among Cdk1, Plk1, Fox and Cdk1 activators. In budding yeast, Cdc28 phosphorylates Fkh2 and Ndd1, while Cdc5 phosphorylates Ndd1. Budding yeast Fkh2 and Ndd1, together with Mcm1, induce *CLB2* and *CDC5* expression. In fission yeast, Plo1 phosphorylates Mbx1 and Mei4 is required for transcription of *cig2*$^+$, *cdc13*$^+$ and *cdc25*$^+$. Fission yeast Fkh2 inhibits expression of *plo1*$^+$; whereas Sep1 and Mbx1 appear to induce it. In mammals, Cdk1 and Plk1 phosphorylate FoxM1, while FoxM1 induces cyclin B, *cdc25*$^+$ and *plk1*$^+$.

forced dephosphorylation at Tyr15 induces virtually all *mei4*-deleted cells into meiotic nuclear divisions, suggesting that Tyr15 phosphorylation of Cdc2 is largely responsible for the arrest of *mei4* mutant cells.[56] Supporting this notion, Tyr15 phosphorylation of Cdc2 is increased in *mei4*-deleted cells.[56,58] In addition, ectopic expression of *cdc25*$^+$ in *mei4*-deleted cells drives the cells to undergo meiotic divisions, suggesting that *cdc25*$^+$ is a critical target of Mei4 in meiotic division.[56] Indeed, both mRNA and protein levels of Cdc25 increase around the first meiotic division, and these levels depend on those of Mei4.[56] Mei4 binds to FLEX elements adjacent to *cdc25*$^+$ both in vivo and in vitro.[56] Expression of mRNA of two B-type cyclins, Cdc13 and Cig2, is also regulated by Mei4, but Cdc13 protein levels are not[56,58,59] (Fig. 3). It has not been done where ectopic expression of *cdc13*$^+$ or *cig2*$^+$ induces meiotic division in *mei4*-deleted cells. Therefore, Mei4 regulates three cell cycle regulators, but in fission yeast the critical target of Mei4 at the first division is probably *cdc25*$^+$ rather than B-type cyclins (Fig. 3). Consistent with this notion, Mei4

is a rate-limiting factor for entry into meiosis I, since overexpression of *mei4*$^+$ induces an earlier start of meiosis I through earlier induction of *cdc25*$^+$.[56] The regulation of Tyr15 phosphorylation of Cdc2 is also a rate-limiting step for entry into meiosis I, since forced dephosphorylation drives cells into meiosis earlier, as observed in *mei4*$^+$-overexpressing cells.[60] Since Mei4 has critical targets for recombination, meiosis I and sporulation, it is likely that Mei4 helps coordinate meiotic events, although the molecular mechanisms of the coordination remain to be elucidated. Although there is a positive-feedback loop in budding yeast of Fkh2-Clb2-Cdc28, it is not known whether Mei4 is regulated by Cdc2.

Expression of fission yeast Fkh2 is regulated in a cell cycle-dependent manner, with mRNA levels peaking at M/G_1 phase.[61] Fkh2 protein is also periodically expressed and phosphorylated during the G_2/M phase.[61] From analysis of *fkh2*-deleted cells, Fkh2 is required for diverse processes including regulation of cell morphology, proper growth, efficient G_2/M transition, normal septation, mating and periodic gene expression in M/G_1 phase.[61-64] Overexpression of Fkh2 inhibits periodic expression of M/G_1 transcription leading to cell death, suggesting that Fkh2 negatively regulates M/G_1 expression.[63]

sep1$^+$ has been identified as one of the genes required for cell separation.[65,66] Like fission yeast Fkh2, Sep1 is required for periodic M/G_1 expression and overexpression of Sep1 is lethal.[61,64,67,68] In contrast to fission yeast Fkh2, *sep1*$^+$ mRNA is not periodically expressed during the cell cycle.[61]

In contrast to the other Fox proteins in fission yeast, the role of Fhl1 is not clear. Fhl1 is not essential, but is involved in proper growth and efficient G_2/M transition.[62] Fhl1 is not required for periodic transcription of M/G_1 genes.[64] The phenotypes of *fhl1*$^+$deleted cells are marginal, suggesting that Fhl1 is not essential for these functions. However, it is possible that Fhl1 performs essential functions in collaboration with other Fox proteins in fission yeast.

