Nuclear Chk1 prevents premature mitotic entry

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Summary

Chk1 inhibits the premature activation of the cyclin-B1–Cdk1. However, it remains controversial whether Chk1 inhibits Cdk1 in the centrosome or in the nucleus before the G2–M transition. In this study, we examined the specificity of the mouse monoclonal anti-Chk1 antibody DCS-310, with which the centrosome was stained. Conditional Chk1 knockout in mouse embryonic fibroblasts reduced nuclear but not centrosomal staining with DCS-310. In Chk1$^{+/myc}$ human colon adenocarcinoma (DLD-1) cells, Chk1 was detected in the nucleus but not in the centrosome using an anti-Myc antibody. Through the combination of protein array and RNAi technologies, we identified Ccdc-151 as a protein that crossreacted with DCS-310 on the centrosome. Mitotic entry was delayed by expression of the Chk1 mutant that localized in the nucleus, although forced immobilization of Chk1 to the centrosome had little impact on the timing of mitotic entry. These results suggest that nuclear but not centrosomal Chk1 contributes to correct timing of mitotic entry.

Key words: Cdk1, Centrosome, Chk1, Nucleus, G2–M transition

Introduction

The cell division cycle is a tightly regulated set of events to distribute complete and accurate replicas of the genome to daughter cells. Checkpoints monitor DNA replication and repair, and thereby couple the completion of these events to the onset of mitosis. In the center of these pathways, there exists a protein kinase cascade from ataxia-telangiectasia and Rad3-related (ATR) to Chk1, the activation of which requires ssDNA and several nuclear proteins, such as replication protein A (RPA), ATR interacting protein (ATRIP), Rad9-Rad1-Hus1 (9-1-1) checkpoint complex, topoisomerase (DNA) II binding protein 1 (TOPBP1), and claspin (Zhou and Elledge, 2000; Chini and Chen, 2004; Kastan and Bartek, 2004; Cimprich and Cortez, 2008; Reinhardt and Yaffe, 2009). ATR phosphorylates Chk1 at Ser317 and Ser345, which then induces autophosphorylation of Chk1 at Ser296. These phosphorylation events are required for the transmission of checkpoint signals to downstream effectors (Zhao and Piwnica-Worms, 2001; Walker et al., 2009; Kasahara et al., 2010). With regard to the cell cycle arrest, Chk1 phosphorylates and inactivates members of the Cdc25 family of dual specificity phosphatases (Neely and Piwnica-Worms, 2003; Boutros et al., 2007). Since these enzymes control cyclin-B1–Cdk1 activation through the dephosphorylation of Thr14 and Tyr15 in the ATP-binding loop of Cdk1 (Nigg, 2001; Doree and Hunt, 2002; Nurse, 2002), activation of Chk1 eventually blocks premature mitotic entry (Lindqvist et al., 2009).

Recent studies have also demonstrated that Chk1 prevents Cdk1 from unscheduled activation even in an unperturbed cell cycle (Kramer et al., 2004; Enomoto et al., 2009). Two models were proposed to explain how Chk1 shields Cdk1 from premature activation. In one model, centrosome-associated Chk1 prevents premature activation of Cdk1 (Kramer et al., 2004; Tibelius et al., 2009). By using the anti-Chk1 monoclonal antibody DCS-310, Kramer and colleagues reported that Chk1 localized to centrosomes in interphase cells but not in prophase cells (Kramer et al., 2004). The 8-day stable induction of GFP-Chk1–PACT (pericentrin-AKAP450 centrosomal targeting domain) inhibited the G2–M transition. Based on these data, they proposed that Chk1 dissociates from the centrosome at the G2–M transition and then cyclin-B1–Cdk1 is activated on the centrosome (Kramer et al., 2004; Tibelius et al., 2009). This centrosomal model is well fit for the observation that active cyclin-B1–Cdk1 was first detected on the centrosome (Jackman et al., 2003). In the other model, nuclear Chk1 prevents premature activation of Cdk1 (Enomoto et al., 2009). By using three independent anti-Chk1 monoclonal antibodies including DCS-310, we have previously demonstrated that Chk1 translocates from the nucleus to the cytoplasm at the G2–M transition (Enomoto et al., 2009). This translocation is regulated by Cdk1-induced Chk1 phosphorylation at Ser286 and Ser301 (Shiromizu et al., 2006), and disturbance of this process results in a delay in mitotic entry (Enomoto et al., 2009). Thus, we have proposed a model in which Cdk1-induced Chk1 phosphorylation leads to the elimination of active Chk1 kinase from the nucleus, which triggers a positive feedback loop of Cdk1 activation in the nucleus. This nuclear model is fits well the observation that Chk1 activation occurs predominantly in the nucleus (Sanchez et al., 1997; Jiang et al., 2003).

