



The DR6 protein from human herpesvirus-6B induces p53-independent cell cycle arrest in G₂/M

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ABSTRACT

HHV-6B infection inhibits cell proliferation in G₂/M, but no protein has so far been recognized to exert this function. Here we identify the protein product of direct repeat 6, DR6, as an inhibitor of G₂/M cell-cycle progression. Transfection of DR6 reduced the total number of cells compared with mock-transfected cells. Lentiviral transduction of DR6 inhibited host cell DNA synthesis in a p53-independent manner, and this inhibition was DR6 dose-dependent. A deletion of 66 amino acids from the N-terminal part of DR6 prevented efficient nuclear translocation and the ability to inhibit DNA synthesis. DR6-induced accumulation of cells in G₂/M was accompanied by an enhanced expression of cyclin B1 that accumulated predominantly in the cytoplasm. Pull-down of cyclin B1 brought down pCdk1 with the inactivating phosphorylation at Tyr15. Together, DR6 delays cell cycle with an accumulation of cells in G₂/M and thus might be involved in HHV-6B-induced cell-cycle arrest.

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1. Introduction

Inhibition of host cell cycle in G₂/M is a well-known strategy used by both RNA and DNA viruses. Although the consequences for the viral infection are not always clear, inhibition of the G₂/M checkpoint has been suggested to increase the viral genome copy number of some DNA viruses (Davy and Doorbar, 2007). The strategies used to accomplish cell cycle arrest are diverse among even closely related viruses, suggesting that the functional phenotype has been important during viral evolution. Despite the different strategies, several of them converge on inhibiting the cyclin B–Cdk1 complex (De Bolle et al., 2004; Li et al., 2011). Parvovirus genomes activate ATM or ATR, or both, resulting in phosphorylation of the Chk1 and Chk2 kinases (Luo et al., 2011). This maintains the phosphatase Cdc25 in a phosphorylated and inactivated state, which results in an inactive cyclin B–Cdk1 complex. An inactive cyclin B–Cdk1 complex is also a hallmark of G₂/M arrest observed during infection with the polyomavirus SV40 (Yuan et al., 2003), or by the presence of viral proteins like Vpr or Vif from HIV-1 (Kino et al., 2005; Sakai et al., 2011), E4 from

HPV-16 (Davy et al., 2005), NS1 from parvovirus (Wan et al., 2010), and σ 1s from reovirus (Poggioli et al., 2000). Despite the G₂/M-arrested phenotype being well documented, the specific mechanisms remain to be defined.

From the perspective of viral evasion, an additional advantage of a G₂/M arrest is the prevention of cell expansion, thereby limiting the adaptive immune response towards the virus. Indeed, ex vivo HIV-1-infected T cells are arrested in G₂, which may limit the host response towards HIV-1 (Zimmerman et al., 2006).

Infections by human herpesvirus (HHV)-6A and -6B are characterized by relatively modest cellular immune responses towards the viruses (Gerdemann et al., 2013) and both of these viruses inhibit cell proliferation. HHV-6A infection blocks the cell cycle in G₂/M in SupT1, HSB2, human embryonic fibroblasts and cord blood mononuclear cells (De Bolle et al., 2004; Li et al., 2011, 2012; Mlechkovich and Frenkel, 2007), whereas HHV-6B induces G₂/M arrest in SupT1 and MOLT3 cells (Mlechkovich and Frenkel, 2007; Oster et al., 2005). A G₁/S-phase arrest has also been observed during HHV-6B infection in MOLT3 and HCT116 wt cells (Oster et al., 2005; Oster et al., 2006). HHV-6A has been shown to alter the E2F1/Rb pathway by inducing Rb degradation. Besides arresting the cell cycle this also results in elevated levels of the transcription factor E2F1, which is active in transcribing genes important for viral DNA synthesis, such as U27 and U79 (Mlechkovich and Frenkel, 2007; Sharon et al., 2014).

Inhibition of cell proliferation can occur through both p53-dependent and -independent pathways (Nascimento et al., 2012).

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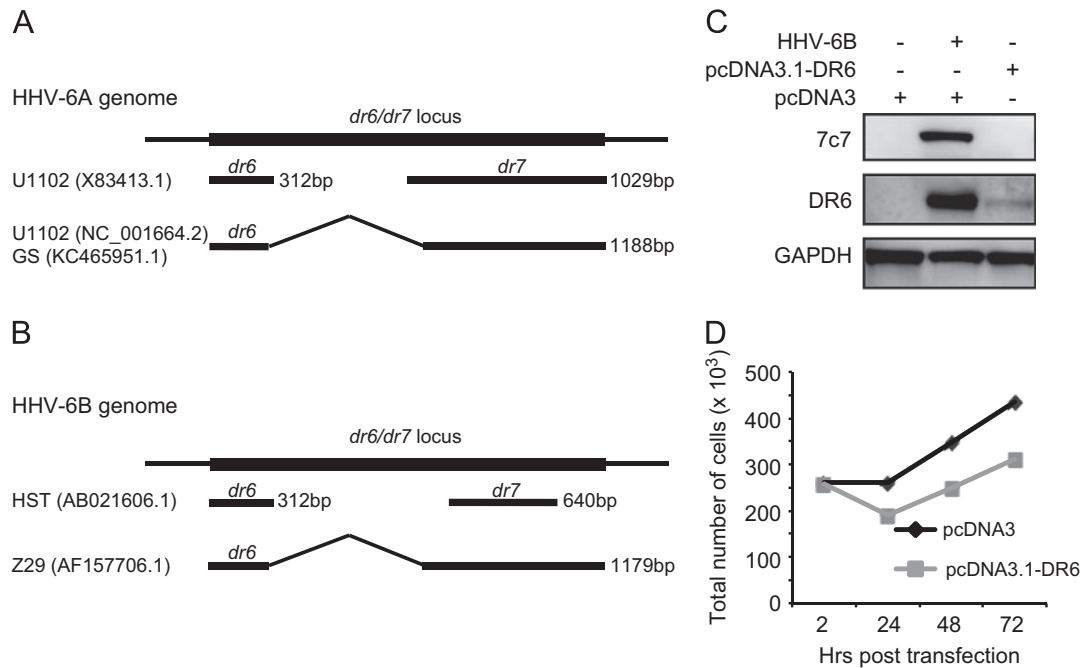


Fig. 1. DR6 overexpression inhibits cell proliferation. Comparison of the *dr6/dr7* locus from (A) HHV-6A strains U1102 (X83413.1), U1102 (NC_001664.2), and GS (KC465951.1), and (B) HHV-6B strains HST (AB021506.1), and Z29 (AF157706.1). (C) Immunoblotting from whole cell lysates from HCT116 wt cells with either pcDNA3 transfection alone, pcDNA3 with HHV-6B infection or pcDNA3.1-DR6 transfection. Immunoblot was probed with anti-7c7 antibody, marker of HHV-6; anti-GAPDH antibody, loading control; and anti-DR6 antibody. A representative of three independent experiments is shown. (D) Total cell count of HCT116 wt cell transfected with either pcDNA3 or pcDNA3.1-DR6 at the time points 2, 24, 48, and 72 h post transfection. A representative of three independent experiments is shown.

