

Proteolytic processing is necessary to separate and ensure proper cell growth and cytokinesis functions of HCF-1

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HCF-1 is a highly conserved and abundant chromatin-associated host cell factor required for transcriptional activation of herpes simplex virus immediate-early genes by the virion protein VP16. HCF-1 exists as a heterodimeric complex of associated N- (HCF-1_N) and C- (HCF-1_C) terminal subunits that result from proteolytic processing of a precursor protein. We have used small-interfering RNA (siRNA) to inactivate HCF-1 in an array of normal and transformed mammalian cells to identify its cellular functions. Our results show that HCF-1 is a broadly acting regulator of two stages of the cell cycle: exit from mitosis, where it ensures proper cytokinesis, and passage through the G₁ phase, where it promotes cell cycle progression. Proteolytic processing is necessary to separate and ensure these two HCF-1 activities, which are performed by separate HCF-1 subunits: the HCF-1_N subunit promotes passage through the G₁ phase whereas the HCF-1_C subunit is involved in proper exit from mitosis. These results suggest that HCF-1 links the regulation of exit from mitosis and the G₁ phase of cell growth, possibly to coordinate the reactivation of gene expression after mitosis.

Keywords: cell cycle/herpes simplex virus/mitosis/proteolysis/siRNA silencing

Introduction

Cell proliferation involves a highly regulated series of events in which the cellular genome is replicated faithfully during a synthesis (S) phase and each of the two resulting copies are segregated properly during mitosis (M). These two critical events are normally separated by two growth phases, G₁ following mitosis and G₂ following S phase. Orderly progression from one phase of the cell cycle to the other is mediated mainly by the regulation of gene expression, and key factors of this process are often transcriptional regulatory proteins.

Here, we elucidate cell cycle functions of the highly conserved and abundant chromatin-associated cell proliferation factor HCF-1, a host-cell factor involved in herpes simplex virus (HSV) immediate-early (IE) gene transcription. In lytic HSV infection, HCF-1 (also known as C1, VCAF and CFF) is incorporated into a multiprotein transcriptional regulatory complex with the viral activator VP16 and the cellular POU-domain transcription factor Oct-1 on HSV IE gene promoters (for a review, see Herr, 1998). Association with VP16 requires the N-terminal 380

amino acids of HCF-1 (LaBoissière *et al.*, 1997; Wilson *et al.*, 1997). This region probably adopts a β -propeller structure and is called the Kelch domain because of its sequence similarity to the *Drosophila* protein Kelch (Wilson *et al.*, 1997). The HCF-1 Kelch domain is sufficient for VP16-induced complex formation as well as to recruit HCF-1 to chromatin in uninfected cells (Wilson *et al.*, 1997; Wysocka *et al.*, 2001a).

In cells, the majority of HCF-1 exists as a heterodimeric complex of N- (HCF-1_N) and C- (HCF-1_C) terminal subunits, which results from the proteolytic processing of the 2035 amino acid (HCF-1₃₀₀) precursor protein and stable association of the resulting subunits (Wilson *et al.*, 1993, 1995; Kristie *et al.*, 1995). This proteolytic cleavage occurs at a series of six 26 amino acid repeats (HCF-1_{PRO} repeats) located near the middle of the HCF-1₃₀₀ protein (Wilson *et al.*, 1995). HCF-1 processing may involve autocatalytic cleavage at the HCF-1_{PRO} repeats (Vogel and Kristie, 2000a), but the functional significance of HCF-1 processing and subunit association is enigmatic.

HCF-1 is known to play some role in cell proliferation based on studies of the temperature-sensitive baby hamster kidney (BHK) cell line tsBN67 (Goto *et al.*, 1997). In tsBN67 cells, the one X-linked copy of the *HCF-1* gene carries a proline to serine substitution at position 134 (P134S) in the HCF-1 Kelch domain. This missense mutation does not grossly alter the stability or processing of HCF-1 at the non-permissive temperature of 40°C (Goto *et al.*, 1997) but it does induce HCF-1 dissociation from chromatin (Wysocka *et al.*, 2001a). This dissociation is followed by a cell proliferation arrest in the G₁/G₀ phase, 36–48 h after shifting to 40°C (Goto *et al.*, 1997). The cell proliferation arrest is accompanied by a conspicuous multinucleated (primarily binucleated) phenotype in ~15% of the arrested cells, indicating an associated cytokinesis defect (Reilly and Herr, 2002). Both tsBN67 defects can be rescued by the HCF-1_N subunit (Wilson *et al.*, 1997) and are suppressed in naturally arising tsBN67 cell growth revertants or by transformation with the DNA tumor virus SV40 early region, which has suggested that these two defects are connected (Reilly and Herr, 2002; Reilly *et al.*, 2002). The mode of HCF-1 action in these two processes, however, is unknown.