The spatiotemporal regulation of several protein kinase activities is considered to have crucial roles in the coordination of

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centrosomal, cytoplasmic, and nuclear events during cell cycle progression and checkpoints (Nigg, 2001; Reinhardt and Yaffe, 2009). Since the centrosome is considered to be a hub in which the cyclin-B1–Cdk1 complex is first activated (Jackman et al., 2003), the centrosomal Chk1 model is attractive. However, this centrosomal model is based largely on the use of one anti-Chk1 antibody, DCS-310 (Kramer et al., 2004; Tibelius et al., 2009). In this present study, we show that the centrosomal reactivity of this antibody reflects the crossreactivity with another protein, Ccdc151, and that Chk1 does not localize to the centrosome. In addition, forced localization of Chk1 to the centrosome has little effect on the entry into mitosis, whereas forced nuclear transport caused a delay in mitotic entry.

Results and Discussion

Chk1 is localized predominantly in the nucleus but not on the centrosome

An anti-Chk1 monoclonal antibody DCS-310 is reported to react with the centrosome in cells fixed with methanol:acetone (1:1) (Kramer et al., 2004; Tibelius et al., 2009). We confirmed that DCS-310 reacts with the centrosome in cells fixed with methanol:acetone (1:1) (Fig. 1B and Fig. 3D) or 1% formaldehyde (Fig. 2E) but not with 3.7% formaldehyde (not depicted). However, especially in methanol–acetone-fixed cells (Fig. 1B and Fig. 3D), nuclear and cytoplasmic DCS-310 signals are much weaker than in cells fixed with 3.7% formaldehyde (Enomoto et al., 2009). In the experiments described here, we used cells fixed in methanol:acetone (1:1) or 1% formaldehyde to evaluate centrosomal DCS-310-staining.

We used Chk1$^{flox/-}$ mouse embryonic fibroblasts (MEFs) in which one *lox*-flanked (flox) *Chk1* allele can be converted into a null allele by Cre–*lox* site-specific homologous recombination (Niida et al., 2005) to examine the effect of conditional Chk1 knockout on the DCS-310-immunoreactivity. For this purpose, we infected Chk1$^{flox/-}$ MEFs with an adenovirus carrying either β-galactosidase (Ad-LacZ; the infection control) or Cre recombinase (Ad-Cre) as described previously (Shimada et al., 2008) and then analyzed them 3 days after the infection. As shown in Fig. 1A, we detected a 54-kDa band that corresponds to mouse Chk1 in the MEFs infected with Ad-LacZ but not with Ad-Cre, demonstrating the success of conditional Chk1 knockout. As shown in Fig. 1B, nuclear and cytoplasmic DCS-310 signals were decreased in Chk1$^{\Delta/-}$ MEFs (infected with Ad-Cre), compared with Chk1$^{flox/-}$ MEFs (infected with Ad-LacZ). However, we observed only marginal differences in the centrosomal staining between these two cells (Fig. 1B); a similar tendency was observed using MEFs fixed with 1% formaldehyde (not shown). These results suggest that, at least on the centrosome, DCS-310 crossreacts with protein(s) other than Chk1.

We next replaced one *CHK1* allele with Myc-tagged Chk1 in stable human colon adenocarcinoma (DLD-1) cells (Fig. 2). As shown in Fig. 2A, we performed homology-directed gene replacement with recombinant adeno-associated virus (rAAV) vector (Kohli et al., 2004; Rago et al., 2007). One *CHK1* allele was targeted by rAAV infection, drug selection and screening by PCR. We confirmed the isolation of heterozygotes, Chk1$^{+/myc}$ cell clones by Southern blot hybridization (Fig. 2B) and by western blotting (Fig. 2C). We treated Chk1$^{+/myc}$ cells with hydroxyurea (HU) in order to activate the DNA replication checkpoint (Fig. 2D). Like wild-type Chk1, Myc-Chk1 (lower and upper bands, respectively) was phosphorylated at Ser345 in HU-treated cells (Fig. 2D), confirming that Myc-Chk1 expressed in DLD-1 cells was functional. In DLD-1$^{+/myc}$ cells fixed with 1% formaldehyde, the nucleus was strongly stained not only by DCS-310 but also anti-