During HHV-6B infection, the majority of the cellular p53 is inactivated and stabilized in the cytoplasm of the cell, most likely due to a decrease in p53 degradation (Oster et al., 2005; Takemoto et al., 2004). Although a minor fraction of p53 may be retained or redistributed to the nucleus, p21 mRNA is not induced during HHV-6B infection (Oster et al., 2008). In contrast, in p53^{-/-} cells, HHV-6B was still able to inhibit cell-cycle progression, suggesting that a p53-independent pathway is sufficient to prevent G₂/M transition.

Specific viral proteins mediating cell cycle arrest observed during HHV-6B infection have not been identified. The HHV-6A and -6B genomes are flanked by direct repeats (DR). Alignments of the *dr6/dr7* gene locus from U1102, GS, HST, and Z29 indicate various alternative splice and translation variants of *dr6* and *dr7* (Fig. 1A and B). The HHV-6A (U1102) direct repeat (DR)7 protein, encoded by *dr7* (1029 bp), originally identified as ORF-1, has been shown to bind and inhibit p53 function and transform NIH-3T3 cells during transient transfection (Kashanchi et al., 1997; Thompson et al., 1994). These DR7-positive NIH-3T3 cells were oncogenic when injected into nude mice (Kashanchi et al., 1997). Moreover, *dr7* (640 bp) from HST has been cloned and its protein product was detected in Reed-Sternberg cells of patients with Hodgkin's lymphoma and was also shown to bind p53 (Lacroix et al., 2010).

Here we report that the protein encoded by *dr6* from HHV-6B has the ability to inhibit G₂/M transition. We demonstrate that this function is p53-independent, but dependent on the N-terminal part of the protein, which is also required for nuclear localization. This identifies the first G₂/M checkpoint-regulating protein in HHV-6B.

2. Results

2.1. DR6 inhibits cell proliferation

The *dr6* (1179 bp) product from HHV-6B (Fig. 1B) was cloned and is referred to as DR6. Transient transfection of HCT116 wt cells with DR6 gave rise to a 44 kDa band on immunoblotting when probed

with anti-DR6 antibodies, similar to what is detected during HHV-6B infection (Fig. 1C). When DR6- and mock-transfected cell cultures were counted over the following 72 h, a decline in the number of DR6-transfected cells was observed within the first 24 h (Fig. 1D). These data demonstrated that overexpression of DR6 inhibited cell proliferation and suggested that DR6 might be a viral protein arresting the cell-cycle progression.

2.2. DR6 suppresses DNA synthesis in HCT116 wt and HCT116 p53^{-/-} cells

The observed decrease in the number of cells transiently transfected with DR6 could be caused by a decrease in cell proliferation or by induction of cell death. To increase DR6 expression efficiency, HCT116 wt and p53^{-/-} cells were transduced with lentiviral particles containing either the DR6 open reading frame or the firefly luciferase (FLUC) open reading frame as a control. [³H]-thymidine pulse for 4 h at 4, 24, 48, and 72 h post transduction (hpt) showed reduced thymidine incorporation in DR6-transduced HCT116 wt cells (Fig. 2A). Seventy-two hpt DR6-transduced cells showed approximately one-third [³H]-thymidine incorporation compared with mock- or FLUC-transduced cells (Fig. 2A).

To examine whether the reduction in [³H]-thymidine incorporation was dependent upon p53, p53^{-/-} HCT116 cells were examined. A similar pattern was observed in the absence of p53, indicating that DR6 suppression of thymidine incorporation was independent of p53 (Fig. 2B). Decrease in the number of cells was not caused by cell death as measured by ATP assay and PARP cleavage (data not shown). DR6 and FLUC protein expression in HCT116 wt and p53^{-/-} cells was verified by immunoblotting at 48 hpt (Fig. 2C and D).

2.3. DR6 suppresses DNA synthesis in a dose-response-dependent manner

To be able to control the expression of DR6, the DR6 full-length open reading frame was stably integrated in the Flp InTM T-RexTM 293

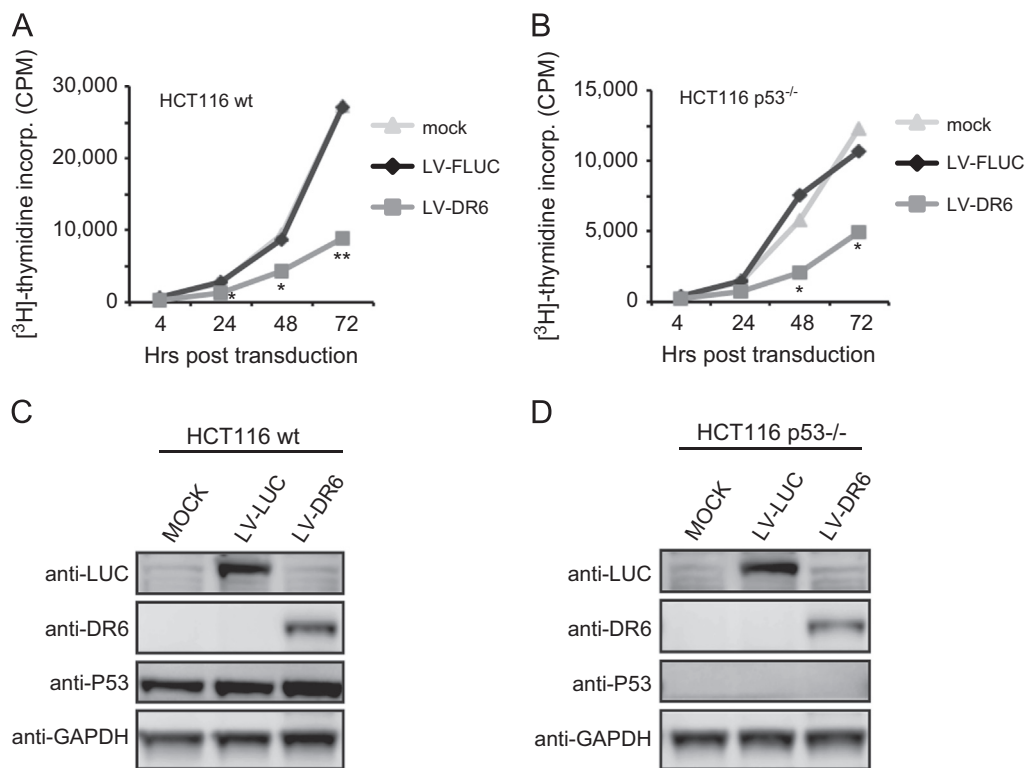


Fig. 2. DR6 inhibition of [³H]-thymidine is independent of p53. [³H]-thymidine incorporation was measured as counts per minute (CPM) from (A) HCT116 wt or (B) HCT116 p53^{-/-} cells that have been either mock-transduced or transduced with lentiviral particles containing Firefly Luciferase (LV-FLUC) or DR6 (LV-DR6). A representative of four independent experiments is shown. (C) and (D) Immunoblotting visualizing DR6, FLUC, and p53 protein expression at 48 h post transduction. GAPDH was used as loading control. A representative of two independent experiments is shown. * indicates $p < 0.05$, ** indicates $p < 0.005$ by Student's t -test.