Although providing a window into the involvement of HCF-1 in cell proliferation, tsBN67 cells possess serious limitations including that this cell line was isolated after chemical mutagenesis and may thus contain additional mutations that cooperate with the loss of HCF-1 activity to induce the cell proliferation arrest. Additionally, the function of HCF-1 processing and the HCF-1_C subunit have not been elucidated with these cells. Furthermore, if HCF-1 function could be analyzed in different cell lines, it would be possible to determine (i) whether HCF-1 is a key regulator of the cell cycle; (ii) whether its function is

restricted to certain cell types; and (iii) whether its function varies between normal and transformed cells.

To address these questions, we have used small-interfering RNA (siRNA) to silence the expression of the *HCF-1* gene in normal and transformed cells. Our results indicate that HCF-1 is a universal regulator of cell proliferation and that HCF-1 processing is required to separate and ensure cell growth and cytokinesis functions of HCF-1 provided by the HCF-1_N and HCF-1_C subunits, respectively.

Results

HCF-1 is depleted efficiently and specifically by siRNA

To determine the function of mammalian HCF-1, we used the siRNA strategy (Elbashir *et al.*, 2001). Figure 1A shows a schematic of HCF-1, along with the *HCF-1* mRNA sequence and corresponding HCF-1 siRNA duplex designed to disrupt HCF-1 synthesis. Successful loss of *HCF-1* mRNA 3 days after two rounds of HCF-1 siRNA treatment but not a parallel treatment with an irrelevant luciferase mRNA-specific siRNA (control) is shown in Figure 1B, using a semi-quantitative RT-PCR analysis. In contrast, β -actin mRNA levels were unaffected (compare lane 3 with lanes 1 and 2, Figure 1B). Furthermore, immunoblot analysis showed that the HCF-1_N and HCF-1_C subunits were also significantly depleted by the same treatment (compare lane 3 with lanes 1 and 2, Figure 1C), whereas the β -actin protein was not affected (bottom panel). Thus, the HCF-1 siRNA induced the disappearance of both HCF-1 subunits by triggering the degradation of the *HCF-1* mRNA. As expected, each strand of the HCF-1 siRNA alone did not affect HCF-1 expression (data not shown).

To evaluate the efficacy of siRNA-induced HCF-1 depletion, HeLa cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to identify nuclei, and with α HCF-1_N antiserum to identify HCF-1-positive cells 3 days after siRNA treatment, as shown in Figure 1D. Whereas all of the untreated cells were positive for HCF-1 (compare a and b), many (but not all) of the HCF-1 siRNA-treated cells showed a sharp decrease in HCF-1 staining (compare c and d, arrowheads). One transfection led to 50% HCF-1-negative cells after 3 days, whereas two transfections separated by 12 h led to 85% HCF-1-negative cells, as shown in Figure 1E. These results indicate that the selected HCF-1 siRNA depletes HeLa cell HCF-1 protein with the efficiency influenced by the number of transfections. Below, we performed two rounds of siRNA treatment to deplete HCF-1 effectively.

HCF-1 depletion prevents progression through S phase

Owing to the P134S missense mutation, tsBN67 cells display a temperature-sensitive cell proliferation arrest (Goto *et al.*, 1997) but retain normal HCF-1 processing and stable HCF-1_{N/P134S} and HCF-1_C subunits. The cellular phenotype resulting from depletion of both HCF-1 subunits is not known, however. To examine such a phenotype, we compared the proliferation rates of HeLa cells either untreated or treated with control or HCF-1 siRNAs as shown in Figure 2A. HCF-1 siRNA-