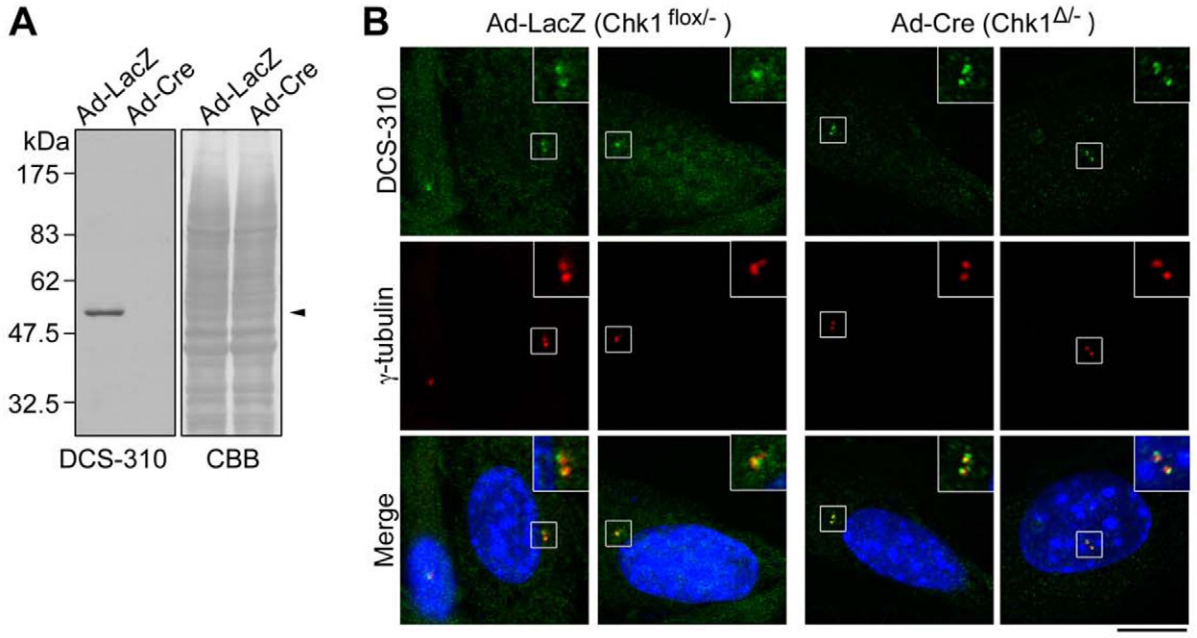

Fig. 1. The centrosome stained positive for Chk1 with the monoclonal antibody DCS-310, even after conditional Chk1 knockout. (**A**,**B**) Chk1$^{flox/-}$ MEFs (Niida et al., 2005) were infected with adenovirus carrying β-galactosidase (Ad-LacZ; infection control) or Cre recombinase (Ad-Cre), as described previously (Shimada et al., 2008). At 3 days after infection, cells were subjected to western blotting (A) or immunocytochemistry (B) with DCS-310. After blotting, transferred membrane was stained with Coomassie Brilliant Blue (CBB; A). Cells were co-stained with anti-γ-tubulin (to detect centrosomes) and DAPI (to detect nuclei; B). Boxed areas (top right) in B show the indicated centrosomes at high magnification. Scale bar: 5 μm.

