Molecular dissection of Cdc6 and the miR-148 family : two stories with common themes

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Chapter 2

p53-dependent regulation of Cdc6 protein stability controls cellular proliferation

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p53-dependent regulation of Cdc6 protein stability controls cellular proliferation

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Activation of the tumor suppressor p53 in response to genotoxic stress imposes cellular growth arrest or apoptosis. We identified Cdc6, a licensing factor of the pre-replication complex, as a novel target of the p53 pathway. We show that activation of p53 by DNA damage results in enhanced Cdc6 destruction by the anaphase-promoting complex. This destruction is triggered by inhibition of CDK2 mediated CDC6 phosphorylation at serine 54. Conversely, suppression of p53 expression results in stabilization of Cdc6. We demonstrate that loss of p53 results in more replicating cells, an effect that can be reversed by reducing Cdc6 protein levels. Collectively, our data suggest that initiation of DNA replication is regulated by p53 through Cdc6 protein stability.

Introduction

Initiation of DNA replication is a tightly regulated process, which is highly conserved in eukaryotes. Key to this process is the licensing of replication origins for DNA replication in which Cdc6 plays a major role (Pelizon, 2003). Cdc6 is recruited to the origins by the origin recognition complex (ORC) during G1 where it serves together with Cdt1 as a loading factor for the minichromosome maintenance (MCM) complex, a putative replicative helicase. Together, this pre-replication complex (pre-RC) renders the genome competent for replication, but initiation of replication only occurs after activation of the complex by cyclin-dependent kinases at the onset of S-phase. This promotes the loading of Cdc45, replication protein A and DNA polymerase-alpha onto chromatin. After initiation of DNA replication, the pre-RCs are converted to post-RCs, which are present in S, G2 and M-phase and are not competent for initiation of replication (Bell and Dutta, 2002; Diffley, 2004; Takisawa et al., 2000).

Cdc6 has been shown to be essential for loading of the MCM-complex onto chromatin and therefore for the initiation of DNA replication in several eukaryotic organisms. First, mutant studies in yeast have demonstrated that association of MCMs to origins is dependent on Cdc6 (Aparicio et al., 1997; Tanaka et al., 1997). Second, it was shown in Xenopus laevis that immuno-depletion of Cdc6 led to loss of MCM origin association (Coleman et al., 1996). Last, ectopic expression of Cdc6 was sufficient to induce stable association of endogenous MCM protein with chromatin in mammalian cells with low Cdc6 levels as a result of serum deprivation (Cook et al., 2002). In addition, immuno-depletion of Cdc6 in human cells by micro-injection of anti-cdc6 antibody could block initiation of DNA replication, which suggests that Cdc6 is also limiting for DNA synthesis in human cells (Hateboer et al., 1998; Yan et al., 1998). Conversely, recombinant Cdc6 protein induced premature entry into S phase in a mammalian cell-free system (Stoeber et al., 1998) and ectopic expression of Cdc6 together with Cdt1 induces rereplication and polyploidy (Vaziri et al., 2003). Thus, Cdc6 is crucial for MCM-loading and therefore involved in regulation of S-phase entry.

In support of an essential function of Cdc6 in initiation of DNA replication, Cdc6 protein abundance is tightly regulated during the cell cycle. In human cells, Cdc6 transcription

is regulated by the E2F transcription factors (Hateboer et al., 1998; Yan et al., 1998). In addition cdc6 is targeted for ubiquitin-mediated proteolysis in early G1 by the E3 ligase-anaphase promoting complex (APC) (Petersen et al., 2000). This is mediated by Cdh1, a protein required for both substrate recognition and activation of the APC in late mitosis/ early G1 (Pfleger et al., 2001; Reed, 2003). Typically, Cdh1 associates with its substrates via their N-termini and requires a well-defined RXXL destruction box (D box) or KEN box (Pfleger and Kirschner, 2000; Pfleger et al., 2001). APC^{Cdh1} dependent proteolysis of Cdc6 requires both a D box and a KEN box (Petersen et al., 2000).

Several studies have shown that mammalian Cdc6 can be phosphorylated in vitro by both CyclinE/CDK2 and CyclinA/CDK2 and an interaction between the cyclins and Cdc6 could be detected (Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999). Moreover, Cdc6 can be phosphorylated in vivo at three N-terminal sites (ser54, ser74 and ser106) that are phosphorylated by CDK2 in vitro (Jiang et al., 1999). Together, this strongly suggests that mammalian Cdc6 is an in vivo CDK2 substrate. However, the role of this phosphorylation in Cdc6 regulation remains controversial. Based on studies with ectopically expressed and tagged wild-type Cdc6 or Cdc6 that was mutated in several potential CDK2 phosphorylation sites, it has been proposed that phosphorylation of Cdc6 by CyclinA/CDK2 in S-phase results in translocation from the nucleus to the cytosol and subsequent degradation (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). Now, it has become evident that only ectopically expressed Cdc6 or the soluble endogenous form is translocated to the cytosol, whereas the chromatin bound form persist through S and G2 phase (Coverley et al., 2000; Mendez and Stillman, 2000). Furthermore, by using a phospho-specific antibody it has been shown that phosphorylated serine-54 Cdc6 maintains high affinity for chromatin during S-phase (Alexandrow and Hamlin, 2004).

The DNA-damage response is essential for maintenance of genome stability. Genotoxic stress activates checkpoints that account for a rapid and prolonged cell cycle arrest, DNA-repair or apoptosis (Iliakis et al., 2003). Primary among the checkpoint genes is the tumor suppressor p53 (Fei and El-Deiry, 2003). In response to DNA damage, such as induced by ionizing radiation (IR), the transcription factor p53 is activated by the ATM-CHK2 pathway. A rapid phosphorylation at serines 15 and 20 leads to release of Mdm2 and stabilization and activation of p53 (Shiloh, 2003). p53 is required for the maintenance of the G1 arrest through activation of its transcriptional target gene p21cip1, induction of apoptosis and for the sustained arrest of cells prior to M phase (Bunz et al., 1998; Fei and El-Deiry, 2003; Motoyama and Naka, 2004). In addition, DNA damage initiates a rapid cell cycle arrest by inactivation of proteins that promote cell cycle progression, such as Cyclin D1 and CDC25A (Agami and Bernards, 2000; Falck et al., 2001).

In this study, we determined the effect of DNA damage on Cdc6 protein levels. We observed a rapid degradation of Cdc6 in response to IR. Interestingly, this irradiation-induced Cdc6 destruction was p53-dependent. Activation of p53 caused inhibition of cyclin E/CDK2 through induction of the cell cycle inhibitor p21cip1. This resulted in loss of Cdc6-serine 54 phosphorylation, which led to accelerated proteolysis by the APC^{Cdh1}.