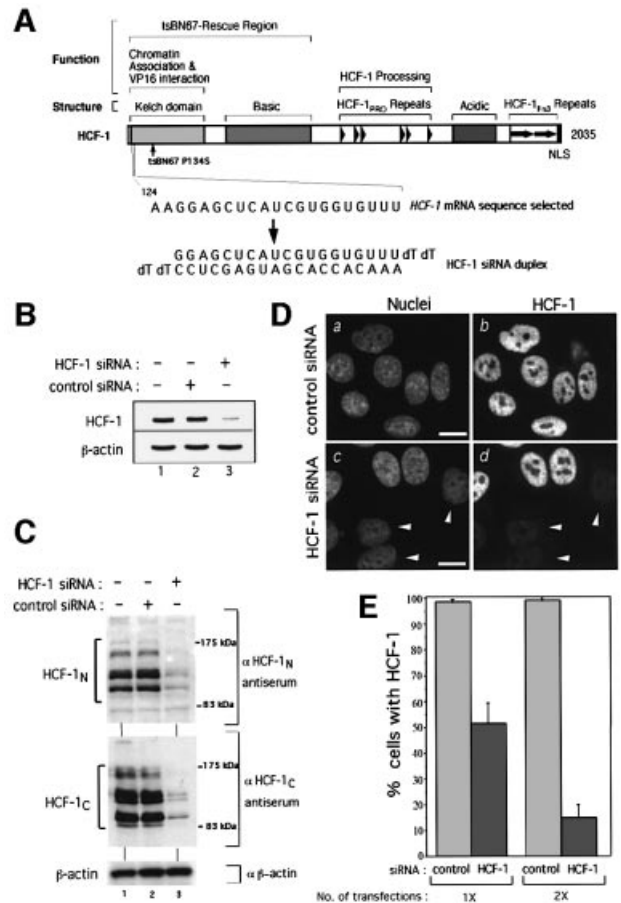


Fig. 1. Human HCF-1 is depleted efficiently and specifically by siRNA. (A) Schematic structure of HCF-1 (Wysocka *et al.*, 2001a). Above are shown (i) structural or sequence elements and (ii) functional regions. The position of the tsBN67 P134S missense mutation is indicated. The selected HCF-1 coding region RNA sequence (nucleotides 124–144; Wilson *et al.*, 1993) and the corresponding HCF-1 siRNA duplex are indicated below. (B) HCF-1 (top) and β -actin (bottom) mRNA levels in untreated HeLa cells (lane 1) or 3 days after treatment with either control (lane 2) or HCF-1 (lane 3) siRNA. (C) Immunoblot analysis of HCF-1_N (top), HCF-1_C (middle) and β -actin (bottom) proteins of untreated HeLa cells (lanes 1) or 3 days after treatment with control (lanes 2) or HCF-1 (lanes 3) siRNA. (D) Control (a and b) or HCF-1 (c and d) siRNA-treated cells were subjected to analysis after paraformaldehyde fixation by nuclear DAPI (a and c) and α HCF-1_N (b and d) staining. Arrowheads point to cells exhibiting HCF-1 depletion. Scale bar: 5 μ m. (E) Percentage of HCF-1-positive cells after one or two transfections with control or HCF-1 siRNA as determined by immunofluorescence. The percentage of HCF-1-positive nuclei was obtained by counting 200 cells per sample in each of three independent experiments.

transfected cells displayed a decrease in cell proliferation 2 days after transfection, whereas control siRNA-transfected cells continued to proliferate at nearly the same rate as mock-treated cells. Thus, as with temperature treatment of tsBN67 cells, depletion of HCF-1 by siRNA in HeLa cells is accompanied by changes in the rates of cell proliferation.

To characterize the proliferative status of individual HCF-1-depleted cells, we identified S phase-competent cells with a long-term bromodeoxyuridine (BrdU) incorporation assay, as shown in Figure 2B. Transfected cells were grown for 2 days and then incubated with BrdU for a further 24 h to ensure that all proliferating cells are labeled

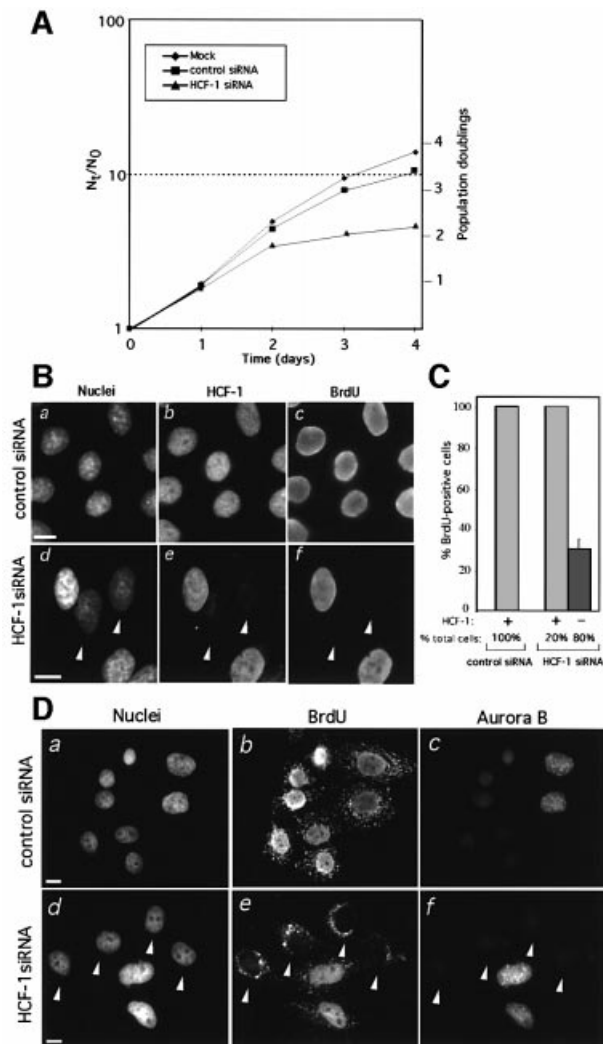


Fig. 2. HCF-1 depletion prevents G₁ progression. (A) Proliferation rates of untreated HeLa cells or after treatment with either control or HCF-1 siRNA. Cells were seeded at a density of 8×10^4 cells per well in 6-well plates the day before transfection. At the indicated times after the first of two transfections, cells were harvested and counted from duplicate plates, and the results averaged. N₀, the number of cells at time 0; N_t, number of cells at each specified time point. (B) Long-term BrdU incorporation assay of HCF-1 or control siRNA-treated HeLa cells. Nuclei were identified by DAPI (a and d), HCF-1 by α HCF-1_N (b and e), and BrdU by α BrdU (c and f) staining, after ethanol fixation. Arrowheads identify HCF-1-negative cells, which lack BrdU incorporation. (C) Quantification of the long-term BrdU incorporation assay of HCF-1-positive and negative HeLa cells after treatment with either control or HCF-1 siRNA. These percentages were determined by counting 200 cell per sample in each of three independent experiments. (D) Detection of Aurora-B in HeLa cells treated with either control (a–c) or HCF-1 (d–f) siRNA. (a and d) nuclear DAPI staining; (b and e) BrdU staining; (c and f) Aurora-B staining. Cells were fixed with paraformaldehyde, for which BrdU staining labels cytoplasmic mitochondria. White arrowheads identify BrdU- and Aurora-B-negative cells. Scale bar: 5 μ m.