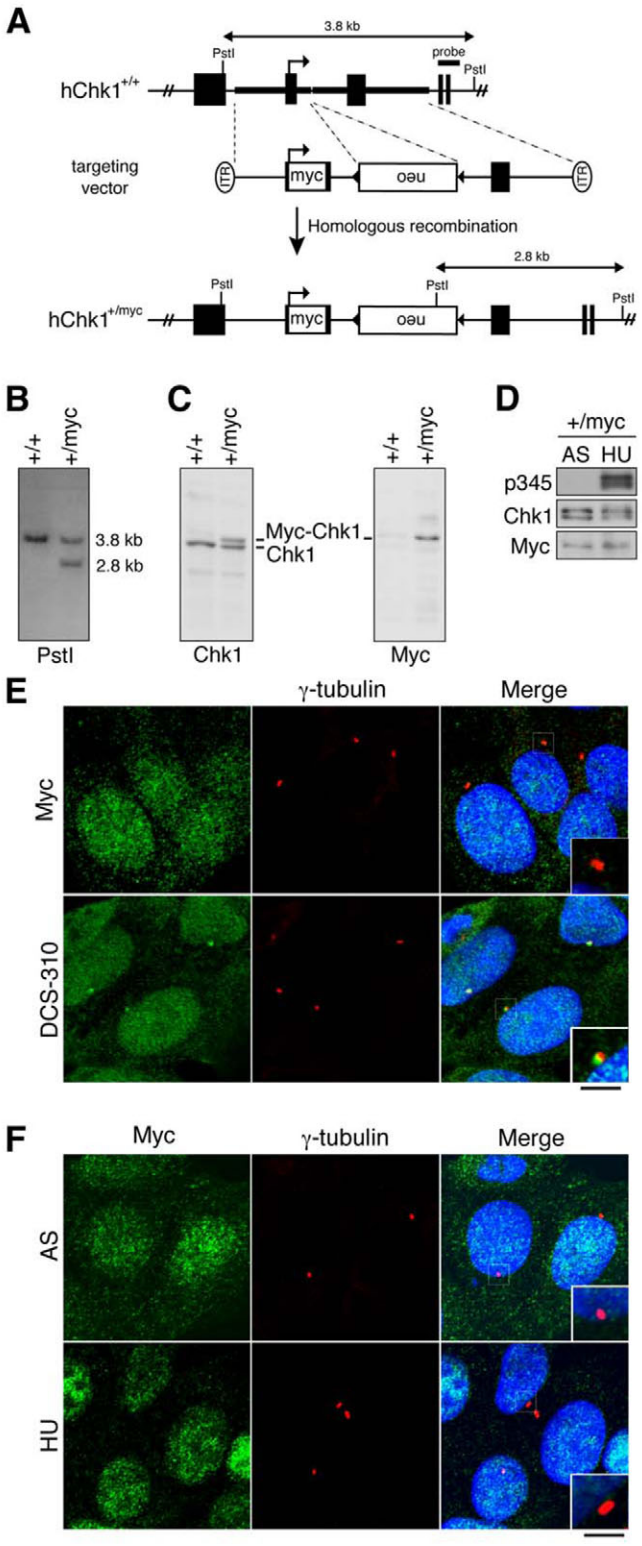

Fig. 2. Chk1 localized mainly in nucleus but not on centrosome. (**A**) Strategy of Chk1$^{+/myc}$ DLD-1 cell generation. The rAAV targeting vector contains sequences from *CHK1* locus flanking a neomycin resistance (*neo*) marker bounded by *loxP* sites. DNA sequence corresponding to Myc-epitope (amino acid sequence; EQKLISEEDL) was inserted between first ATG and the second codon on exon 2. The diagram indicates the positions of Chk1-derived sequences in the targeting vector and of the labeled hybridization probe relative to *Pst*I sites. The predicted restriction map of targeted *CHK1* allele is also shown (hChk1$^{+/myc}$). (**B**,**C**) Established heterozygotes (+/myc) were subjected to Southern (B) and western (C) blot analyses. The Southern blot hybridization to *Pst*I-digested genomic DNA shows single band of ~3.8 kb in wild-type (+/+) and two bands of ~3.8 kb and ~2.8 kb with similar intensity in heterozygotes (+/myc). Positions of Myc-tagged Chk1 (Myc-Chk1) and wild-type Chk1 (Chk1) are indicated (C). (**D**) Chk1$^{+/myc}$ DLD-1 cells were treated with 3 mM hydroxyurea (HU) or left untreated (AS) for 16 hours. Then, cells were subjected to western blotting with indicated antibodies. (**E**,**F**) Chk1$^{+/myc}$ DLD-1 cells were subjected to immunocytochemistry with anti-Myc or DCS-310, together with anti-γ-tubulin and DAPI. HU treatment was performed as described above. Scale bars: 5 μm.

Myc antibody (Fig. 2E). However, unlike DCS-310, the centrosome was hardly stained by anti-Myc antibody (Fig. 2E). Since Chk1 had been reported to accumulate in the nucleus (Sanchez et al., 1997; Jiang et al., 2003) or on the centrosome (Loffler et al., 2007; Niida et al., 2007) in response to the checkpoint activation, we also stained HU-treated Chk1$^{+/myc}$ cells with anti-Myc antibody. Regardless of the HU treatment, we hardly detected anti-Myc signals on the centrosome (Fig. 2F). However, HU treatment enhanced nuclear Myc signals (Fig. 2F). Since these signals were detected only at low or background levels in parental (Chk1$^{+/+}$) DLD-1 cells (supplementary material Fig. S1), the majority of observed Myc signals in Chk1$^{+/myc}$ cells reflected the staining of Myc-tagged Chk1 but not of endogenous Myc. Thus, these results suggest that Chk1 is localized predominantly in the nucleus but not on the centrosome.

We then searched for DCS-310-reactive centrosomal protein(s) using a protein array system (Goshima et al., 2008). Among 19,900 proteins, we identified 18 proteins (including Chk1) as being DCS-310-reactive (supplementary material Fig. S2 and Table S1). In order to examine which protein(s) localize to the centrosome, we introduced each Flag-tagged protein into HeLa cells. As shown in Fig. 3A,B (also see supplementary material Fig. S3), two independent proteins, bicaudal D homolog 2 (Bicd2) (Splinter et al., 2010) and the non-characterized protein coiled-coil domain containing 151 (Ccdc151), were validated as candidate centrosomal proteins, although Flag-tagged Chk1 was localized predominantly in the nucleus but not on the centrosome. RNA interference (RNAi) experiments showed that depletion of Ccdc151 specifically reduced DCS-310 immunoreactitity on the centrosome (Fig. 3C,D). All these results suggest that centrosomal DCS-310 immunoreactivity reflects the existence of Ccdc151 but not Chk1 on the centrosome.

Nuclear but not centrosomal Chk1 prevents Cdk1 from premature activation

For functional analyses of Chk1, we established Tet-On HeLa cells in which each type of Myc-tagged Chk1 (Myc-Chk1) is expressed in a doxycycline (Dox)-dependent manner. After the addition of 0.3 μg/ml Dox, the expression level of Myc-Chk1 fused to PACT (Myc-Chk1–PACT) was comparable to that of Myc-Chk1 wild-type (WT), Chk1 kinase-inactive mutant (K38M) or of a mutant in which two serine phosphorylation sites of Cdk1 were changed to alanine (S286/301A) (Fig. 4A). Under this condition, Myc-Chk1–PACT localized predominantly on the centrosome, whereas other types of Myc-Chk1 mutant were localized predominantly in the nucleus but not on the centrosome (Fig. 4B). We next evaluated the effect of each protein expression on the G2–M transition and cyclin-B1–Cdk1 activation in the following method (summarized in Fig. 4C). As shown in Fig. 4D, each Tet-On cell line was synchronized at the G1–S boundary by the double-thymidine block