Results

p53 dependent down-regulation of Cdc6 following DNA damage

Since the licensing factor Cdc6 is critical for formation of the pre-RC and the initiation of DNA replication, we examined the effect of IR on the Cdc6 protein levels. Primary human BJ fibroblasts were treated with increasing amounts of IR and the cells were harvested at various time-points. We found reduced Cdc6 protein levels within 2 hours after exposure to IR and 2 Gy was sufficient to elicit this response (Fig. 1A). Control immuno-stainings showed an increase in

the p53 transcription factor and its target $p21^{Cip1}$ at these doses, demonstrating that p53 was both stabilized and activated after DNA damage in these cells. Similar down-regulation of Cdc6 was observed in other cell types, such as MCF-7 cells (Fig. 1B). However, this cancer cell line required a higher dose of 5-20 Gy to activate a DNA damage response that resulted in both Cdc6 down-regulation and stabilization of p53 (data not shown). Furthermore, Cdc6 down-regulation following irradiation in MCF-7 cells was observed 2-4 hours following IR and at these time-points no significant change in cell cycle profile was observed (Supplementary Fig. 1A). Interestingly, the reduction in Cdc6 levels following IR in MCF-7 cells was preceded the accumulation of $p21^{Cip1}$ (Fig. 1B). This is likely to be the result of p53-independent DNA damage responses, such as cyclin D1 and Cdc25A destruction, operating early after DNA damage to inhibit CDK2 activity (Agami and Bernards, 2000; Mailand and Diffley, 2005). Indeed, the reduction in Cdc6 levels following radiation of MCF-7 cells correlated well with the reduction in CDK2 activity, which was almost completed within 2 hrs after IR

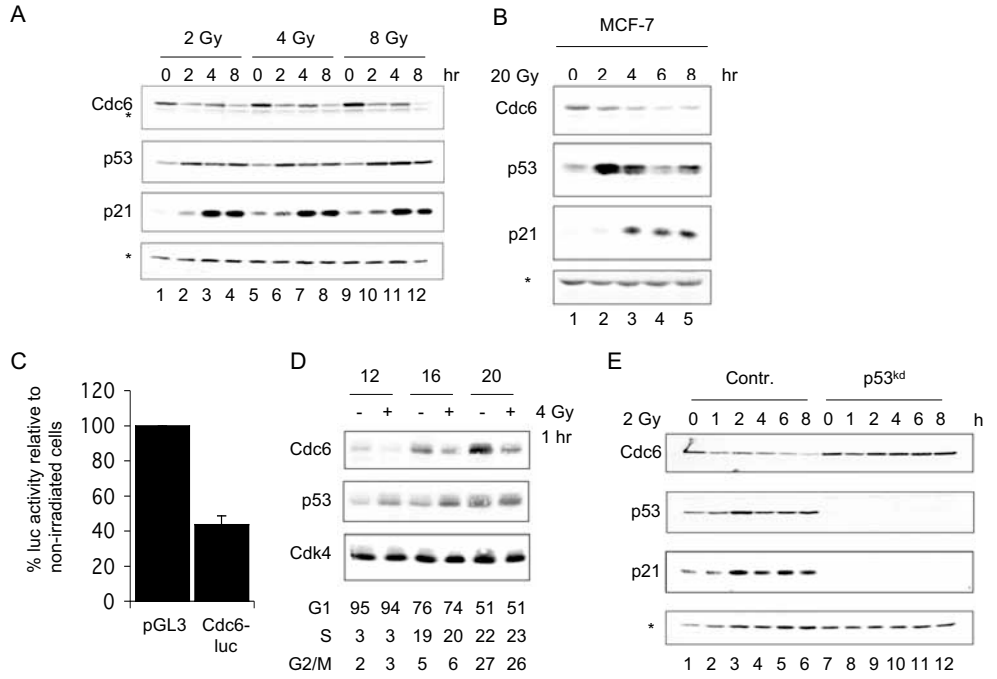

Figure 1. p53 dependent regulation of Cdc6. A Primary human BJ fibroblasts were subjected to 2, 4 and 8 Gy of IR and harvested 0, 2, 4, and 8 hours later. Whole cell extracts (WCE) were made, separated on 10% SDS-PAGE and immuno-blotted to detect Cdc6, p53 and p21 proteins. Asterisks mark background bands. **B** MCF-7 cells irradiated with 20 Gy were treated as described in A. **C** MCF-7 cells were transfected with Cdc6-Luciferase and control pGL3-promoter vector constructs (n=3). 72 hrs after transfection, cells were irradiated (20 Gy) and harvested 2 hrs later. Luciferase activities were measured, normalized to co-transfected Renilla luciferase and compared to non-irradiated cells. **D** Primary human BJ fibroblasts were serum-starved for 72 hours, released with addition of 10% FCS, irradiated (4Gy) 1 hour prior to the harvest at the indicated time points. In parallel, cells were collected for flow cytometric analysis (Supplementary Fig 1). **E** Polyclonal pools of primary human BJ fibroblasts stably carrying the retroviral vector pRETRO-SUPER (pRS) or pRS-p53kd, which mediates the inhibition of p53 expression through RNAi, were subjected to 2 Gy of irradiation. WCE were prepared at the indicated time-points and protein levels of Cdc6, p53 and p21 were examined by immuno-blotting. Contr., control.

(Supplementary Fig. 1B). To estimate the quantity of Cdc6 protein reduction following IR, we constructed a firefly Cdc6-luciferase fusion construct driven by the SV40 promoter and transfected it into MCF-7 cells together with a Renilla-luciferase control. Treatment with 20 Gy irradiation resulted in a reduction of 60% Cdc6-luciferase activity after 4 hours, whereas no change in control luciferase activity was observed (Fig. 1C). Collectively, these results indicate that Cdc6 protein level is reduced in response to DNA damage.

Next, we assessed whether Cdc6 protein levels are reduced in G1 phase of the cell cycle when pre-RCs are formed. We synchronized primary human BJ fibroblasts in G0 by serum starvation and then released them to enter synchronously G1 phase around 12 hours later and S phase around 16 and 20 hours later (supplementary Fig. 1C). Cells were irradiated (4 Gy) 12, 16 and 20 hours after the release, harvested one hour later and Cdc6 protein level was compared to untreated cells by immuno-blot analysis. Clearly, potent down-regulation of Cdc6 following IR occurred when cells were almost exclusively in G1 phase of the cell cycle (12 hr), indicating that Cdc6 protein levels are reduced when it is functional (Fig. 1D). Also here, Cdc6 down-regulation preceded any detectable cell cycle changes induced by IR, suggesting that Cdc6 down-regulation in G1 contributes to the IR-induced cellular responses (Fig. 1D and Supplementary Fig. 1C).

The p53 protein is a central player in the DNA damage response. We therefore investigated whether the decrease in Cdc6 protein level following IR is p53-dependent. We transduced primary human BJ cells with the pRS-p53^{kd} that stably suppresses p53 expression through RNA interference (RNAi) (Voorhoeve and Agami, 2003) and monitored Cdc6 protein level following IR. Interestingly, immuno-blot analysis showed that IR-induced Cdc6 down-regulation was completely abrogated in p53^{kd} cells (Fig. 1E). Control immuno-labeling showed the absence of p53 activation and induction of its transcriptional target p21^{Cip1} following IR in the p53^{kd} cells. Quantification of the Cdc6 reduction in control BJ cells revealed that Cdc6 protein level was reduced to 10-30% following 2 Gy of irradiation, whereas no reduction in cdc6 level was observed in p53kd cells (data not shown). These results demonstrate that the p53 pathway controls Cdc6 protein level in human cells in response to DNA damage.

Enhanced Cdc6 proteolysis following DNA damage

As a transcription factor, p53 can directly repress gene expression (Nakade et al., 2004) and may therefore switch-off Cdc6 transcription when activated. However, no changes in Cdc6 mRNA level after IR were found by northern blot analysis (Fig. 2A). Furthermore, Cdc6 protein expressed from a heterologous CMV promoter was reduced after DNA damage to a similar extent as the endogenous protein (data not shown). These results indicate that the IR-induced Cdc6 down-regulation occurs at the post-transcriptional level.