tetracycline-inducible expression cell system. GFP was used as a control for protein expression. A four-fold tetracycline titration from 500 to 2 ng/ml showed an expected decrease in protein expression on immunoblotting 48 h post tetracycline induction (hpti). DR6 and GFP proteins were induced at tetracycline levels of 500–31 ng/ml; however, between 31 and 7.8 ng/ml the protein expression shifts to a minimal level, suggesting that the tetracycline repressor system is slightly leaky (Fig. 3A). DR6 suppression of [³H]-thymidine incorporation correlated with tetracycline-induced expression of DR6 in a dose-dependent manner (Fig. 3B). Compared with GFP at 48 hpti, the [³H]-thymidine incorporation was reduced by approximately one-third for cells treated with 500 or 125 ng/ml of tetracycline, by one-fourth for cells treated with 31 ng/ml, and not reduced for cells treated with only 7.8 or 2 ng/ml (Fig. 3B). This indicated that the barely detectable DR6 levels at low tetracycline concentrations were not functional in the [³H]-thymidine incorporation assay (Fig. 3A).

Based on the titration of tetracycline-induced expression, further experiments with the Flp InTM T-RexTM 293 system used a tetracycline concentration of 100 ng/ml to ensure a level of DR6 expression that was functional. [³H]-thymidine incorporation assays at 6, 24, 48, and 72 hpti on Flp InTM T-RexTM 293-host, -GFP and -DR6 cells showed the same pattern in host and GFP cells, independent of tetracycline addition. As expected, DR6 cells showed a significantly lowered [³H]-thymidine incorporation at 48 h and 72 h upon tetracycline treatment (Fig. 3C). These data further corroborated that the presence of DR6 was sufficient to prevent cell-cycle progression as measured by [³H]-thymidine incorporation.

2.4. The N-terminal domain of DR6 is necessary for suppression of DNA synthesis

To further study functional domains of DR6, we cloned a Δ N-truncated version of DR6, termed DR6 Δ N, with a deletion of the

first 198 bp except for ATG. DR6 Δ N has a 984 bp open reading frame and an expected protein size of 37 kDa. Protein expression, stability and molecular mass were confirmed by immunoblotting at 48 hpti with anti-DR6 antibody that recognizes the C-terminal part of the protein (Fig. 4A).

A stably integrated DR6 Δ N in the Flp InTM T-RexTM 293 tetracycline-inducible expression cell system was generated. From cell lysates at 4, 24, 48, and 72 hpti, cytoplasmic (CY) and nuclear extracts (NE) of DR6- and DR6 Δ N-tetracycline induced cells were separated, immunoblotted and probed with anti-DR6 antibody. Whereas DR6 was evenly distributed between the cytoplasm and the nucleus, with a slight majority in the nucleus at 72 h, the vast majority of the DR6 Δ N protein localized to the cytoplasm with no tendency towards nuclear translocation (Fig. 4B). When examined by the [³H]-thymidine incorporation assay, DR6 Δ N protein was unable to reduce [³H]-thymidine incorporation (Fig. 4C). These observations strongly suggested that the N-terminal part of the protein was necessary for efficient nuclear localization and suppression of cellular DNA synthesis.

2.5. DR6 expression delays the cell cycle progression in G₂/M

To further address the question of which part of the cell cycle was arrested by DR6, cell populations were separated in G₀/G₁-, S- and G₂/M-phases using Click-iT[®] EdU Alexa Fluor[®] 488 Flow Cytometry Assay Kit, which detects newly synthesized DNA, and FxCycle[™] Far Red stain, which stains the amount of DNA. A schematic representation of the separations in the cell cycle obtained by this method is shown in Fig. 5A, and the percentages of Flp InTM T-RexTM 293-host and -DR6 cell populations in each cell cycle phase were determined at 24 and 48 hpti (Fig. 5B).

Cell cycle phases were plotted as phase indices with and without induction of DR6, and DR6 Δ N expression (Fig. 5C).