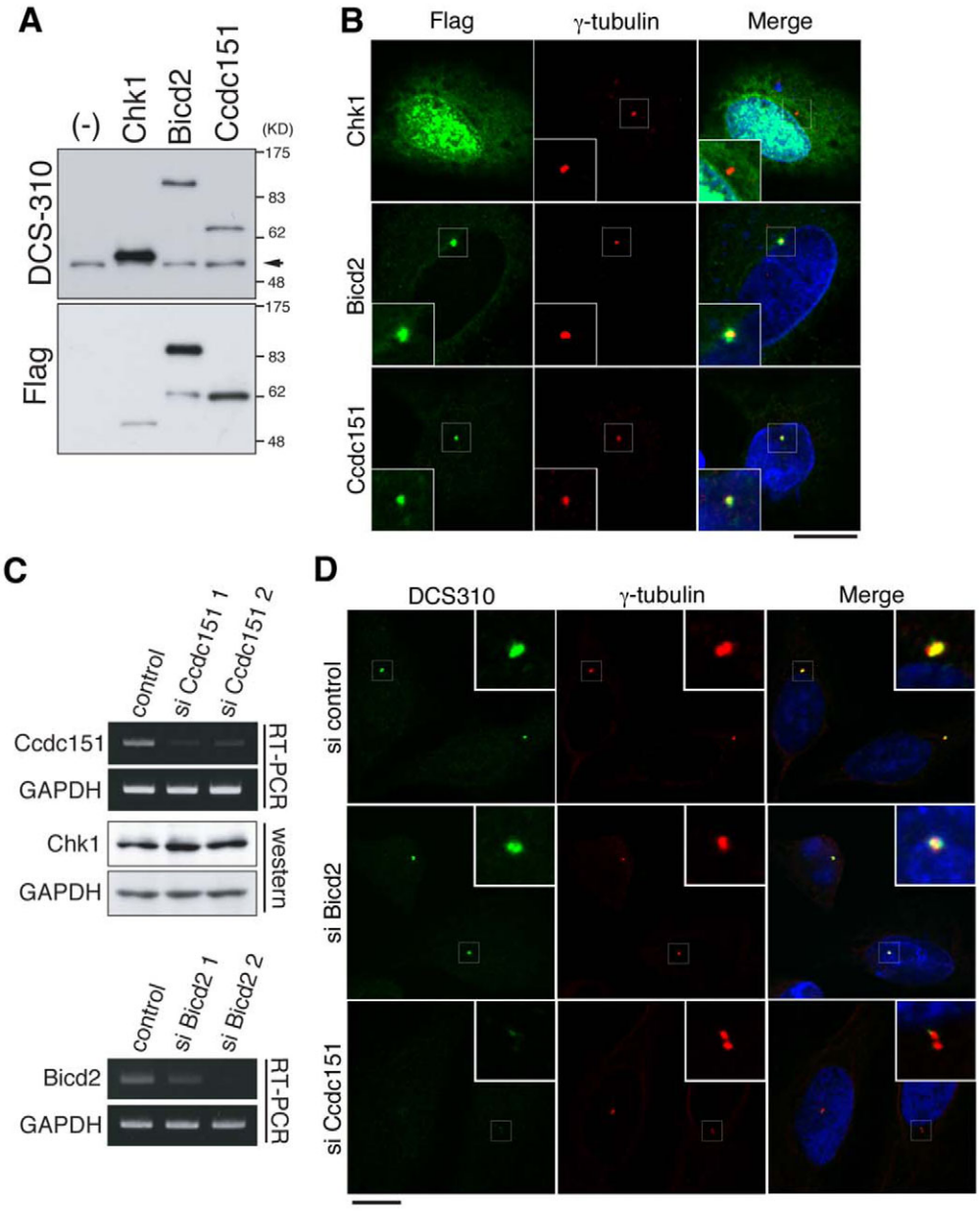

Fig. 3. Centrosomal staining of anti-Chk1 antibody DCS-310 indicates the presence of Ccdc151 but not Chk1 on centrosomes. (**A**,**B**) HeLa cells were transfected with pDEST12.2 (Invitrogen) each carrying a Flag-tagged protein. As a negative control, we used pDEST12.2 carrying only Flag (–). After transfection, cells were subjected to western blotting (A) or immunocytochemistry (B). The arrowhead in A indicates the position of endogenous Chk1. (**C**,**D**) HeLa cells were transfected with siRNA as indicated. At 72 hours after transfection, cells were subjected to RT-PCR, western blotting (C) or immunocytochemistry (D). Since we observed only marginal differences in immunocytochemistry between two sequences targeted to each protein (not depicted), data by using one target sequence are indicated (D). Scale bars: 5 μm.