as they pass through S phase. Cells subsequently were stained (i) with DAPI to identify nuclei; (ii) with α HCF-1_N antiserum to distinguish the HCF-1-positive and -negative cells; and (iii) with α BrdU antiserum to identify proliferating cells. Proliferating siRNA control-treated cells all contained HCF-1 and incorporated BrdU (Figure 2B, compare a–c). In contrast, siRNA-induced HCF-1-

depleted cells failed to incorporate BrdU (arrowheads), whereas accompanying HCF-1-positive cells incorporated BrdU (compare positive and negatively stained cells in e and f). As shown in Figure 2C, quantification of BrdU-positive and negative cells shows that all of the HCF-1-positive cells in both the control (100% of the total cells) and HCF-1 siRNA-transfected (20% of total cells) samples incorporated BrdU, indicating that this assay can effectively measure S phase-competent cells. In the HCF-1 siRNA-treated sample, however, of the 80% of the cells that were HCF-1 negative, 70% failed to incorporate BrdU (Figure 2C), showing an evident albeit incomplete defect in entering or progressing through S phase in the absence of HCF-1.

HCF-1-depleted cells are arrested in the G₁ phase

To determine if individual arrested HCF-1-depleted cells are in a specific phase of the cell cycle, we took advantage of the differential cell cycle phase-specific staining of Aurora-B kinase in HeLa cells (Crosio *et al.*, 2002): by immunofluorescence, Aurora-B kinase is not detected in G₁/S and displays a punctate nuclear distribution in G₂. We used the G₂-specific Aurora-B staining pattern to distinguish G₁ and G₂ phase cells. As shown in Figure 2D, all control siRNA-treated cells incorporated BrdU (panel b) and showed either background non-Aurora-B G₁/S staining with generally smaller nuclei, or G₂ punctate nuclear Aurora-B staining with generally larger nuclei (compare six G₁/S phase cells on the left and two G₂ cells on the right in a–c). In HCF-1 siRNA-treated cells, BrdU-negative cells appeared (arrowheads, panel e). These cells correspond to HCF-1-depleted cells (see Figure 2B and C). All of these BrdU-negative cells also exhibit the G₁/S phase-specific absence of Aurora-B staining (panel f). Because these cells failed to enter S phase at any time during the long-term BrdU labeling, these results suggest that HCF-1 depletion leads to a G₁ phase arrest.

Inactivation of the pRb protein family is not sufficient to rescue the cell cycle arrest induced by HCF-1 depletion

The G₁ phase arrest induced by the depletion of HCF-1 in HeLa cells is reminiscent of the temperature-induced arrest of tsBN67 cells. The HCF-1 siRNA HeLa cell proliferation arrest is intriguing because inactivation of the pRb protein family in tsBN67 cells by the SV40 early region (*SV40e*) can suppress the effect of the P134S HCF-1 mutation (Reilly *et al.*, 2002), and in HeLa cells the pRb protein family is already inactivated by the human papillomavirus E7 protein (Masters, 2002). These results suggest that the effect of the P134S mutation is less severe than that of HCF-1 depletion. To test this hypothesis, we asked whether HCF-1 depletion in tsBN67_{SV40e} cells disrupts their proliferation.

After hamster HCF-1 siRNA transfection, cells were grown for 3 days at permissive (33.5°C) or non-permissive (40°C) temperature, which was sufficient to deplete HCF-1 as determined by immunoblot analysis (data not shown), and then incubated in the presence of BrdU for 24 h. As shown in Figure 3, consistent with previous results (Goto *et al.*, 1997; Reilly *et al.*, 2002), HCF-1-positive tsBN67 cells incorporated BrdU at 33.5°C but not at 40°C (compare samples 1 and 3), whereas the HCF-1-positive