FoxM1. FoxM1, found in mammals, is a member of the Fox family of transcription factors and is structurally similar to fission yeast Mei4 and Sep1 and budding yeast Hcm1,[69,70] (Figs. 1 and 2). FoxM1 is closely involved in regulation of the cell cycle, aging and cancer and is now a novel therapeutic target in cancer.[69-73] Expression of FoxM1 is detected only in cells that are proliferating, being severely reduced in quiescent or terminally-differentiated cells.[74] FoxM1 accumulates mainly in the cytoplasm at G_1/S, while it is localized to the nucleus during G_2/M.[30] Study of FoxM1 knock-out mouse models reveals that FoxM1 plays a minor role in the early stage of embryogenesis, but is required later during organogenesis, and also prevents endoreplication.[75] In addition, cells deficient in FoxM1 show a slight delay in G_1/S and a severe delay in G_2, as well as chromosome mis-segregation and failure of cytokinesis.[76-79] The target of FoxM1 in the G_1/S transition is likely to be the c-Jun N-terminal kinase (JNK1) gene that controls expression of genes for the G_1/S transition, since expression of JNK1 rescues the G_1/S but not the G_2/M cell cycle delay in FoxM1-deficient cells.[77]

Like yeast Fox proteins, FoxM1 controls G_2/M progression by inducing expression of the Cdk1 activators cyclin B and Cdc25,[76] (Fig. 3). The defect of G_2/M transition in FoxM1-deficient cells is partially rescued by overexpression of cyclin B.[76] These facts suggest that poor induction of cyclin B is responsible for the defect of G_2/M transition in FoxM1-deficient cells and that cyclin B is a critical target of FoxM1 at the G_2/M transition. In fission yeast, regulation of Tyr15 phosphorylation of Cdk1 is a rate-limiting step for G_2/M transition both in mitosis and meiosis, whereas fission yeast cyclin B is an essential but not rate-limiting factor for entry into mitosis.[2] In contrast to fission yeast, in budding yeast regulation of Tyr15, phosphorylation of Cdk1 is less important for cell cycle progression.[80] In mammals, both cyclin B and Cdc25 are important for mitotic control.[2] Therefore, as regulators of nuclear division, FoxM1-related proteins mainly regulate *cdc25*$^+$ in fission yeast, cyclin B in budding yeast and both in mammals. In general, regulation of the Cdk activators cyclin B and Cdc25 by FoxM1-related proteins is conserved from yeast to human.

In addition, FoxM1 regulates expression of CENP-F, whose inactivation results in chromosome mis-segregation.[76] Like FoxM1, CENP-F is required for proper chromosome segregation and probably for the spindle assembly checkpoint.[53] However, it is not clear whether CENP-F is responsible for proper chromosome segregation in FoxM1-deficient cells, since it has not been done that ectopic expression of CENP-F in these cells rescues the chromosome mis-segregation. As mentioned above, FoxM1 is structurally related to fission yeast Mei4 and Sep1 and budding yeast Hcm1. Therefore, this Fox group of proteins primarily regulates expression of genes required for entry into mitosis and chromosome segregation.