(DTB) method. At the release of the second thymidine block, we added 0.3 μg/ml Dox to the growing medium for the induction of each Myc-Chk1. From 6–13 hours after the release, cells were collected and then subjected to immunocytochemistry (to evaluate mitotic index; Fig. 4F) or in-vitro H1 kinase assays by using anti-Cyclin B1 immunocomplex (to evaluate the activity of cyclin-B1–Cdk1 complex; Fig. 4G). Without Dox addition (Myc-Chk1 induction), we observed only marginal changes in the timing of mitotic entry among the established cell lines (Fig. 4E). WT expression delayed the timing not only of mitotic entry (Fig. 4F) but also of cyclin-B1–Cdk1 activation (Fig. 4G), compared with the K38M mutant. The timing was more delayed when mutant S286/301A was expressed (Fig. 4F,G), confirming the existence of a positive feedback loop between Cdk1 and Chk1 (Enomoto et al., 2009). However, we observed only marginal effects when Myc-Chk1–PACT was expressed, similar to the experiments with the K38M mutant (Fig. 4F,G). These results suggest that forced immobilization of Chk1 to the centrosome has little impact on the timing of mitotic entry and activation of Cdk1.

These observations appeared to contrast with the previous report that stable induction of GFP-Chk1–PACT inhibited the G2–M transition. We consider that this discrepancy might be partly caused by the difference in the expression level of Chk1–PACT. In the case of higher expression of Myc-Chk1–PACT (by use of 1–2 μg/ml Dox), Myc-Chk1–PACT was not restricted to the centrosome; it also localized to the nucleus (Fig. 4H,I). Under this condition, the expression of Myc-Chk1–PACT also delayed the timing of mitotic entry, like WT expression (Fig. 4J). These results imply one possibility, namely that the localization of mutant Chk1–PACT is highly dependent on its level of expression and that reflects the phenotype.

We also evaluated the effect of the expression of Myc-Chk1 WT with three repeated sequences of NLS at the C-terminus (Myc-Chk1-3×NLS). The expression level of Myc-Chk1-3×NLS was