Next, we tested whether the rapid decrease in Cdc6 protein level after IR is a result of increased proteolysis. We used the protein-synthesis inhibitor cycloheximide (CHX) to monitor Cdc6 half-life. To allow proper cell cycle response to DNA damage, CHX was added to cells 2 hours after the IR treatment. (If CHX was added right after the radiation treatment, Cdc6 stability remained unchanged following IR, supplementary Fig. 2). In MCF-7 cells, Cdc6 protein level decreased more rapidly in irradiated cells compared to non-irradiated cells (Fig. 2B) and quantification analysis showed that the half-life of Cdc6 was reduced from 90 min to 30 min upon IR treatment (supplementary Fig. 3A). Also in synchronized G1/S primary BJ fibroblasts we found that the half-life of Cdc6 protein is reduced from 25 min to 12.5 min following DNA damage (Fig. 2C and supplementary Fig. 3B). Since Cdc6 down-regulation following IR appeared to be p53-dependent, we monitored Cdc6 protein stability in control and p53^{kd} MCF-7 cells 2 hours after IR (Fig. 2D and supplementary Fig. 3C). Indeed, Cdc6

maintained its stability following IR in p53kd MCF-7 cells (half-life 90 min) compared to control irradiated cells (half-life 25 min). The protein stability of cyclin D1 following IR was not increased by the reduced level of p53, thereby showing the specificity of Cdc6 stabilization (Fig. 2D). Last, this p53-dependent Cdc6 degradation was mediated by the proteasome since it was abrogated when MCF-7 cells were irradiated in the presence of the proteasome inhibitor MG-132 (Fig. 2E). Taken together, these results demonstrate that the proteasome machinery mediates the accelerated proteolysis of Cdc6 following genotoxic stress and that Cdc6 protein abundance following IR is mainly controlled by p53.

Enhanced destruction of Cdc6 following DNA damage is mediated by the E3-ligase APC^{Cdh1}

In early G1, the N-terminal KEN and RxxL (D-box) destruction motifs of Cdc6 (Fig. 3A) mediate its degradation by the E3-ligase anaphase-promoting complex (APC) (Petersen et al., 2000). To determine whether Cdc6 destruction following IR involves the APC, we suppressed the expression of Cdh1 and Cdc20 by RNAi. Both proteins act as substrate recognition and activating modules for the APC (Reed, 2003). Inhibition of Cdh1 expression completely abrogated IR-induced Cdc6 destruction, whereas the Cdc20 knockdown had no effect (Fig. 3B). The reduced expression of both Cdh1 and Cdc20

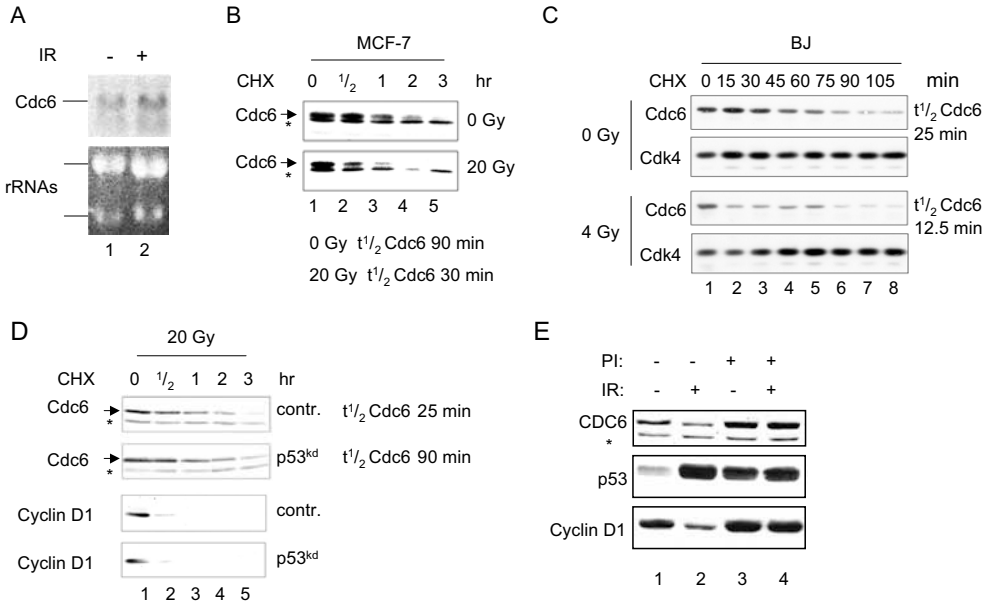

Figure 2. Increased Cdc6 proteolysis following DNA damage. A MCF-7 cells were subjected to IR treatment (20 Gy) and collected for northern blot analysis 4 hours later. Blots were hybridized with a Cdc6 cDNA probe to detect Cdc6 mRNA. **B** Irradiated (20Gy) or non-irradiated MCF-7 cells were treated with cycloheximide (CHX) 2 hours after IR. Cells were harvested at the indicated time-points after CHX addition and immuno-blotted against Cdc6. Asterisks indicate background bands. Half-life values were determined by quantification analysis **C** Serum starved BJ cells were released for 13 hours, treated as described above and immuno-blotted to detect Cdc6 and CDK4. Quantification of Cdc6 protein level was used to determine the half-life. **D** pRS and pRS-p53^{kd} cells were irradiated and treated with CHX 2 hours later. Cells were harvested at the indicated time-points and quantifications of half-life were calculated **E** MCF-7 cells were treated with or without 20 Gy irradiation (IR) in the presence or absence of proteasome inhibitors (PI). PI was added 30 min prior to the IR treatment. WCE were immuno-blotted against Cdc6, p53 and cyclin D1. A cross-reacting band (asterisk) was used as loading control.

was not accompanied with any significant change in cell cycle profile or p53 activation following IR (Supplementary Fig. 4A and data not shown).
The involvement of the APC^{Cdh1} in the p53-dependent regulation of Cdc6 suggests that also the KEN or the RxxL destruction motifs play a role in IR-induced Cdc6 destruction. Therefore, we constructed Cdc6-GFP chimeras with mutations in these destruction motifs. Similar to endogenous Cdc6, the stability of the transfected Cdc6-GFP fusion protein is reduced upon IR treatment (Fig. 3C lanes 1-4 and quantified in Fig. 3D). Mutating the KEN motif to AAA (KENmut) or the RxxL motif to AxxA (RxxLmut) partly prevented IR-induced Cdc6 destruction (lanes 5-12 and Fig. 3d). However, mutating both destruction motifs (R+K) completely abrogated Cdc6 destruction in response to IR (lanes 13-16). This demonstrates that both the KEN and the RxxL destruction motifs are involved in the p53-dependent Cdc6 destruction following IR through the APC^{Cdh1}.