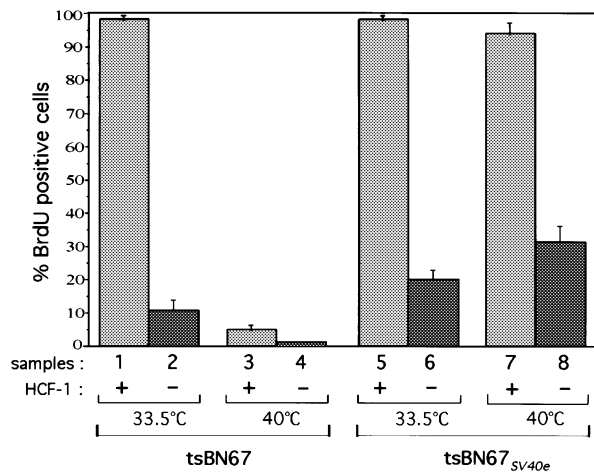


Fig. 3. Inactivation of the pRb protein family is not sufficient to rescue the cell cycle arrest induced by HCF-1 depletion. Quantification of long-term BrdU incorporation in HCF-1-positive (odd numbered samples) and negative (even numbered samples) tsBN67 (samples 1–4) and tsBN67^{SV40e} (samples 5–8) cells after treatment with hamster-specific HCF-1 siRNA. The percentages were obtained by counting 200 cells per sample in each of three independent experiments. Control siRNA-treated tsBN67 and tsBN67^{SV40e} cells displayed the same BrdU incorporation pattern as the HCF-1-positive HCF-1 siRNA-transfected cells shown in the figure (data not shown).

tsBN67^{SV40e} cells incorporated BrdU at both temperatures (compare samples 5 and 7). In contrast, HCF-1 depletion induced a cell cycle arrest in tsBN67 cells at both 33.5 and 40°C (compare samples 2 and 4). Interestingly, the HCF-1 depletion also led to the same arrest of tsBN67^{SV40e} cells (samples 6 and 8). These results indicate that the mutant P134S HCF-1 protein retains one or more activities that can cooperate with the *SV40e* region to promote cell proliferation. Interestingly, HCF-1-depleted tsBN67^{SV40e} cells display an elevated number of BrdU-positive cells at 33.5 and 40°C as compared with the parental tsBN67 cells (compare samples 2, 4, 6 and 8); indeed the level of BrdU-positive tsBN67^{SV40e} cells is very similar to that in HCF-1-depleted HeLa cells (compare Figures 2C and 3), suggesting that inactivation of the pRb protein family still bypasses some cell proliferation requirements for HCF-1 in HCF-1-depleted cells.

HCF-1 depletion causes a cell cycle arrest in normal as well as in oncogenically transformed cells

The ability to disrupt S phase entry by siRNA in both HeLa and tsBN67 cells—cell lines from different species, tissues and oncogenic transformation state—indicates that the role(s) of HCF-1 in cell proliferation is not restricted to specific cells. Because the genetic and growth properties of HeLa and tsBN67 cells are altered, we asked whether HCF-1 depletion causes cell proliferation defects in normal cells. We therefore treated mouse embryo fibroblasts (MEFs) with mouse-specific HCF-1 siRNA and analyzed them by the long-term BrdU incorporation assay. By performing immunofluorescence on individual cells, we were able to detect 5–10% HCF-1-depleted MEFs after HCF-1-siRNA treatment. As shown in Figure 4A (a–c), all HCF-1-positive MEFs incorporated BrdU, whereas HCF-1-depleted cells (arrowheads) did

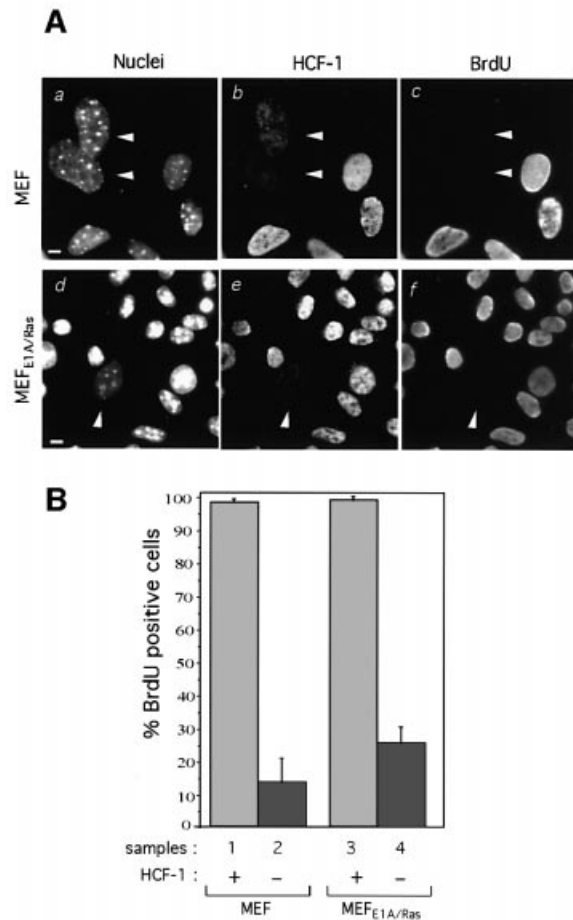


Fig. 4. HCF-1 depletion causes a cell proliferation arrest in primary and oncogenically transformed cells. (A) Long-term BrdU incorporation by MEF (a–c) and MEF^{E1A/Ras} (d–f) cells after treatment with mouse HCF-1 siRNA. Nuclear DAPI (a and d), α HCF-1_C (b and e) and α BrdU (c and f) staining are shown. Arrowheads point to HCF-1 depleted cells, which are negative for BrdU incorporation. (B) Quantification of BrdU-positive cells in HCF-1 siRNA-treated MEF (samples 1 and 2) and MEF^{E1A/Ras} (samples 3 and 4) cells. The percentage of cells incorporating BrdU was obtained by counting 200 cells per sample in each of three independent experiments. Scale bar: 5 μ m.

not, indicating that loss of HCF-1 in normal cells also induces a cell cycle arrest. [For unexplained reasons, HCF-1-depleted MEFs often displayed enlarged nuclei (see panel a).] Quantification (Figure 4B, samples 1 and 2) shows that essentially all of the HCF-1-positive cells but <15% of the HCF-1-depleted cells entered S phase, suggesting that HCF-1 is important for cell cycle progression in normal cells.