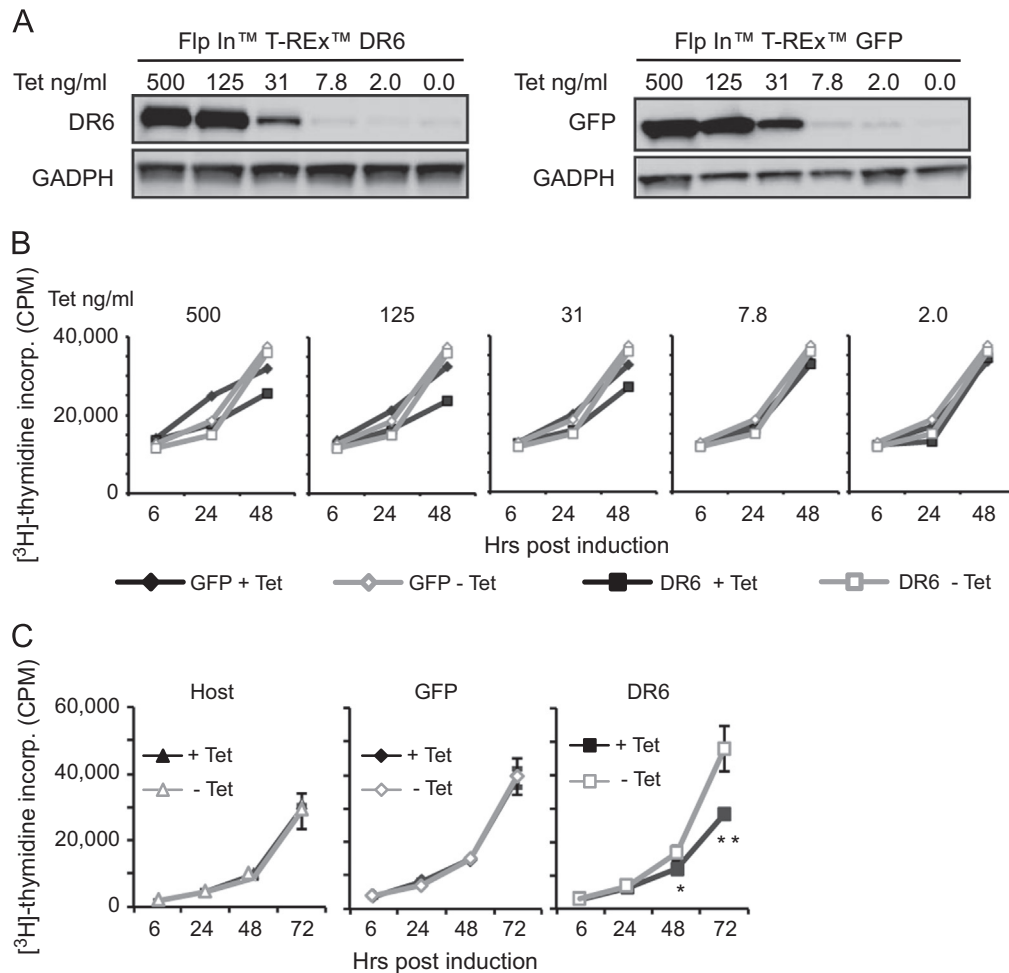


Fig. 3. DR6 inhibits [³H]-thymidine incorporation in a dose-response dependent manner. (A) Immunoblotting showing tetracycline titration on Flp In™ T-Rex™-293-GFP or -DR6 tet-inducible cell lines. DR6 or GFP proteins were visualized 48 h post induction. GAPDH was used as loading control. A representative of two independent experiments is shown. (B) [³H]-thymidine incorporation was measured from Flp In™ T-Rex™-293-GFP or -DR6 cells at 6, 24, or 48 h post tetracycline-induced expression. Cells were either mock-treated with 0 ng/ml tetracycline, or treated with a four-fold titration of tetracycline ranging from 500 to 2 ng/ml. (C) [³H]-thymidine incorporation of Flp In™ T-Rex™ 293-host, -GFP or -DR6 uninduced or induced by 100 ng/ml tetracycline. A representative of three independent experiments is shown. * indicates $p < 0.05$, ** indicates $p < 0.005$ by Student's *t*-test.

An increase in the percentage of cells in G₂/M after 24 hpti was detected in cells induced to express DR6. Host and DR6 ΔN cells showed the same phase index pattern independent of tetracycline treatment. This suggested that the effect observed for DR6 only occurred when the protein was able to enter the nucleus. In the time after accumulation of cells in the G₂/M-phase, cells slowly moved into the G₀/G₁-phase, which was seen as a slight decline in the G₂/M-phase index at 24–48 h and corresponded to a slight increase in the G₀/G₁-phase index. Together, these data demonstrated that DR6 expression caused a G₂/M delay that subsequently gave rise to a reduced DNA synthesis.

2.6. DR6 expression enhances the formation of a cyclin B1–pCdk1 (Tyr15) complex

One of the essential events for G₂/M transition is dephosphorylation of pCdk1(Tyr15), which is in complex with cyclin B1. During G₂ arrest pCdk1(Tyr15) is bound to cyclin B1 and retained in the cytoplasm to prevent initiation of mitosis. Therefore, we examined cyclin B1 localization and cyclin B1–pCdk1(Tyr15) complex status in DR6-expressing, G₂/M-phase-delayed cells. Flp In™ T-Rex™ 293-GFP and -DR6 cells were fixed to coverslips and treated with tetracycline at 4, 24, 48, and 72 h to induce G₂/M-phase delay. At 48 and 72 hpti cyclin B1 was more abundant in the

cytoplasm in DR6-expressing cells compared with untreated and GFP-expressing cells (Fig. 6A) and this correlated with an increase in cyclin B1 on immunoblotting (Fig. 6B). This suggested that the G₂/M-phase delay might be associated with an accumulation of cyclin B1 in the cytoplasm. Furthermore, an increase in cyclin B1–pCdk1(Tyr15) complex formation was seen after 48 hpti (Figs. 6B and C). Pull-down of cyclin B1 showed increased cyclin B1 levels in DR6-expressing cells. When cyclin B1 pull-down was probed with anti-pCdk1(Tyr15) antibody, an increased amount of pCdk1(Tyr15) protein was detected, indicative of increased association between the two proteins. Examination of lysates after pull-down experiments indicated that a fraction of both cyclin B1 and pCdk1(Tyr15) was removed from the lysates compared with the lysates prior to the pull-down experiments (data not shown). This demonstrated that DR6 enhanced the accumulation of cyclin B1–pCdk1(Tyr15), which represents an inactive form of cyclin B1.

3. Discussion

During DR6 overexpression a growth-promoting function, as seen for DR7 from HHV-6A, might be expected. On the contrary, a total cell count indicated a reduced cell proliferation of DR6-transfected cells when compared with mock-transfected cells