Inhibition of CDK2 activity results in enhanced Cdc6 proteolysis

Activation of the p53 transcription factor following genotoxic stress results in up-regulation of the cell cycle inhibitor $p21^{Cip1}$. We therefore asked whether $p21^{Cip1}$ is required for the p53-dependent destruction of Cdc6 and transfected MCF-7 cells with knockdown constructs targeting $p21^{Cip1}$, $p27^{Kip1}$ (a homologue of $p21^{Cip1}$ that is not regulated by p53) and p53. Suppression of $p21^{Cip1}$ expression, but not $p27^{Kip1}$, completely abrogated Cdc6 destruction after IR (Fig. 4A lanes, 9-16). This effect was in correlation with CDK2 activity, as $p21^{Cip1}$, but not $p27^{Kip1}$,

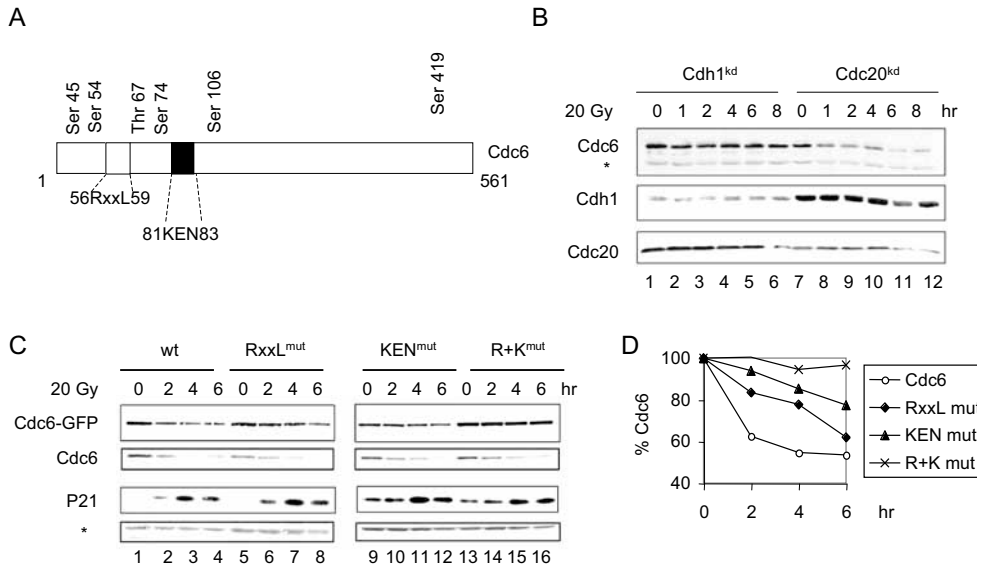

Figure 3. Enhanced destruction of Cdc6 following DNA damage is mediated by the E3-ligase APC^{Cdh1}. A A schematic representation of the Cdc6 protein with its five putative CDK2 N-terminal phosphorylation sites and the APC destruction boxes; RxxL and KEN motifs. **B** MCF-7 cells were transiently electroporated with pSUPER (pS) and RNAi constructs that target Cdh1 and Cdc20. Cells were subjected to IR treatment 60 hours after transfection and subsequently harvested for immuno-blotting analysis to detect Cdc6, Cdh1 and Cdc20 at the indicated time points. The asterisk marks a background band. **C** The same experimental settings as in b, only that cells were electroporated with Cdc6-GFP chimera (wt) or mutants of the RxxL and the KEN-box destruction motifs. R+K contains mutations in both the RxxL and KEN-box motif. WCE were immuno-blotted against Cdc6 and p21. The asterisk indicates a background band and shows equal loading. **D** Quantification analysis of the different Cdc6 proteins shown in Figure 3C. Mut., mutant.

knockdown cells maintained significant CDK2 activity following IR treatment (Supplementary. Fig. 4b). The protection from IR-induced destruction of Cdc6 in cells with suppressed expression of $p21^{Cip1}$ was comparable to that observed in $p53^{kd}$ cells (Fig. 4A, lanes 4-12). This effect was specific to Cdc6 as the enhanced degradation of cyclin D1, which is p53-independent, was still sustained in the $p21^{Cip1}$ knockdown cells (data not shown).

In response to IR, accumulation of $p21^{Cip1}$ inhibits CDK2 activity. It was described that mammalian Cdc6 is phosphorylated in vivo at three N-terminal sites (S54, S74 and S106) (Jiang et al., 1999), which can be phosphorylated by Cyclin E/CDK2 complex in vitro (Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999). To address the role of CDK2 in regulating Cdc6 stability, we treated cells expressing the Cdc6-luciferase chimera protein with increasing amounts of the CDK2 inhibitor roscovitin and monitored luciferase activity 1 hour later. Roscovitin treatment resulted in rapid reduction in luciferase activity suggesting a direct connection between CDK2 activity and Cdc6 protein stability (Fig. 4B). In a second experiment, we transfected MCF-7 cells with control, pS-p53kd or pS-$Cdh1^{kd}$ RNAi vectors, treated

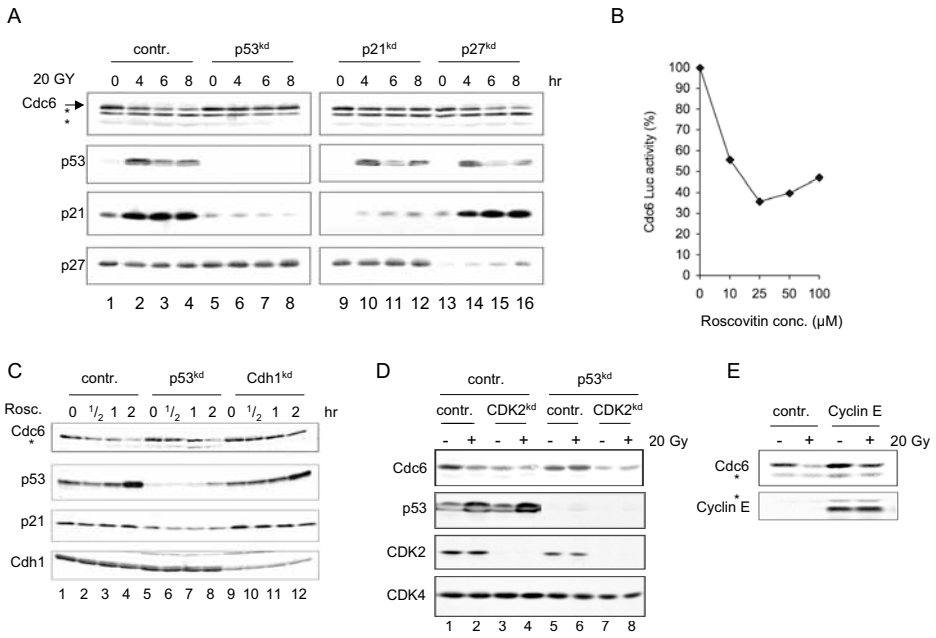

Figure 4. Inhibition of CDK2 activity results in enhanced Cdc6 proteolysis. A MCF-7 cells were electroporated with pS, pS-$p53^{kd}$, pS-$p21^{kd}$ and pS-$p27^{kd}$ and subjected to IR treatment 60 hours after transfection. WCE were prepared at indicated times-points and immuno-blot analysis was performed to detect protein levels of Cdc6, p53, p21 and p27. Asterisks indicate cross-reacting bands. **B** MCF-7 cells expressing the Cdc6-luciferase chimera protein were treated with the indicated amounts of Roscovitin and harvested for luciferase analysis 1 hour later. **C** MCF-7 cells were electroporated with a control construct and RNAi constructs targeting p53 and Cdh1 and treated with Roscovitin 60 hours later. WCE were prepared several time-points after treatment and immuno-blot analysis was performed to detect the indicated proteins. **D** MCF-7 and MCF-7-p53kd cells were electroporated with CDK2-siRNA oligos, irradiated 60 hours later (20 Gy) and harvested 4 hours later. WCE were made and immuno-blotted with the indicated antibodies. **E** MCF-7 cells were electoporated with cyclin E and control expression vectors. WCE were made and immuno-blotted to detect Cdc6 and Cyclin E proteins. Asterisks indicate cross-reacting bands.

them with roscovitin and harvested the cells for immuno-blot analyses at various time-points. Figure 4c shows that also endogenous Cdc6 was rapidly reduced upon roscovitin treatment in control-transfected cells (lanes 1-4). In addition, Cdc6 was down-regulated upon roscovitin treatment in $p53^{kd}$ cells (lanes 5-8), although these cells are impaired in their ability to enhance degradation of Cdc6 following IR (Fig. 1E and 4A). This indicates that CDK2 is downstream of p53 in the IR-induced Cdc6 degradation. In contrast, Cdc6 remained stable upon roscovitin treatment in cells with suppressed Cdh1 levels (lanes 9-12), suggesting that the APC^{Cdh1} is downstream of CDK2 in this pathway. Notably, this experiment also demonstrates that the resistance of Cdc6 to IR in $p53^{kd}$ cells is not a result of increased mRNA levels.