To determine whether defined oncogenic cellular transformation could relieve the requirement for HCF-1 function, we tested the effects of siRNA-induced HCF-1 depletion in low-passage adenovirus E1A and oncogenic Ras V12-transformed MEFs (MEF^{E1A/Ras}). As shown in Figure 4A (d–f), even though the efficiency of HCF-1 depletion was low, we observe in individual MEF^{E1A/Ras} cells that these potent oncogenes do not relieve the requirement for HCF-1, although quantification shows that, as with tsBN67^{SV40e} and tsBN67 cells, there is some induction of S phase entry compared with the wild-type

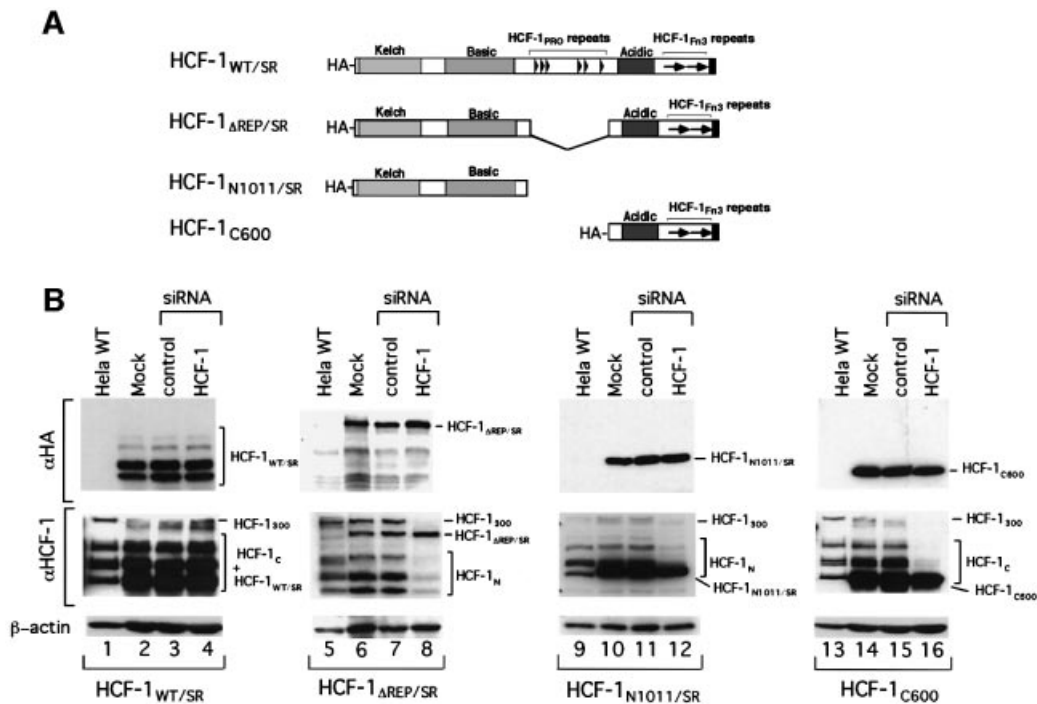


Fig. 5. Silent mutations in the *HCF-1* mRNA sequence targeted by the HCF-1 siRNA duplex prevent ectopic HCF-1 depletion by siRNA. (A) Schematic structures of the four siRNA-resistant (SR) HCF-1 recombinant proteins. (B) Immunoblot analysis of untreated wild-type HeLa cells (lanes 1, 5, 9 and 13) or HeLa cells synthesizing the different HCF-1_{SR} recombinant proteins either untreated (lanes 2, 6, 10 and 14) or treated with control (lanes 3, 7, 11 and 15) or HCF-1 (lanes 4, 8, 12 and 16) siRNA. HCF-1₃₀₀, HCF-1 precursor. The HCF-1_{SR} proteins were visualized alone (αHA tag antiserum, top panel) or together with endogenous HCF-1 proteins (middle panel) using the αHCF-1_N (lanes 5–12) or αHCF-1_C (lanes 1–4 and 13–16) antisera. β-actin, loading control (bottom panel).

MEFs (25% versus 13%, samples 2 and 4, Figure 4B). These results show that HCF-1 is a broad, if not universal, key promoter of cell growth and proliferation in mammalian cells.