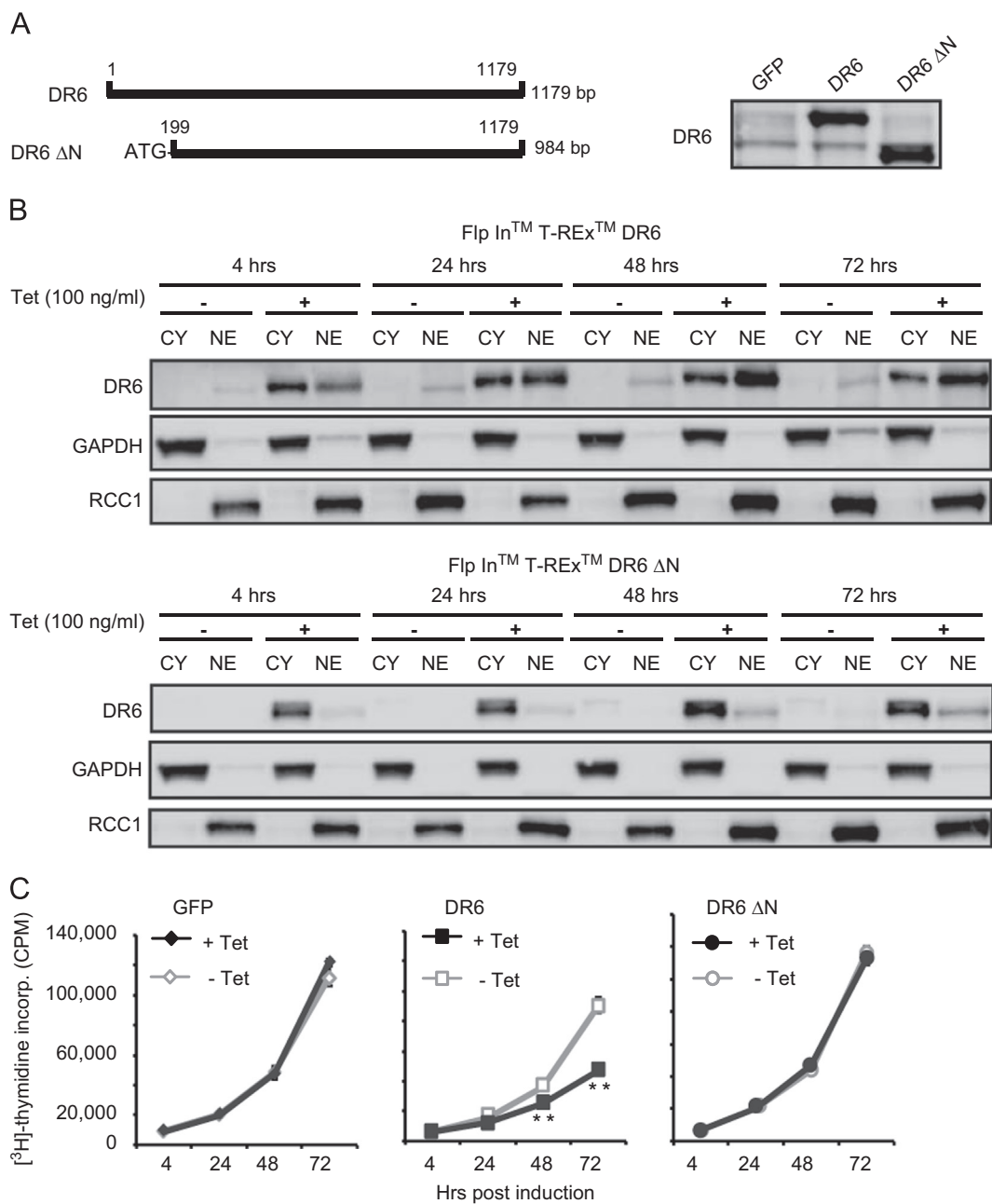


Fig. 4. DR6 is dependent on an N-terminal sequence for inhibition of [³H]-thymidine incorporation. (A) A schematic representation of the cloned DR6 (1179 bp) and DR6ΔN (984 bp) DNA. Stable protein products were verified by immunoblotting from both Flp InTM T-RExTM 293-DR6 and -DR6 ΔN after treatment with 100 ng/ml tetracycline. The membrane was probed with anti-DR6 antibody. (B) Immunoblotting of tetracyclin-induced (100 ng/ml) or uninduced Flp InTM T-RExTM-293-DR6 and -DR6 ΔN cell lysates separated into cytoplasmic (CY) and nuclear fractions (NE). Immunoblots were probed with anti-DR6 antibody and anti-RCC1 antibody as nuclear separation control, and anti-GADPH antibody as cytoplasmic separation control. (C) [³H]-thymidine incorporation of Flp InTM T-RExTM-293-GFP, -DR6 and -DR6 ΔN with or without tetracycline induction (100 ng/ml). A representative of three independent experiments is shown. Statistics: ** indicates *p* < 0.005 by Student's *t*-test.

(Fig. 1B). Moreover, we were unable to demonstrate an association between DR6 and p53 by either pull-down experiments or confocal microscopy co-localization analyses of cells overexpressing DR6 and p53 during HHV-6B infection ((Schleimann et al., 2009) and unpublished observations). In agreement with this, we observed that DR6 suppressed DNA synthesis in a p53-independent manner (Fig. 2). Alignments of the *dr6/dr7* gene locus, from U1102, GS, HST and Z29, indicate a variety of alternative splice or translation variants, which may give rise to different protein products. This could be a possible explanation for the discrepancy between our data on DR6 and the previously published data on DR7/ORF-1.

Generating an inducible DR6-expressing cell line allowed us to demonstrate a dose-response effect of DR6 expression. This effect appears to be specific for DR6, since it is not seen during induction of an assumed irrelevant protein such as GFP. Nevertheless, whether the DR6-induced cell-cycle arrest is a direct or an indirect effect remains to be determined. DR7 has been characterized as a transcription factor of HIV-LTR, and it is possible that the observed effect is caused by transcription of one or more genes involved in cell-cycle control (Kashanchi et al., 1994; Thompson et al., 1994). Although DR6 clearly delays cell-cycle progression, we do not know whether DR6 is critical for inhibiting cell proliferation during HHV-6B infection. To address this we attempted to knock

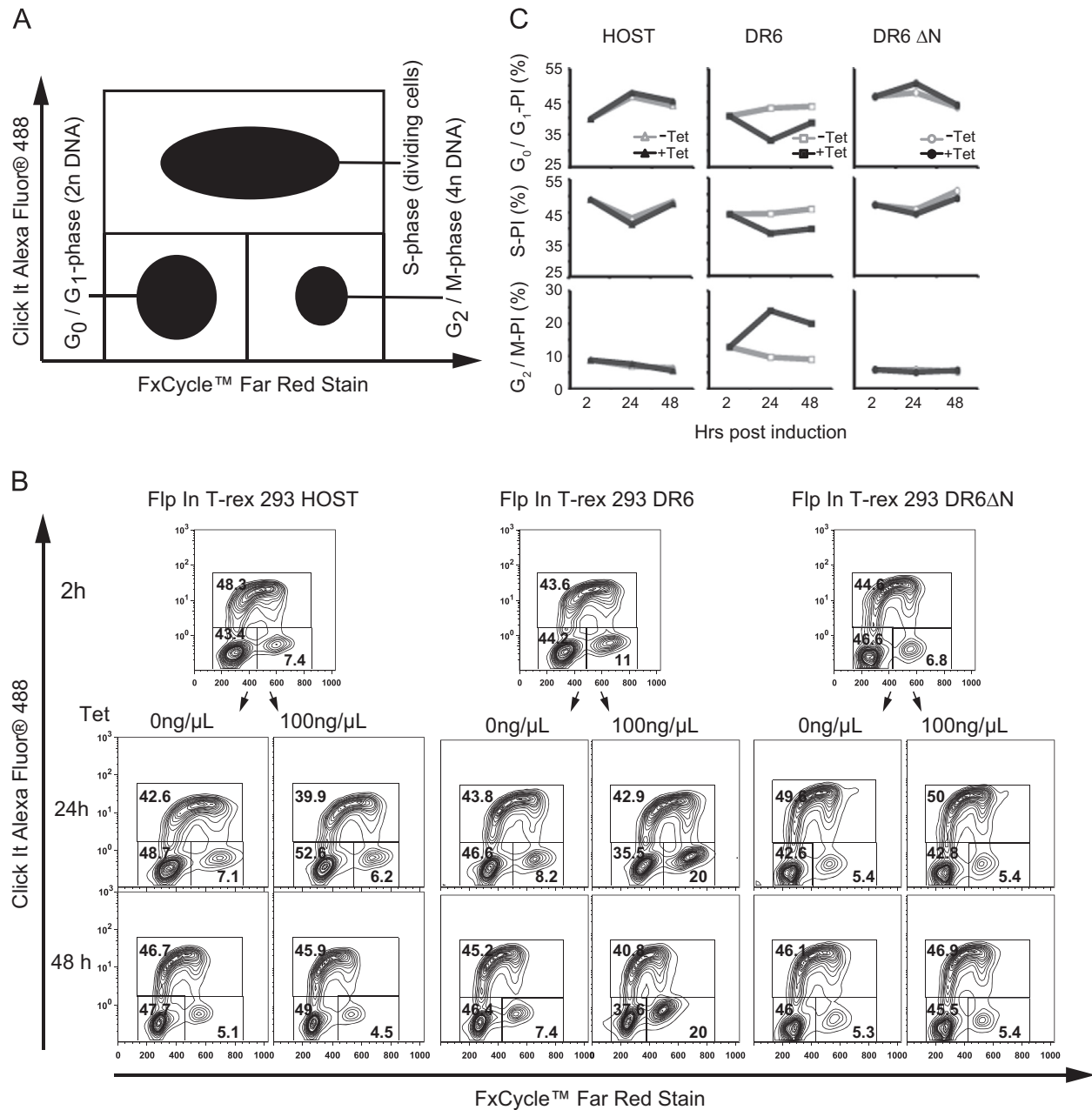


Fig. 5. DR6 expression induces accumulation of cells in G₂/M. (A) A schematic representation of cell cycle-phase separation into G₀/G₁- S- and G₂/M-phases by flow cytometry. (B) Flow cytometry of untreated or 100 ng/ml tetracycline-treated Flp In™ T-REx™-293-host, -DR6, and -DR6 ΔN cells. Time points of 2, 24, and 48 h post induction are shown. Percentages of cells in each cell cycle phase are given. (C) Cell cycle phase-indices of untreated or 100 ng/ml tetracycline-treated Flp In™ T-REx™-293-host, -DR6, and -DR6 ΔN cells. Time points of 2, 24, and 48 h post induction are shown. A representative of two independent experiments is shown.