To further substantiate the role of CDK2 in Cdc6 destruction following DNA damage, we assessed the effect of IR on Cdc6 protein level in MCF-7 and MCF-7-$p53^{kd}$ cells transfected with control or CDK2 siRNAs. In agreement with the observed Cdc6 protein reduction upon roscovitin treatment, Cdc6 protein level was reduced in response to low CDK2 levels in both control and $p53^{kd}$ cells (Fig. 4D, lanes 1, 3, 5 and 7), Indicating that CDK2 plays an essential role in maintaining Cdc6 protein level. Furthermore, Cdc6 protein was down-regulated in control cells within 2 hours after irradiation, whereas it remained unaffected in irradiated $CDK2^{kd}$ cells (Fig. 4D, lanes 1-4). As expected, no change in Cdc6 levels was observed in $p53^{kd}$ cells after irradiation (lanes 5-8). Last, our model predicts that ectopic activation of CDK2 should confer significant resistance to Cdc6 from the enhanced destruction following DNA damage. To test this hypothesis we over-expressed cyclin E, an activator of CDK2, in cells that were treated with IR. Figure 4e shows that cyclin E over-expression results in accumulation of Cdc6 in untreated cells and significant resistance to DNA damage effects. Altogether, these results demonstrate the direct requirement of CDK2 inhibition in the p53-dependent destruction of Cdc6 protein after IR.

Phosphorylation of serine-54 by CDK2 inhibits p53-dependent Cdc6 proteolysis

It has been proposed that phosphorylation of Cdc6 results in translocation from the nucleus to the cytosol (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). In stark contrast, our results indicate that phosphorylation of Cdc6 by CDK2 leads to its stabilization. Therefore, we aimed to map this stabilizing phosphorylation site by mutating putative CDK2 phosphorylation sites to aspartic acid, thereby mimicking their phosphorylated state. The N-terminus of Cdc6 harbors three sites that can be phosphorylated in vivo, serines 54, 74 and 106 (Jiang et al., 1999). We chose to mutate S54 and S74, as they are the sites nearest to the destruction motifs (Fig. 3A). We made Cdc6-GFP fusion constructs that contain S54D and S74D mutations and examined them in a transient transfection assay in MCF-7 cells. Mutation of S54 to aspartic acid (S54D) completely abrogated the p53-induced Cdc6 destruction, whereas the S74D mutant was degraded to an extent that was comparable to wt Cdc6-GFP and to the endogenous protein (Fig 5A. and supplementary Fig. 4C). Importantly, the expression of the S54D mutant did not affect the upstream p53 response, as $p21^{Cip1}$ levels accumulated in these cells after DNA damage (Fig 5A). This result suggests that the phoshorylation of S54 is critical for Cdc6 stability in non-stressed cells. To examine this point we mutated serine 54 to alanine (S54A), a change mimics unphosphorylated state of Cdc6 at this position. Introduction of S54A into cells as a GFP chimera protein resulted in very little expression compared with wt Cdc6 and its stable S54D mutant (Fig. 5B, lanes 1, 3 and 5). Treatment with the proteasome inhibitor MG-123 resulted in the accumulation of both wt and S54A Cdc6 whereas S54D remained unchanged (lanes 2, 4 and 6). This result indicates that in non-stressed cells, phosphorylation of at serine 54 protects Cdc6 from rapid destruction.

To further substantiate the connection between S54 phosphorylation and the p53-

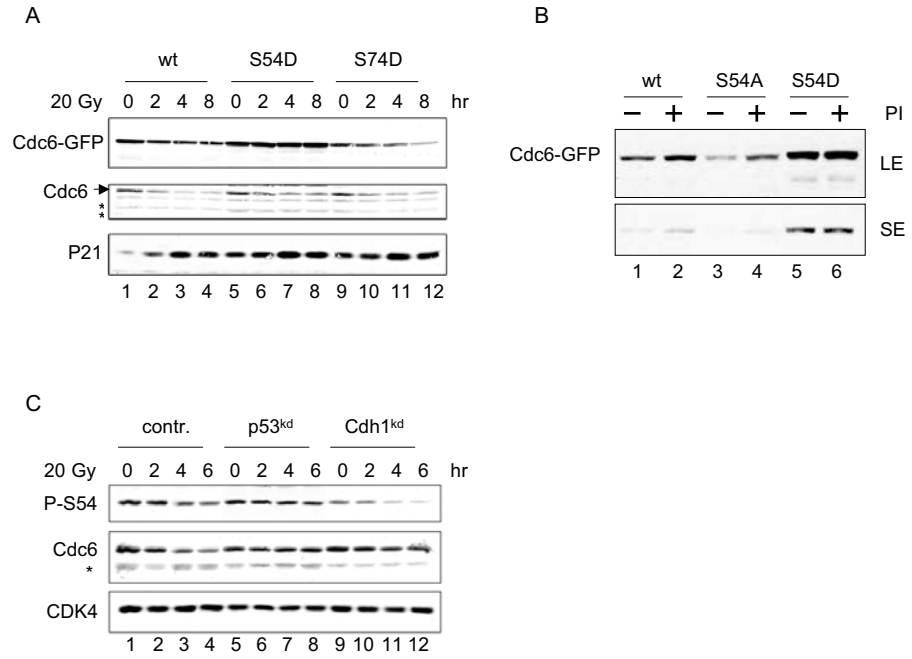

Figure 5. Phosphorylation of serine-54 by CDK2 inhibits p53-dependent Cdc6 proteolysis. A MCF-7 cells were electroporated with wt Cdc6-GFP or S54D and S74D Cdc6-mutant constructs and the experiment was performed as described in Figure 3C. **B** MCF-7 cells were electroporated with wt, S54A and S54D Cdc6-GFP constructs. Forty eight hrs after transcfection, the proteasome inhibitor MG-123 (PI) was added, WCE were separated on 10 SDS-PAGE and immuno-blotted to detect Cdc6. For comparison, long and short (LE and SE, respectively) exposures are shown. **C** MCF-7 cells were electroporated with pS, pS-p53^{kd} and pS-Cdh1^{kd}, irradiated (20 Gy) and harvested at the indicated times. WCE were immuno-blotted with anti-phospho-S54-Cdc6, Cdc6 or control Cdk4 antibodies. Asterisk marks a non-specific band.