Silent mutations in the *HCF-1* mRNA sequence targeted by the HCF-1 siRNA protect from siRNA-induced silencing

To identify the regions of HCF-1 required to promote cell proliferation, a set of four recombinant flu hemagglutinin (HA) epitope-tagged siRNA-resistant (SR) HCF-1 molecules were stably expressed in HeLa cells. To prevent destruction of the recombinant *HCF-1* mRNAs containing the sequence targeted by the HCF-1 siRNA, we used the strategy of Lassus *et al.* (2002): nine silent mutations were introduced into the HCF-1 sequence corresponding to the siRNA duplex, creating siRNA-resistant HCF-1 (HCF-1_{SR}) proteins. As expected, these silent mutations did not affect rescue of the tsBN67 cell proliferation defect (data not shown). Figure 5A shows the structure of the four HA-HCF-1_{SR} proteins: (i) HCF-1_{WT/SR} representing the wild-type protein; (ii) HCF-1_{ΔREP/SR} lacking the HCF-1_{PRO} repeats and deficient in HCF-1 proteolytic processing; (iii) HCF-1_{N1011/SR} representing the HCF-1_N subunit; and (iv) the naturally siRNA-resistant HCF-1_{C600} representing the HCF-1_C subunit.

Each of these four HeLa cell lines was subjected to siRNA treatment and analyzed by immunoblot as shown in Figure 5B. The αHA antiserum showed that each of the HCF-1_{SR} proteins is resistant to siRNA treatment (top panels, compare lanes 2–4, 6–8, 10–12 and 14–16),

whereas HCF-1 antisera showed that the endogenous HCF-1 subunits disappeared after HCF-1 siRNA treatment for the HCF-1_{ΔREP/SR} (middle panel, lanes 7 and 8), HCF-1_{N1011/SR} (lanes 11 and 12) and HCF-1_{C600} (lanes 15 and 16) cell lines; for the HCF-1_{WT/SR} sample, it was not possible to ascertain the loss of endogenous HCF-1 owing to the co-migration of the HCF-1_{WT/SR} subunits.

The HCF-1_N subunit is necessary and sufficient to promote G₁ phase progression and S phase entry

We next examined the ability of the different HA-HCF-1_{SR} proteins to rescue the S phase entry defect in endogenous HCF-1-depleted cells, by performing the long-term BrdU incorporation assay as shown in Figure 6. The control siRNA had no effect on BrdU incorporation in any of the cell lines (data not shown). Owing to the inability to identify HCF-1-depleted cells with the HCF-1_{WT/SR} and HCF-1_{ΔREP/SR} cell lines, the quantification in Figure 6 includes all cells irrespective of their HCF-1 depletion status. Thus, ~35% of the wild-type HeLa cells were S phase competent (sample 1), which represents the background in this assay. Expression of the HCF-1_{WT/SR} protein restored the ability of the cells to incorporate BrdU, confirming that the S phase defect is indeed caused by HCF-1 depletion (sample 2). HCF-1 processing is not required to rescue the S phase defect because the HCF-1_{ΔREP/SR} protein restored BrdU incorporation to near wild-type levels (compare samples 2 and 5).

In previous experiments with tsBN67 cells, the HCF-1_{N1011} but not the HCF-1_{C600} construct could rescue

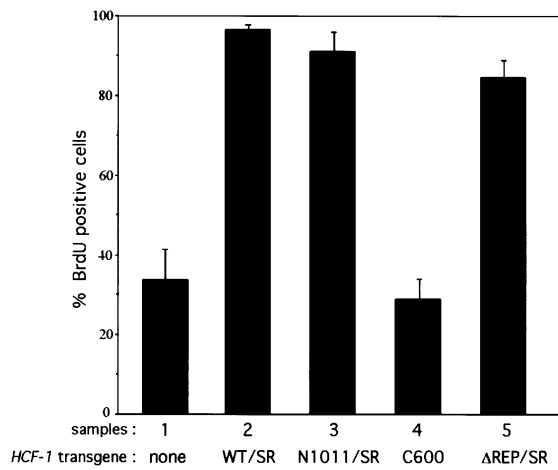


Fig. 6. The HCF-1_N subunit is necessary and sufficient to promote G₁ progression and entry into S phase. Quantification of the long-term BrdU incorporation in parental HeLa cells (sample 1) or HeLa cells synthesizing HCF-1_{SR} proteins (samples 2–5) performed as described in Materials and methods is shown. The percentage of cells incorporating BrdU was obtained by counting 200 cells per sample in each of three independent experiments.

cell proliferation at non-permissive temperature (Wilson *et al.*, 1997). In these experiments, however, it was not possible to determine whether the HCF-1_N subunit alone is sufficient to rescue cell cycle progression because the P134S mutant protein in tsBN67 cells is synthesized at near wild-type levels and processed normally. By using siRNA, however, we can deplete the HCF-1_N and HCF-1_C subunits simultaneously (see Figure 1C) and address the roles of the two HCF-1 subunits independently. Interestingly, expression of the HCF-1_N subunit alone (i.e. HCF-1_{N1011/SR}), but not the HCF-1_C subunit (i.e. HCF-1_{C600}), was sufficient to restore BrdU incorporation to wild-type levels (compare samples 3 and 4). Thus, the HCF-1_N subunit is necessary and sufficient to promote G₁ phase progression and S-phase entry.