down DR6 using an ON-TARGETplus™ SMARTpool DR6 siRNA (Dharmacon, Thermo Fisher Scientific, Lafayette, CO.). Unfortunately, this did not significantly reduce the level of DR6. Next, we generated lentiviral particles expressing seven different DR6 shRNAs. However, these constructs were also insufficient in knocking down DR6. Thus, we were unable to demonstrate the significance of DR6 during HHV-6B infection. Nevertheless, the localization of DR6 during overexpression is similar to its localization during infection, suggesting that our data reflects the function of DR6 during infection.

DR6 localizes in replication compartments during HHV-6B infection (Schleimann et al., 2009), and inhibits cell proliferation when overexpressed in several cell lines. DR6 delays cells in the G₂/M-phase with an accumulation of cyclin B1 in the cytoplasm and causes an increase in cyclinB1–pCdk1(Tyr15) complex formation

(Figs. 5 and 6). These data are consistent with data showing G₂/M-phase arrest during HHV-6A and -6B infection (De Bolle et al., 2004; Li et al., 2011; Oster et al., 2005). Li et al. have recently shown that viral replication is enhanced when a G₂/M arrest is induced (Li et al., 2013) and this may occur by switching E2F1 towards transcription of viral genes (Sharon et al., 2014). Thus, a possible role of DR6 during HHV-6B infection might be that DR6 functions as a chaperone facilitating the assembly of viral replication units or facilitating cell proliferation arrest in order to enhance viral replication.

It appears that HHV-6A is not dependent on full-length DR6, since deletion of the first exon of DR6 does not prevent virus replication in culture (Borenstein et al., 2010). The first exon of DR6 comprises 304 bp and we find that a truncation of approximately two-thirds of this exon (198 bp) prevents translocation to

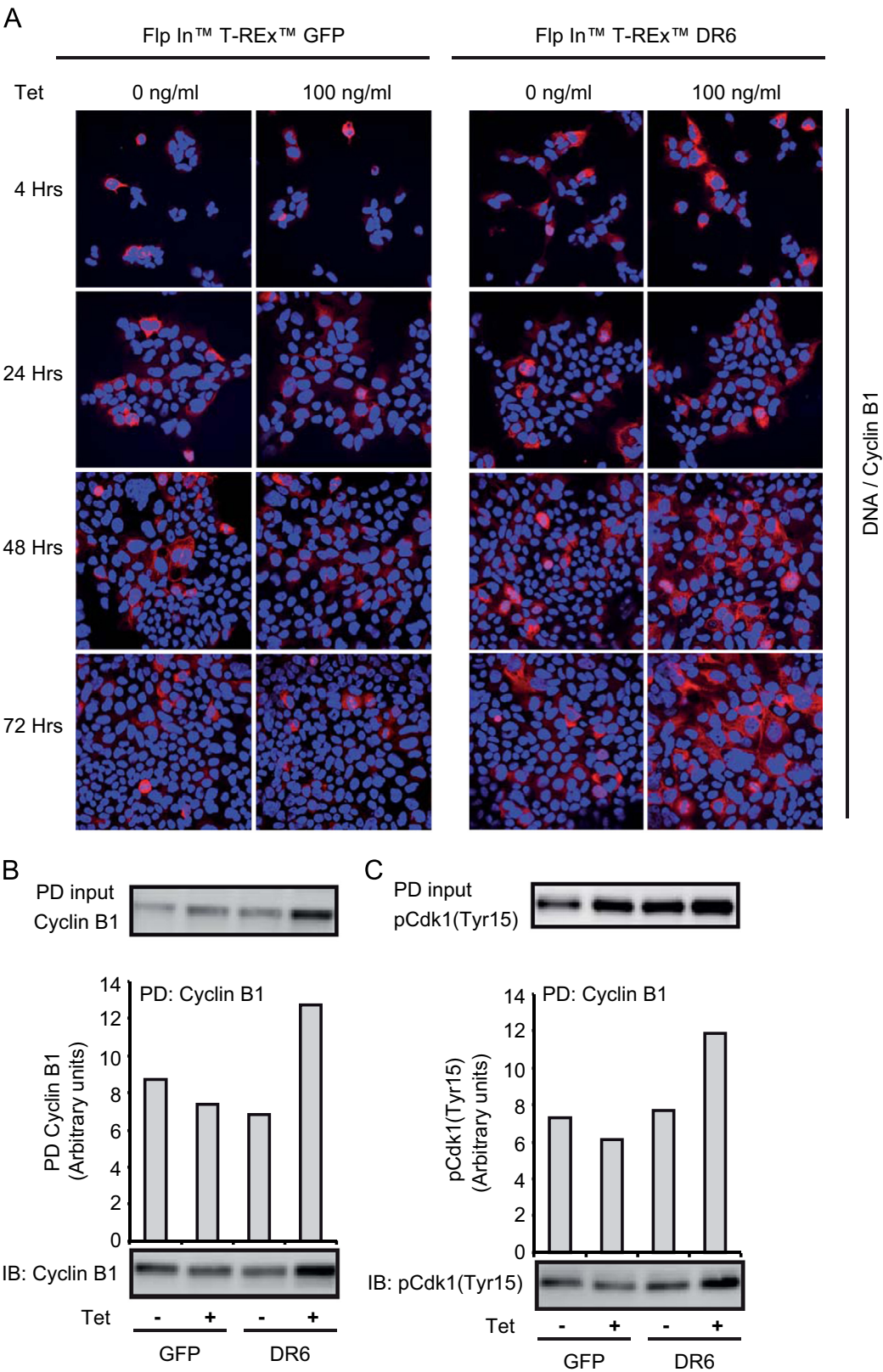


Fig. 6. DR6 expression induces accumulation of cyclin B1. (A) Confocal laser scanning microscopy of the localization of cyclin B1. Flp InTM T-RExTM-293-GFP and -DR6 cells were treated with and without tetracycline at 100 ng/ml. Cells were examined at 4, 24, 48, and 72 h post induction. DNA was stained with DAPI. A representative of two experiments is shown. (B) and (C) Immunoblotting and band intensity quantification of pull-down (PD). Pull-down was performed with anti-cyclin B1 antibody at 48 h post tetracycline-induced expression. Immunoblots (IB) of whole-cell lysates (PD input) and PD were probed using antibodies against anti-cyclin B1 (B) or anti-pCdk1(Tyr15) (C). A representative of two experiments is shown.

the nucleus (Fig. 4B). We cannot exclude the possibility that DR6 might exert a function in the cytoplasm depending on the N-terminal sequence; however, Fig. 4A indicates that the best inhibition is

achieved at 72 h where most of the DR6 have translocated to the nucleus, consistent with the nuclear function of DR6. Although the N-terminal part of DR6 might be necessary for G₂/M delay in HHV-6B

infections, it is not necessarily in conflict with the observation that HHV-6A can still replicate.