dependent destruction of Cdc6, we used an anti-phospho-S54 specific Cdc6 antibody and monitored the phosphorylation state of endogenous Cdc6. We transfected MCF-7 cells with pS, pS-p53^{kd} or pS-Cdh1^{kd} constructs and subjected them to IR treatment. Importantly, we detected phosphorylated-S54 Cdc6 in whole cell extracts from cycling control-transfected cells and these levels were reduced following IR (Fig. 5C, lanes 1-4). In the cells transfected with either pS-p53^{kd} or pS-Cdh1^{kd}, Cdc6 protein level remained high following DNA damage, however the level of phosphorylated S54 was only stable after IR in the p53kd cells (lanes 4-12). In the cdh1^{kd} cells the level of S54 phosphorylated Cdc6 was still reduced. This result is in full agreement with a pathway consisting of upstream p53-dependent regulation of Cdc6 stability by phosphorylation and downstream APC^{Cdh1}-mediated degradation. Last, we examined the effect of cyclin E over-expression or Cdh1^{kd} on the stability of S54D-Cdc6 in the absence of DNA damage. Supplementary Fig. 4D shows that S54D mutant was unaffected by both treatments, suggesting that S54 phosphorylation is a major determinant of Cdc6 stability. Collectively, these results reveal that inhibition of S54 phosphorylation is the mechanism by which p53 regulates Cdc6 stability following DNA damage. The importance of this pathway is further extended by the fact that both Cdc6 S54 and the RxxL and KEN destruction boxes are evolutionary conserved in mice.

p53-dependent regulation of Cdc6 protein stability controls cellular proliferation

Apart from its role in DNA damage responses, p53 regulates cellular proliferation in non-stressed cells as well, since its suppression by RNAi accelerates proliferation of primary human cells (Voorhoeve and Agami, 2003) and MCF-7 cells (data not shown). Therefore, we examined the effect of reduced p53 expression on the cell cycle profile of MCF-7 cells. Also here we observed that suppression of endogenous p53 expression resulted in more proliferating cells (6 % increase in S-phase and 10% decrease in G1, Fig. 6A). This higher fraction of S-phase population could be due to earlier assembly of the pre-RC in G1. Since Cdc6 is regulated by p53 in the DNA damage response, we examined the level of Cdc6 in proliferating cells with suppressed p53 expression. Interestingly, we found a higher level of Cdc6 protein in pRS-p53^{kd} cell extracts compared to control cells (Fig. 6B). This suggests that Cdc6 is more stable in p53-knockdown cells. To test this directly, we treated MCF-7 cells with CHX and observed that the protein turnover of Cdc6 is slower in the p53^{kd} cells (115 min),

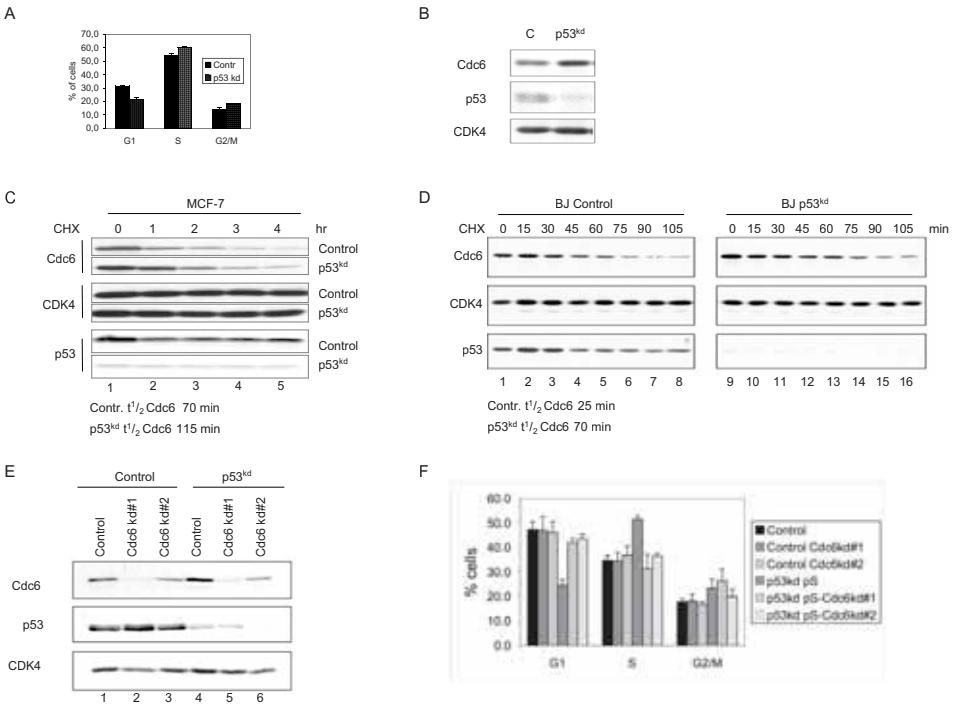

Figure 6. p53-dependent regulation of Cdc6 protein stability controls cellular proliferation. A Cell cycle analysis of polyclonal control or p53-knockdown MCF-7 cells. BrdU-labeled cells were analyzed by fluorescence activated cell sorting (FACS). The result is the mean of three independent experiments. **B** Whole cell lysates of control and p53^{kd} MCF-7 cells were immuno-blotted to detect Cdc6, p53 and CDK4 protein. **C** pRS and pRS-p53^{kd} MCF-7 cells were treated with CHX as described in Fig 2B. WCE were labeled with Cdc6, p53 and control CDK4 antibodies. **D** Serum starved BJ cells that were released for 13 hours were treated as described in Fig. 2C **E** The polyclonal pools of pRS and pRS-p53^{kd} cells were electroporated with Cdc6 knockdown constructs (#1 and #2), harvested after 72 hours and immuno-blotted to detect Cdc6, p53 and as loading control CDK4 proteins. **F** The same experimental setting as in Fig. 5E, but cells were subjected to flow cytometric analysis. The result is the mean of three independent experiments.

compared to control cells (70 min, Fig. 6C). Control immuno-blot analyses of CDK4 and p53 showed equal loading and the extent of the p53 knockdown. The same qualitative result was also seen in synchronized G1-phase primary BJ cells where Cdc6 stability was raised from 25 min in normal cells to 70 min in the BJ-p53^{kd} cells (Fig. 6D). To investigate whether Cdc6 stability is important for the increased proliferation of p53^{kd} cells, we transfected control and stable polyclonal p53^{kd} cells with two effective knockdown constructs targeting Cdc6. Immuno-blot analysis showed reduction of Cdc6 expression in pS-Cdc6^{kd} transfected cells in both cell types (Fig 6E). Notably, the Cdc6^{kd} had no significant effect on p53 levels. Flow cytometric analysis of the same cell populations repeatedly revealed that inhibition of Cdc6 expression altered the cell cycle profile of only p53^{kd} cells by increasing the fraction of cells in G1 and decreasing the fraction of cells in S phase (Fig. 6F). In contrast, no significant change was detected in the cell cycle profile of the control MCF-7 cells. This indicates that the increased Cdc6 levels observed in the absence of p53 are required for the enhanced proliferation of these cells. Collectively, our data implies that Cdc6 is part of a p53 protein-network that regulates cellular proliferation.