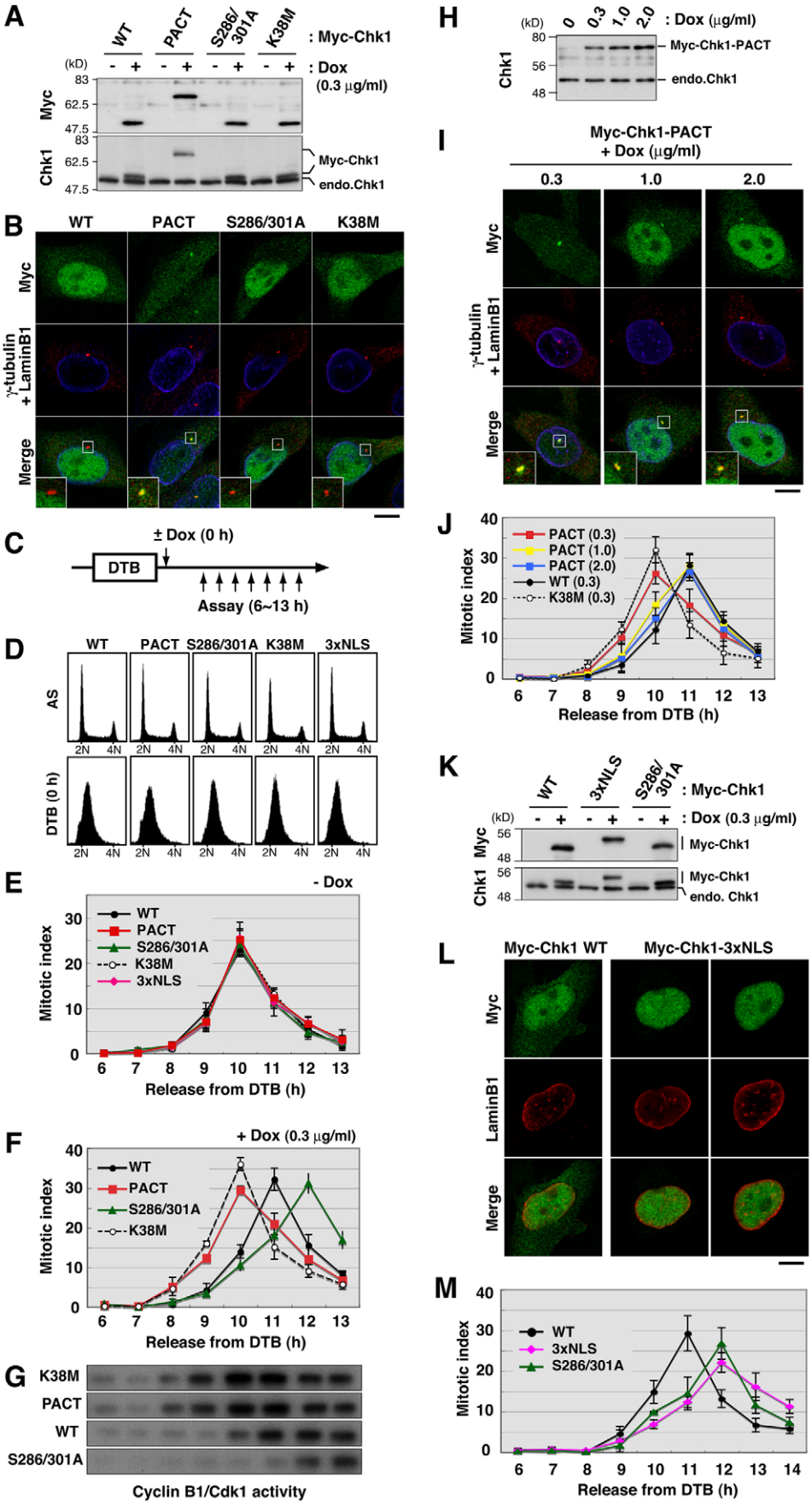

Fig. 4. Nuclear but not centrosomal Chk1 prevents Cdk1 from unscheduled activation. (**A**–**M**) Each Tet-ON cell line was incubated with (+) or without (–) doxycycline (Dox) for 16 hours after which the cells were subjected to western blotting (A,H,K) or immunocytochemistry (B,I,L). Positions of endogenous Chk1 (endo. Chk1) or exogenous Myc-Chk1 are indicated (A,H,K). Cells were co-stained with anti-γ-tubulin (B,I) and anti-lamin-B1 (to detect nuclear membrane; B,I,L). To evaluate the effect of each Myc-Chk1 expression on mitotic entry (F,J,M) and Cdk1 activity (G), the following experiments were performed as indicated (C). Each Tet-On cell line was synchronized at the G1–S boundary using the double thymidine block (DTB) method. To confirm the synchrony of each cell line by DTB, some cells were subjected to FACS analyses (Time 0; D). As a control, FACS analyses were also performed using random culture cells (AS; D). Upon release of second thymidine block, Dox was added to the growth medium to induce Myc-Chk1. Between 6 hours and 13 hours after release, cells were collected and subjected to immunocytochemistry (F,J,M) or in vitro H1 kinase assays using anti-Cyclin B1 immunocomplex (G). As a control, the timing of mitotic entry without Myc-Chk1 induction was analyzed (Dox addition; E). Plotted data of mitotic indices at each time point represent mean ± s.e.m. of 200 cells from three independent experiments (E,F,J,M). Cyclin-B1–Cdk1 activity in each sample was visualized through autoradiography of H1 (G). Scale bars: 10 μm.

comparable to that of Myc-Chk1 WT or mutant Chk1 S286/301A (Fig. 4K). Myc-Chk1-3×NLS was localized at higher levels than Myc-Chk1 WT in the nucleus (Fig. 4L). Like that of S286/301A, the expression of Myc-Chk1-3×NLS delayed mitotic entry more than the expression of Myc-Chk1 WT (Fig. 4M). Since the S286/301A mutant was localized predominantly in the nucleus even during the first mitotic phase prophase (Enomoto et al., 2009), all these observations suggest that nuclear Chk1 prevents Cdk1 from unscheduled activation before the G2–M transition.

In this study, we demonstrate that Chk1, when localized in the nucleus but not on the centrosome, inhibits premature Cdk1 activation. Together with our previous study (Enomoto et al., 2009), our findings here strongly support a model according to which the ATR-Chk1 pathway monitors the integrity of genomic DNA in the nucleus before mitosis. In support of this notion, all known regulators of this pathway are localized in the nucleus (see Introduction) and Chk1 accumulates in the nucleus in response to the checkpoint activation (Sanchez et al., 1997; Jiang et al., 2003). Since Chk1 did not translocate to the centrosome even in HU-treated cells (Fig. 2F), Chk1 did not directly inhibit centrosomal cyclin-B1–Cdk1 activation, which was first detected at the G2–M transition (Jackman et al., 2003). Accumulating data has demonstrated a new checkpoint pathway mediated by MAPK (p38) and MAPK-activated protein kinase-2 (MAPKAP kinase-2; MK2) that operates parallel to Chk1 and is activated downstream of ataxia telangiectasia mutated (ATM) and ATR (Bulavin et al., 2001; Manke et al., 2005; Raman et al., 2007; Reinhardt et al., 2007; Reinhardt and Yaffe, 2009). A recent study also revealed that Chk1 controls nuclear events but that the p38–MK2 pathway regulates cytoplasmic events in checkpoint responses (Reinhardt et al., 2010). Interestingly, p38 has been reported to phosphorylate and inhibit Cdc25B (Bulavin et al., 2001), which was proposed to function as a centrosomal Cdc25 (Gabrielli et al., 1996; Karlsson et al., 1999; De Souza et al., 2000; Lindqvist et al., 2005). Therefore, the p38–MK2 pathway is likely to transmit nuclear checkpoint signals generated by Chk1 to other cellular components such as the centrosome.

Our present study documents the importance of spatiotemporally regulated localization of Chk1 in the mitotic entry and paves the way for future studies that evaluate the coordination of centrosomal, cytoplasmic, and nuclear events during the checkpoint response.