Cdk1, Plk and Fox Proteins are Required for Cell Cycle Regulation

As mentioned above, some of the Fox proteins control transcription of the Cdk1 activators. In addition, other links between Cdk1, Plk and Fox proteins have been found in yeast and humans (Figs. 3 and 4). In budding yeast, the Cdc28 kinase complexed with S phase cyclin Clb5 phosphorylates Fkh2 during S phase, whereas the Cdc28 kinase complexed with G_2/M cyclin Clb2 phosphorylates Ndd1 during G_2 phase.[32,81] As described earlier, Fkh2 and Ndd1 appear to function as activators of G_2/M transcription. However, these proteins have distinct functions. Phosphorylation of the C-terminal domain of Fkh2 by Cdc28 kinase seems to relieve transcriptional repression, since while cells lacking Ndd1 are non-viable, when the C-terminal domain of Fkh2 or whole Fhk2 is deleted, cell viability is restored.[33,34,41,42] Supporting this notion, cells lacking both Fkh2 and Ndd1 show increased expression of *CLB2* cluster genes in G_1 and decreased expression in G_2, in addition to loss of periodic expression.[34] These observations suggest that Fkh2 functions as a repressor at G_1 and that Fkh2 is converted to an active form, in association with Ndd1, by Cdk1 phosphorylation during S/G_2 phase (Fig. 4). These results also support a model of Fkh2 as a platform to regulate the recruitment of factors, including Ndd1, that activate transcription. Therefore, cells lacking both Ndd1 and the C-terminal domain of Fkh2 might activate transcription by