DR6 does not encode a known nuclear localization motif. Thus, the N-terminal part of the protein may contain a novel unknown nuclear localization signal or may be necessary for protein–protein interaction in the case of DR6 being transported to the nucleus by an unknown cellular protein. Although a deletion may also alter the conformation of a protein, the stability of the protein was confirmed by its presence in immunoblotting. Moreover, recognition of native DR6 Δ N can be achieved by an antibody raised against a DR6 sequence. Anyhow, only crystallization data will provide a definitive answer to whether DR6 Δ N may fold differently.

In conclusion, we show that DR6 from HHV-6B (PL-1) delayed cells in the G₂/M-phase within the first 24 h by a mechanism independent of cellular p53 expression. The G₂/M-phase delay was accompanied by an accumulation of cyclin B1 and an increased cyclin B1–pCdk1(Tyr15) complex formation. We speculate that this delay in cell cycle is advantageous for viral genome production and hence formation of new viral particles.

4. Materials and methods

4.1. Cell lines and virus stock

The human epithelial colon carcinoma cell line HCT116 wt and p53^{−/−} (a gift from B. Vogelstein and K.W. Kinzler, Johns Hopkins University School of Medicine, Baltimore, MD, USA) was grown in McCoy's medium (Gibco, CA, USA), supplemented with 10% inactivated fetal calf serum (iFCS) (Sigma-Aldrich, Saint Louis, USA), glutamine (0.2 g/l), HEPES (10 mM), penicillin (0.2 g/l), and streptomycin (0.2 g/l) (all from the Substrate Department, Bartholin Building, Aarhus University, Denmark).

Flp InTM T-REXTM 293 host cell line containing a stably integrated FRT site (pFRT/lacZeo) and tetracycline repressor (pcDNATM6/TR) was grown in Dulbecco's Modified Eagles Medium (DMEM), High Glucose, GlutamaxTM, HEPES (Gibco) supplemented with 15% iFCS (Sigma-Aldrich), penicillin (0.2 g/l), and streptomycin (0.2 g/l) (from the Substrate Department, Aarhus University). To sustain the tetracycline repressor site the cells are grown in 10 μ g/ml Blastidin (Life Technologies, Carlsbad, CA, USA) and to sustain the Flp In site the cells are grown in 100 μ g/ml Zeocin (Life Technologies).

HHV-6B, strain PL1 (generated by Dr. P. Lusso, Milan, Italy), was propagated as previously described (Schleimann et al., 2009). Viral titer was determined by the Reed-Muench assay to 3200 TCID₅₀.

4.2. Cloning of DR6 and DR6 Δ N

The Direct Repeat 6 full-length gene (DR6) was cloned into the pcDNATM3.1D/V5-His-TOPO (Life Technologies) vector using forward primer: 5'-caccatgacacgcgacacgcag-3' and reverse primer: 5'-ctaaccaggtacgctctttg-3'. This vector is referred to as pcDNA3.1-DR6. Furthermore, dr6 was cloned into a third-generation lentiviral packaging vector (pCCL-WPS-PGK-GFP-WHV) and pcDNATM5/FRT/TO vector using forward primer: 5'-atgacaacgcgacacgcag-3' and reverse primer: 5'-ctaaccaggtacgctctttg-3'. These vectors are referred to as pCCL-DR6 and pcDNATM5/FRT/TO-DR6. Insertion into the vectors was performed using restriction enzymes XbaI, Apal and Quick LigationTM kit (New England Biolabs (NEB), Ipswich, MA) for pCCL-DR6 and HindIII and Apal and Quick LigationTM kit (NEB) for pcDNATM5/FRT/TO-DR6. In addition, a DR6-truncated version (DR6 Δ N) was generated by PCR using forward primer: 5'-atggaaactcatcggtacgta-3' and reverse primer: 5'-ctaaccaggtacgctctttg-3'. The dr6 Δ N was cloned into the pcDNATM5/FRT/TO vector using HindIII and Apal and Quick LigationTM kit (NEB) and referred to as

pcDNATM5/FRT/TO-DR6 Δ N. All PCR reactions were carried out under the same conditions with an initial heat shock starting at 95 °C for 5 min, after which 35 amplification cycles were carried out with a denaturation step at 94 °C for 30 s, a primer annealing step at 55 °C for 30 s, and a final elongation step at 68 °C for 60 s.

4.3. Production of lentiviral particles

For the production of lentiviral particles, 293T cells were seeded at a density of 5×10^4 cells/cm² in 10-cm dishes 1 day before transfection. Cells were transfected using 250 mM CaCl₂ with 3.75 μ g pMD2.G, 3 μ g pRSV-Rev, 13 μ g pMDGP-Lg/RRE, and 13 μ g pCCL-DR6 or pCCL-FLUC. At 48 and 72 h after transfection the viral supernatants were harvested and passed through 0.45 μ m filters to remove cellular debris (Sarstedt, Nümbrecht, Germany). The resulting lentiviral particles were designated LV-DR6 and LV-FLUC. The lentiviral supernatants were ultracentrifuged for 2 h (4 °C at 25,000 rpm) in a SW28 rotor (Beckman Coulter, CA, USA). Virus pellets were re-suspended overnight in PBS at 4 °C in a volume of 1/300 of the original volume.

4.4. Transduction of lentiviral particles

HCT116 wt and HCT116 p53^{−/−} cells were seeded at 3×10^6 cells per flask in a T25 cell culture flask to obtain maximum transduction efficiency at 80–90% confluence the following day. Cells were mock-, LV-FLUC- or LV-DR6-transduced and 8 μ g/ml polybrene in 5 ml culture medium. 4 h post transduction, an additional 4 ml of medium was provided. 24 h post transduction, the cells were seeded for further analyses.

4.5. Generation of the stably inducible Flp InTM T-REXTM 293-DR6, -DR6 Δ N and -GFP cell lines

The pcDNATM5/FRT/TO-DR6, -DR6 Δ N and -GFP vectors were each mixed with a recombinase containing vector (pOG44) in a 1:9 ratio and then Fugene-transfected into the Flp InTM T-REXTM 293 host cell. The next day the cells were split and the culture medium was supplemented with 100 μ g/ml hygromycin instead of Zeocin to select cells that had integrated the DR6 gene. To induce DR6 protein expression cells were treated with 100 ng/ml tetracycline (Sigma-Aldrich).

4.6. [³H]-Thymidine incorporation assay

For thymidine incorporation assay, cells were seeded at a concentration of 2000 cells/200 μ l per well in a 96-well flat-bottom plate. Cells were incubated at 37 °C in 4, 24, 48, or 72 h including incubation with 1 μ Ci [³H]-thymidine/well (Perkin Elmer, Waltham, MA, USA) for the last 4 h. Post incubation the plates were stored at −20 °C until the cells were harvested and the incorporated [³H]-thymidine was counted on a 1450 Micro- β -scintillations counter (Perkin Elmer).