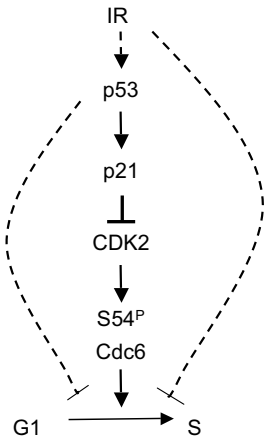

Figure 7. Model. A schematic model depicts the p53 pathway that regulates Cdc6, and thereby S-phase entry, both under normal tissue culture conditions (solid lines) and following IR (dashed lines).

Discussion

In response to genotoxic stress, cells activate checkpoints that prevent DNA replication and cell cycle progression (Bartek and Lukas, 2001). Our results establish a novel connection between DNA damage and the formation of preRCs in G1 phase (Fig 7). Cdc6, an essential protein of the preRC, is rapidly degraded upon genotoxic stress. The enhanced degradation of Cdc6 following IR is not complete, which can be a result of a small population of Cdc6 that is protected from degradation or that the enhanced is restricted to certain phases of the cell cycle. A central role in this destruction pathway is played by the p53 tumor suppressor gene, which controls the rate by which Cdc6 is degraded by the APC^{Cdh1}. Cdc6 is a licensing factor of DNA replication and once bound to the ORC-complex at origins of replication it recruits together with Cdt1 the MCM-complex. Our data implies that IR-induced down-regulation of Cdc6 contributes to a checkpoint that prevents DNA replication in cells with damaged DNA. Interestingly, DNA damage also induces the destruction of the licensing factor Cdt1, but in a p53-independent manner (Higa et al., 2003; Hu et al., 2004; Kondo et al., 2004). Therefore, we propose that the destruction of both Cdc6 and Cdt1 acts in concert to execute a tight regulation of pre-RC assembly following genotoxic stress.

We also found that p53-dependent regulation of Cdc6 protein stability plays a role in non-genotoxic stressed cells. Under normal tissue culture conditions Cdc6 protein stability is increased in p53^{kd} cells. We show that reducing Cdc6 protein level by siRNAs in p53^{kd} cells results in a reduction of replicating cells and a cell cycle distribution that is comparable to cells with functional p53. However, reducing Cdc6 protein levels in control MCF-7 cells did not affect their proliferation, which suggests that cells

without functional p53 are more susceptible to reduction in Cdc6 levels.

It is generally thought that initiation of DNA replication is regulated in a two-step process to ensure that the DNA is replicated only once in each cell cycle (Bell and Dutta, 2002). First, pre-replication complexes are assembled at origins of replication and second the origins are fired. Notably, the regulation of these events varies in different organisms. In mammals, CDKs appear to have an inhibiting role in pre-RC assembly. For both Cdt1 and Orc1 it has been proposed that they are targeted for proteolysis in a CDK-dependent manner (Liu et al., 2004; Mendez et al., 2002; Nishitani et al., 2004). In addition, it has been proposed that cyclinA/CDK2 activity results in Cdc6 translocation from the nucleus to the cytosol and subsequent proteolysis (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). In contrast to these observations, we found that phosphorylation of Cdc6 by cyclinE/CDK2 results in stabilization of the protein. We established that one amino acid (serine 54) mediates p53-induced Cdc6 destruction. Once serine 54 is phosphorylated by CDK2, Cdc6 becomes more resistant to APC^{Cdh1} degradation. Our results are in agreement with a report showing that only ectopically expressed Cdc6 is translocated to the cytosol and that serine 54 phosphorylated Cdc6 remains chromatin bound in S-phase (Alexandrow and Hamlin, 2004). Therefore, we propose that phosphorylation of S54 by cyclinE/CDK2 is a novel stabilizing modification of the Cdc6 protein, which can be modulated by the p53 pathway.

Our data implicates that cyclinE/CDK2 activity might have a positive role in DNA replication licensing by stabilizing Cdc6. This is consistent with in vitro studies, which show that cyclin E cooperates with Cdc6 to make mammalian G1 nuclei competent for replication (Coverley et al., 2002). Furthermore, Cdc6 and cyclin E appear to have a synergistic effect on inducing S-phase entry upon co-transfection in human cells (Cook et al., 2002; Hateboer et al., 1998). In addition, it was shown that overexpression of cyclin E could stabilize Cdc6 protein during megakaryocytic endoreplication (Bermejo et al., 2002). In these cells cyclin E could not stabilize an unphosphorylatable Cdc6 mutant in which all five N-terminal putative CDK2 phosphorylation sites were mutated to alanines. Significantly, it was also shown that cyclin E ablation in mice results in DNA replication defects (Geng et al., 2003; Parisi et al., 2003). Cells that re-enter the cell cycle from quiescence fail to load MCM2-7 and endoreplication in trophoblast giant cells and megakaryocytes is impaired. In view of our results it will be interesting to examine whether these defects are a consequence of reduced Cdc6 stabilization in these cells.

Both CDKs and the APC are key regulators of initiation of DNA replication (Diffley, 2004). Intriguingly, proteolysis of both the licensing factor Cdc6 and geminin, the inhibitor of the licensing factor Cdt1 are regulated by the APC (McGarry and Kirschner, 1998). It is at present unclear how the APC-dependent degradation of an activator and an inhibitor of DNA replication are coordinated to ensure that Cdc6 and Cdt1 are present in the same time frame to allow for efficient pre-RC assembly. Our results shed new light on this. Similar to geminin, Cdc6 is degraded in the end of G2/M and in early G1 phase of the cell cycle (Petersen et al., 2000). We propose that the increased activity of cyclin E/CDK2 during G1 protects Cdc6 from APC^{Cdh1}-dependent destruction, whereas geminin is still degraded. This provides the cells with a time frame in which both Cdt1 and Cdc6 can bind to the origins and recruit the MCM-complex to assemble the pre-RC.
In conclusion, our findings give novel insights in the regulation of Cdc6 in the assembly of pre-RCs. We established that phosphorylation of Cdc6 at S54 by cyclinE/CDK2 stabilizes the protein by protecting it from APC^{Cdh1}-mediated destruction. The rapid and p53-dependent destruction of Cdc6 in response to DNA damage suggests that it guards the genome for mutations by blocking new origin firing.

Methods

Materials and antibodies

Irradiation was performed with a 2 x 415 Ci 137Cs Source. The proteasome inhibitor MG-132 was purchased from Sigma and used at a final concentration of 10 mM. Cycloheximide (Sigma) was used at a final concentration of 25 µg/ml and Roscovitin (Sigma) at 25µM. The siRNA oligos against luciferase and CDK2 were described (Elbashir et al., 2001; Tetsu and McCormick, 1999). The antibodies used in this report were directed against human Cdc6 (180.2), p-Cdc6 (Ser 54), p53 (DO-1), p21 (C-19), Cyclin D1 (M-20), CDK4 (C-22), Cdc20 (H-175) and CDK2 (M-20) from Santa Cruz. Further Kip1/p27 (Transduction Lab.), Cdh1 (Abcam) ORC1 (Abcam) and MCM2 (Abcam) antibodies were used.

Constructs

Cdc6 expression constructs were cloned by PCR in pcDNA3.1 vector (Invitrogen), pGL3 (Promega) or pEGFP-N3 (Clontech). Cdc6 mutants were generated by site directed mutagenesis using PCR and constructs were verified by DNA sequence analysis. We used pSuper (pS) constructs targeting p53, Cdh1 and Cdc20 that were described previously (Brummelkamp et al., 2002b). The other 19 nt target knockdown sequences used were:

$p21^{Cip1}$: GACCATGTGGACCTGTCAC

$p27^{Kip1}$: GGGCAGCTTGCCCGAGTTC

Cdc6#1: ATGTCCAAACCGTAACCTG

Cdc6#2: CCTATGCAACACTCCCCAT

For stable expression siRNAs constructs were cloned in the retroviral 480-puro vector (Voorhoeve and Agami, 2003).