Materials and Methods

Generation of the Chk1$^{+/myc}$ DLD-1 and HeLa Tet-On cell lines

The colon adenocarcinoma (DLD-1) cell line was purchased from ATCC (#CCL-221). For the construction of targeting vectors, genomic regions of the *CHK1* locus were amplified from DLD-1 genomic DNA by using primers of 5′ and 3′ homology arms. Then, site-directed mutagenesis (Stratagene, La Jolla, CA) was performed for the addition of a DNA sequence corresponding to Myc epitope (amino acid sequence; EQKLISEEDL). As shown in Fig. 2A, both homology arms and *loxP–neo–loxP* were ligated into the pAAV-MCS vector. Recombinant adeno-associated viruses (rAAVs) were produced according to the manufacturer's protocol (Stratagene). Chk1$^{+/myc}$ DLD-1 cells were established as described previously (Kohli et al., 2004; Rago et al., 2007). Each HeLa Tet-On cell line was generated as reported previously (Ikegami et al., 2008; Enomoto et al., 2009).

Transfection

For the expression of 18 DCS-310-reactive proteins in HeLa cells, pDEST12.2 carrying each Flag-tagged protein was constructed through the homologous recombination between pDEST12.2 and 'FLJ' cDNA clones (Goshima et al., 2008) using Gateway technology (Invitrogen, Carlsbad, CA). Transfection was performed with Lipofectamine^{TM} reagent (Invitrogen) according to the manufacturer's protocol.

All small interfering RNA (siRNA) duplexes were purchased from QIAGEN (Valencia, CA). Target sequences were as follows: Bicd2 siRNAs, 5′-GGAGCUGU-CACACUACAUG-3′ (sequence 1) and 5′-GGUGGACUAUGAGGCUAUC-3′ (sequence 2) (Splinter et al., 2010); Ccdc151 siRNAs, 5′-GGAGACUAAGGCACUG-GAA-3′ (sequence 1) and 5′-CAA GGCCUAUCUAAUGGACGA-3′ (sequence 2); and non-silencing siRNA (as a negative control), 5′-UUCUCCGAACGUGUCACGU-3′. Transfection was performed with a mixture of each siRNA (final concentration 25 nM) and Lipofectamine^{TM} RNAiMAX reagent (Invitrogen) according to the transfection procedure.

Antibodies

Antibodies against the following proteins or tags were used in the study: Chk1 (DCS310), γ-tubulin (GTU-88), Flag (M2; Sigma, St Louis, MO); Chk1 phospho-Ser345 (133D3), Myc (9B11; Cell Signaling Technology, Beverly, MA); CyclinB1 (GNS-1; BD Transduction Laboratories, San Diego, CA); rabbit laminB1, GAPDH conjugated to HRP (Abcam, Cambridge, UK).

Immunocytochemistry

Before fixation, cells were grown on glass coverslips. For immunostaining with DCS-310 (except that shown in Fig. 2) or anti-Flag, cells were fixed in –20°C methanol/acetone (1:1) for 7 minutes as described previously (Kramer et al., 2004). In other experiments, cells were treated with 1% (Fig. 2) or 3.7% (Fig. 4) formaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 minutes and then with –20°C methanol for 10 minutes. Cells were incubated with primary antibodies for 1 hour and then with appropriate Alexa-Fluor-conjugated secondary antibodies (Invitrogen) for 30 minutes at room temperature. DNA was also stained with 0.5 μg/ml DAPI for 5 minutes. Each fluorescence image was captured as a single optical section using a Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY).

In vitro H1 kinase assays using anti-Cyclin B1 immunocomplex

The cyclin-B1–Cdk1 complex was purified as an anti-cyclin-B1 immunocomplex as described previously (Ikegami et al., 2008; Enomoto et al., 2009). This immunocomplex was incubated with histone H1 as described previously (Kasahara et al., 2010). Cyclin-B1–Cdk1 activity in each sample was visualized through the autoradiography of histone H1.

Reverse-transcriptase PCR

Reverse-transcriptase (RT)-PCR was performed using the following primers as described previously (Goto et al., 2006): Ccdc151, 5′-CAGGAGAC-CATCAGTCAGCTC-3′ (forward) and 5′-GCAGGTACACGCTGGTAATGT-3′ (reverse); Bicd2, 5′-ACTCGGAGATGAGTGCTTTGA-3′ (forward) and 5′-CACAACGTCCTAAAACCCAGA-3′ (reverse); and GAPDH, 5′-GGCATGGC-CTTCCGTGTTCCT-3′ (forward) and 5′-TCCTTGCTGGGGTGGGTGGTC-3′ (reverse).

FACS analysis

For fluorescence-activated cell sorting (FACS) analysis that shows the DNA content in each group, ~10^6 treated cells were collected by trypsinization, resuspended in buffer solution (CycleTEST^{TM} PLUS kit; Becton-Dickinson, San Diego, CA) and stored at –80°C. Then, we treated cells according to the manufacturer's protocol (CycleTEST^{TM} PLUS kit) and analyzed them using a Becton-Dickinson FacsScan and CellQuest software.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/13/2113/DC1

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