4.7. Cell lysis and immunoblotting

Cells were lysed for 30 min in 1 \times lysis buffer (Cell Signaling, Technology Inc., Beverly, MA, USA) supplemented with 1 mM phenylmethanesulphonylfluoride (PMSF), 5 mM sodium fluoride (NaF) and Complete Mini Protease Inhibitor (Roche Diagnostics, IN, USA) according to the manufacturer's recommendations. Lysates were centrifuged at 4,200g for 5 min, followed by a 10 min centrifugation at 20,000g to remove cell debris completely. Protein concentrations of the lysates were measured by the Bradford method and 30 μ g protein of each lysate was separated on an XT Criterion 10% gel (Bio-Rad, CA, USA) with 1 \times XT MOPS running

buffer (Bio-Rad) for 1 h at 175 V (constant) and subsequently electro-transferred to a nitrocellulose membrane at 300 mA (constant) for 2 h.

Nuclear and cytoplasmic extracts were prepared from Flp InTM T-RExTM 293-DR6 and -DR6 Δ N treated with or without 100 ng/ml tetracycline to induce protein expression (ProteoExtract Subcellular Proteome Extraction Kit, Calbiochem, Merck, Darmstadt, Germany).

The following antibodies were used for immunoblotting: mouse anti-7c7 antibody (1:600) (Argene Biosoft, Verniole, France); rabbit anti-DR6 antibody (1:1000) (Genscript, Piscataway, NJ, USA) were custom-made to the C-terminal 14 amino acid peptide from DR6, as previously described in Schleimann et al. (2009); rabbit anti-GAPDH antibody (1:2000) (Santa Cruz Biotechnology, CA, USA); rabbit anti-GFP antibody (1:500) (Santa Cruz Biotechnology); goat anti-RCC1 antibody (1:500) (Santa Cruz Biotechnology); rabbit anti-cyclin B1 antibody (1:1000 in 5% BSA in 1 \times TBS/0.1% Tween20) (Cell Signaling); rabbit anti-pCdc2(Tyr15) antibody (1:1000 in 5% BSA in 1 \times TBS/0.1% Tween20) (Cell Signaling); horseradish peroxidase-conjugated polyclonal swine anti-rabbit antibody (P0217); and horseradish peroxidase-conjugated polyclonal rabbit anti-mouse antibody (P0260) (all from Dako, Glostrup, Denmark). All of the peroxidase-conjugated antibodies were used at 1:2000 dilution in 5% skimmed milk or 5% BSA in TBS with 0.1% Tween 20 (Sigma-Aldrich). Membranes were developed with Chemiluminescence femto (Pierce, Thermo Scientific, IL, USA) and Image Reader Las-4000 (Science Imaging Scandinavia AB, Sweden).

4.8. Cell cycle assay

Using Click-iT[®] EdU Alexa Fluor[®] 488 Flow Cytometry Assay Kit (Life Technologies) and FxCycleTM Far Red stain (Life Technologies) cell populations were separated in G₀/G₁-, S- and G₂/M-phases. Flp InTM T-RExTM 293-host, -GFP, or -DR6 cells were seeded in T25 culture flasks with or without 100 ng/ml tetracycline and incubated at 37 °C in 2, 24, 48, or 72 h including 10 μ M EdU during the last 2 h of incubation. EdU (5-ethynyl-2'-deoxyuridine) is a thymidine analog modified with an ethynyl moiety with an alkyne. Cells were fixed, permeabilized, and treated with Alexa Fluor[®] 488 coupled to azide, which reacts with the alkyne. Finally, cells were treated with FxCycleTM, a DNA stain, according to manufacturer's guidelines. Cell cycle analyses were performed on an FC500 flow cytometer (Beckman-Coulter).

4.9. Confocal microscopy

Flp InTM T-RExTM 293-GFP or -DR6 was transferred to poly-L-lysine-coated slides and incubated with or without 100 ng/ml tetracycline for 4, 24, 48, and 72 h followed by fixation in 4% formalin/PBS (pH 7.5). Cells were washed twice in PBS, permeabilized in 0.2% Triton X-100 (Sigma-Aldrich)/PBS and blocked in 5% BSA (Sigma-Aldrich)/PBS. Cyclin B1 was visualized using rabbit anti-cyclin B1 antibody (Cell Signaling) (1:200) and the secondary antibody was a goat anti-rabbit F(ab')₂ antibody conjugated with Alexa Fluor 488 (Life Technologies) (1:400). Nuclear staining was performed with the DNA dye 49, 6-diamidino-2-phenylindole dihydrochloride (DAPI), (Sigma-Aldrich). For imaging, a 488 nm line of a multiline argon laser, and the 633 nm line of the helium-neon laser on a confocal laser scanning microscope (LSM710, Zeiss, Jena, Germany) were used with a 63 \times oil-immersion objective with a numerical aperture of 1.4.

4.10. Pull-down assay

Flp InTM T-RExTM 293-GFP or -DR6 cells were seeded in T75 culture flasks with or without tetracycline at a concentration of

100 ng/ml for 48 h. Cells were washed once with PBS and lysed directly in the incubation flask in 1 ml of a 1% CHAPS lysis buffer supplemented with 1 mM sodium orthovanadate (Na₂VO₄), 1 mM phenylmethanesulphonylfluoride (PMSF), 5 mM sodium fluoride (NaF) and Complete Mini Protease Inhibitor (Roche Diagnostics) according to the manufacturer's recommendations. Lysates were centrifuged at 20,000g for 15 min to remove cell debris. Antibody crosslinking to beads was prepared from 3 μ g rabbit cyclin B1 antibody (Cell Signaling) or 3 μ g control rabbit IgG (Cell lab, Beckman-Coulter) coupled to 30 μ l protein A Dynabeads (Life Technologies). The mixture was rotated for 1 h at 4 °C, washed twice in PBS and antibodies were cross-linked to beads using 7.5 mg/ml dimethyl pimelimidate (DMP) pH 8.5 (Sigma-Aldrich) for 30 min at room temperature (RT). DMP-crosslinking was repeated three times separated by 5 min washes in 0.2 M triethanolamine in PBS at RT, after which the cross-linking was quenched twice using 50 mM ethanolamine in PBS for 5 min at RT. Remaining non-cross-linked antibody was eluted with 1 M glycine. Elution was performed twice for 10 min at RT. The mixture was washed three times with 1% CHAPS buffer for 5 min at RT. Finally the antibody-conjugated beads were incubated with 1 ml of protein lysate overnight at 4 °C, followed by 3 washing steps in PBS. Bound protein was eluted with 5 volumes of 4 \times sample buffer (Bio-Rad) and 1 volume of reducing agent (Bio-Rad).

Author contributions

Experimental concept and design: MHS, CTW, EK-O, PH. Performance of the experiments: MHS, SH, ASH, BB. Analysis of the data: MHS, EK-O, PH. Preparation of the manuscript: MHS, PH.

Competing interests

The authors have declared that no competing interests exist.

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