Cell culture and transfection

MCF-7 and BJ cells were grown in DMEM and 10% fetal bovine serum. MCF-7 cells carrying the murine ecotropic receptor were infected with ecotropic retroviral supernatants as described (Brummelkamp et al., 2002a) to generate a polyclonal pool of cells. MCF-7 cells were transfected by electroporation as described (Agami and Bernards, 2000) and about 90% of transfection efficiency was obtained. Cells were electroporated and seeded, washed 16 hours later and harvested 48 hours after washing.

Western blot, cell cycle profile analysis and luciferase assay

For western blots, whole cell extracts were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Milipore). Western blots were developed using enhanced chemiluminescence (Amersham Bioscience, Inc.). Densitometric quantification of western blots was done by using Tina 2.09 software. For bromodeoxyuridine (BrdU) labeling, cells were incubated with 2.5 mM BrdU 40 min prior to harvesting. Cells were fixed ON at 4°C in ethanol. Fixed cells were treated 30 min at 37°C with RNase A (0.5 mg/ml), washed with PBS, incubated 20 min in 5M HCl/0.5% Triton solution and neutralized with 0.1 M Na2B4O7. Cells were sequentially stained with anti-BrdU antibodies (DAKO) and FITC-conjugated goat-anti-mouse antibodies (Molecular Probes). Prior to the flow cytometric analysis, cells were resuspended in 50µg/ml propidium iodide in PBS. In each assay 10,000 single cells were analyzed using the Cell Quest program (Becton Dickinson). Firefly luciferase and Renilla luciferase (internal control) activities were measured by employing the Dual Luciferase Reagent Assay Kit (Promega).

Northern blot analysis

RNA was isolated from cells using Trizol (Invitrogen) and northern blot analysis was performed exactly as described (Brummelkamp et al., 2002b) and membranes were hybridized with a probe made from the Cdc6 cDNA.

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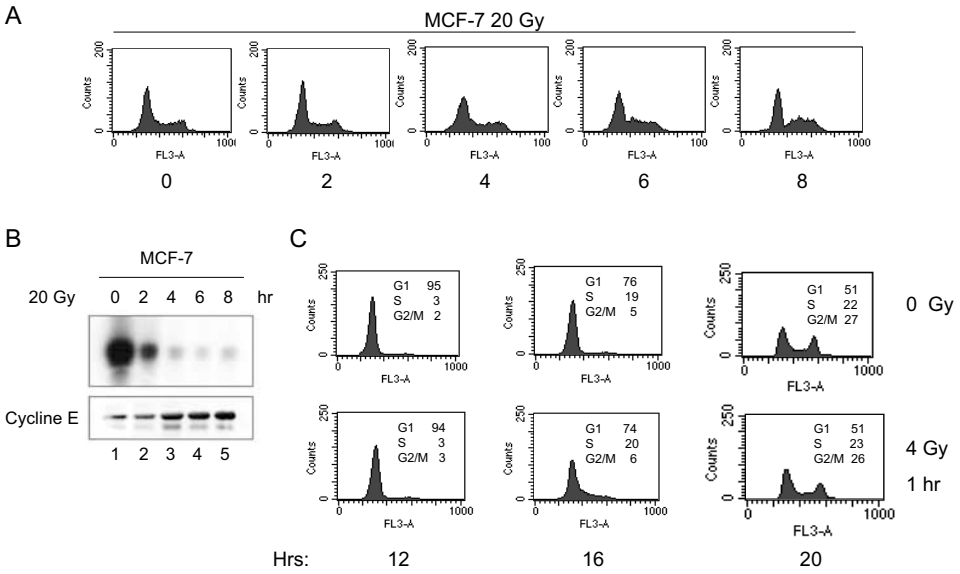
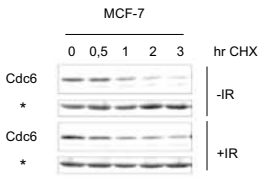
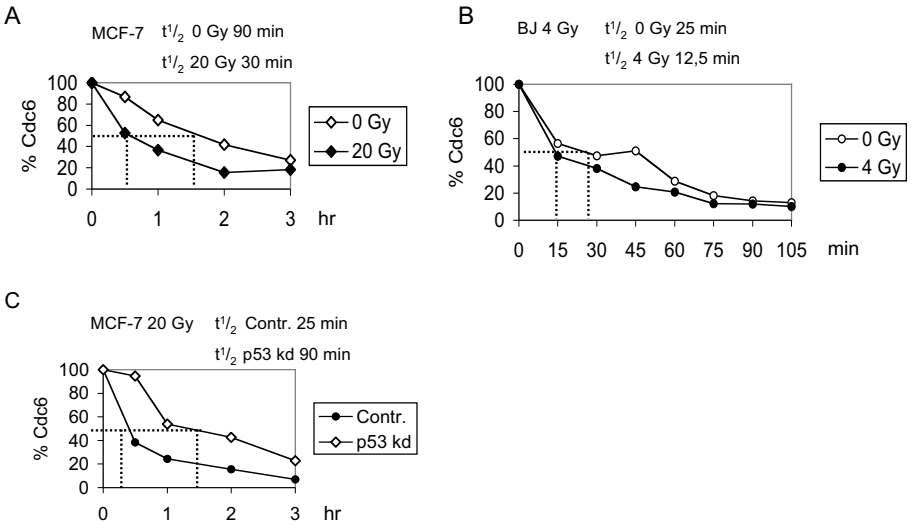


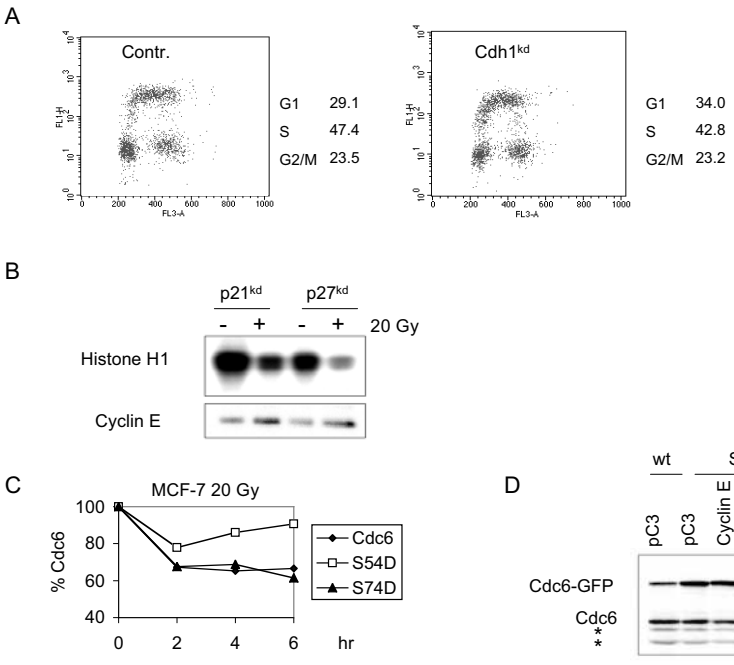
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4