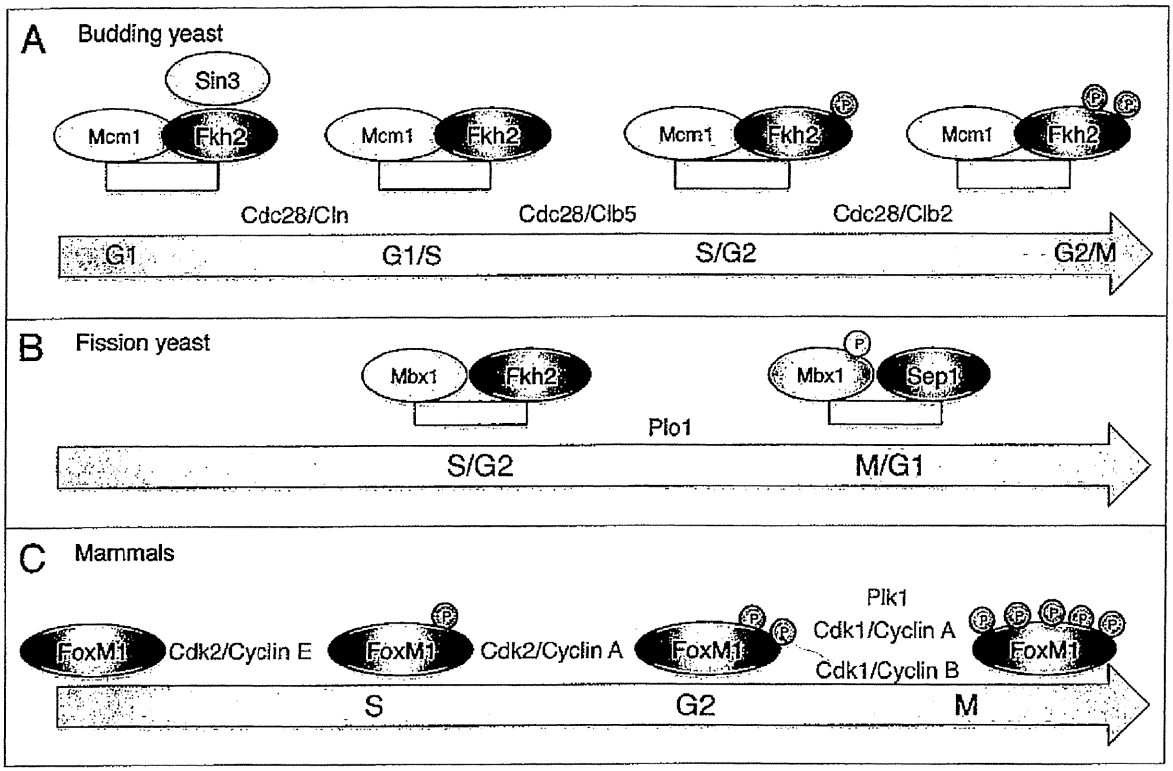

Figure 4. Cell cycle-dependent control of Fox proteins by Cdk and Plk. (A) In budding yeast, Cdc28-G_1 cyclin (Cln) complexes are required to remove the Sin3 histone deacetylase complex from the *CLB2* promoter during G_1. Fkh2 is phosphorylated by Cdc28-S cyclin (Clb5) during S phase, and by Cdc28-G_2/M cyclin (Clb2) during G_2/M phase. Transcription from the *CLB2* promoter increases around G_2/M. (B) In fission yeast, Plo1 phosphorylates Mbx1 during M phase. Fkh2 represses M/G_1 transcription during S/G_2 phase, whereas Sep1 activates it during M/G_1 phase. (C) FoxM1 is phosphorylated by Cdk2/cyclin E and subsequently by Cdk2/cyclin A, during S phase. During G_2 phase, Cdk1 associated with cyclin B or cyclin A phosphorylates Fox M1, and these phosphorylations appear to be a priming event for phosphorylation by Plk1 during G_2/M phase. FoxM1 transcriptional activity increases from S to G_2/M.

recruiting another activator to the truncated Fkh2. The association of Fkh2 with Ndd1 is mediated through the FHA domain of Fkh2 and is enhanced by Cdc28 phosphorylation of these proteins.[34,41,43] In addition, Ndd1 is phosphorylated by Cdc5 and this phosphorylation is required to regulate normal temporal expression of the *CLB2* cluster, including *CDC5* itself, creating a positive-feedback loop.[82] Similar regulation has also been found in fission yeast, in which Plo1 phosphorylates Mbx1 and regulates cell cycle transcription.[83] Thus, it is likely that control by Plk phosphorylation of Fox-associated proteins may be conserved among eukaryotes. Additionally, the Cdc28 kinase, Fkh2, Ndd1 and Clb2 constitute a positive-feedback loop and this loop maintains G_2/M gene expression until the cells exit mitosis (Fig. 3).

It is not known whether Ndd1-like proteins exist in fission yeast and mammals. As mentioned above, the FHA domain of budding yeast Fkh2 is required for Ndd1 association. The FHA domain is not present in FoxM1, Mei4, Sep1 or Hcm1, which are structurally similar to each other (Figs. 1 and 2). A close structural homolog of Ndd1 do not exist in these organisms. However, the possibility cannot be excluded that a functional homolog does exist in these organisms.

Repression of the *CLB2* cluster by Fkh2 is mediated by the Sin3 histone deacetylase complex[84] (Fig. 4). It has also been reported that the Isw2 chromatin-remodeling ATPase is involved in this process.[85] In support of a notion of opposing effects of both Sin3 and Isw2 on Ndd1 function, *ndd1* lethality may be bypassed in cells deleted for *SIN3* or *ISW2*. During G_1, Sin3 binds to the *CLB2* promoter. Due to Cdc28 being complexed with G_1 cyclins, Sin3 is removed at the onset of the S phase. Then, Ndd1 associates with the *CLB2* promoter, this being dependent on Cdc28 complexing with S cyclin. These results suggest that Cdc28 associated with different cyclins regulates expression of genes of the *CLB2* cluster by removing or recruiting Fkh2-associated factors.

Like the budding yeast Fkh2-Ndd1-Mcm1 system, fission yeast Fkh2, Sep1 and the MADS box protein Mbx1 control periodic gene expression, including that of *fkh2*$^+$ itself and *plo1*$^+$ in M/G_1 phase (Fig. 4).[61,64,68] The lethality induced by overexpression of *fkh2*$^+$ is rescued by *sep1*$^+$ deletion, but the lethality caused by overexpression of *sep1*$^+$ is not rescued by *fkh2*$^+$ deletion, suggesting that Sep1 acts downstream of Fkh2 and that Sep1 is required for Fkh2 function.[64] Mbx1 is similar to budding yeast Mcm1 and is required for proper cytokinesis.[64] Although *mbx1*$^+$-deleted cells still show M/G_1 periodic expression, it appears that Mbx1 regulates M/G_1 expression through Plo1.[83] Plo1 is required for M/G_1 transcription and directly phosphorylates Mbx1.[83] Fkh2 and Plo1 associate with the promoter region of the periodically-expressed