HCF-1-depleted cells arrest with a population of multinucleated cells

As mentioned above, temperature-arrested tsBN67 cells display a multinucleated, primarily binucleated, phenotype in ~15% of arrested cells (Reilly and Herr, 2002). Three days after transfection, we examined the siRNA-treated HeLa cells for a similar phenotype, as shown in Figure 7. Like wild-type HeLa cells, the control siRNA-treated cells were clustered and displayed a compact shape with few multinucleated cells (Figure 7A, a). In contrast, the HCF-1 siRNA-treated HeLa cells displayed elongated shapes and many multinucleated, predominantly binucleated, cells (e.g. see arrowhead in b), indicating that, as with cell growth, the loss of HCF-1 function in different cell types can lead to a multinucleation defect, probably arising from improper cytokinesis.

Fluorescent staining with (i) DAPI to identify the nuclei, (ii) αHCF-1_C antiserum to detect HCF-1 and (iii) α-tubulin antiserum to determine the cell borders revealed that the multinucleated cells were depleted of HCF-1 (Figure 7B, see arrowhead), indicating that the multinucleation phenotype is indeed due to the inactivation of HCF-1.

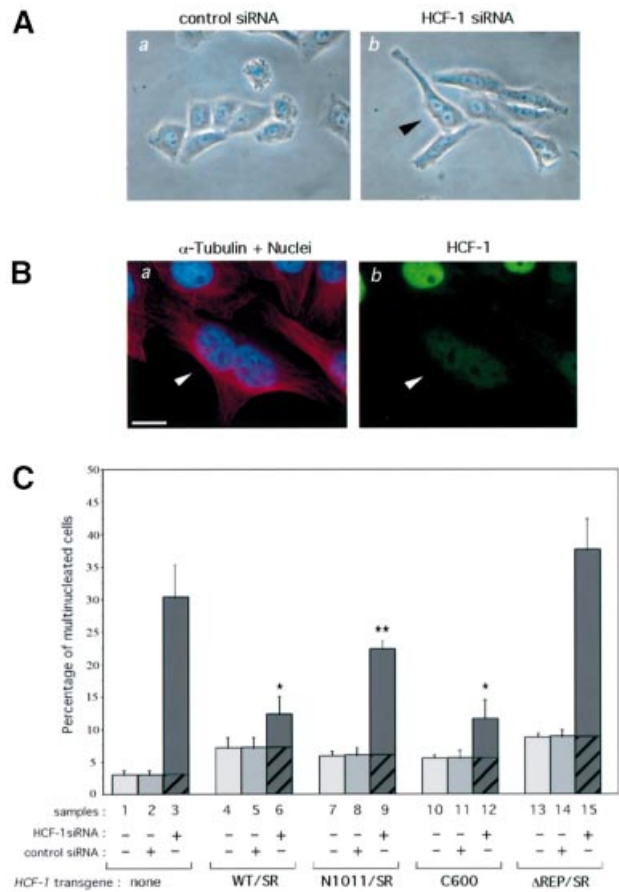


Fig. 7. HCF-1 depletion causes a cytokinesis defect that is rescued by the HCF-1_C subunit. (A) Representative phase-contrast images of HeLa cells are shown after treatment with control (a) or HCF-1 (b) siRNA. The black arrowhead points to an elongated and binucleated cell. (B) Immunofluorescence analysis of a binucleated cell after HCF-1 siRNA treatment. (a) α-tubulin (red) and DAPI (blue) staining; (b) αHCF-1_N (green) staining. Arrowhead, HCF-1-depleted binucleated cell. Scale bar: 5 μm. (C) Quantification of multinucleated cells of parental HeLa cells (samples 1–3) or HeLa cells synthesizing the HCF-1_{SR} proteins (samples 4–15), either untreated (samples 1, 4, 7, 10 and 13), or treated with control (samples 2, 5, 8, 11, and 14) or HCF-1 (samples 3, 6, 9, 12, and 15) siRNA. Multinucleated cells were identified by immunofluorescence using DAPI, and αHA and α-tubulin antisera. The percentage of multinucleated cells was obtained by counting 200 cells per sample in each of three independent experiments. *P*-value of <0.02 (*) or <0.06 (**) as determined by Student's *t*-test.

The HCF-1_C subunit ensures proper cytokinesis

Using the same α-tubulin–DAPI fluorescence assay as in Figure 7B, we quantitated the levels of multinucleation in the siRNA-treated cells 3 days after transfection. Analysis of the parental HeLa cells shows that ~3% of untreated or control siRNA-treated cells are multinucleated (compare samples 1 and 2 in Figure 7C), whereas 25–35% of the HCF-1 siRNA-treated cells were multinucleated (sample 3). Thus, loss of HCF-1 function in different cell types (HeLa versus BHK) and by different mechanisms (HCF-1 siRNA versus HCF-1 temperature sensitivity) leads to a pronounced defect in mitosis, probably cytokinesis, in many but not all cells.

To determine which regions of HCF-1 are involved in cytokinesis, we assayed the ability of the HCF-1_{SR} proteins to rescue HCF-1 siRNA-induced multinucleation.

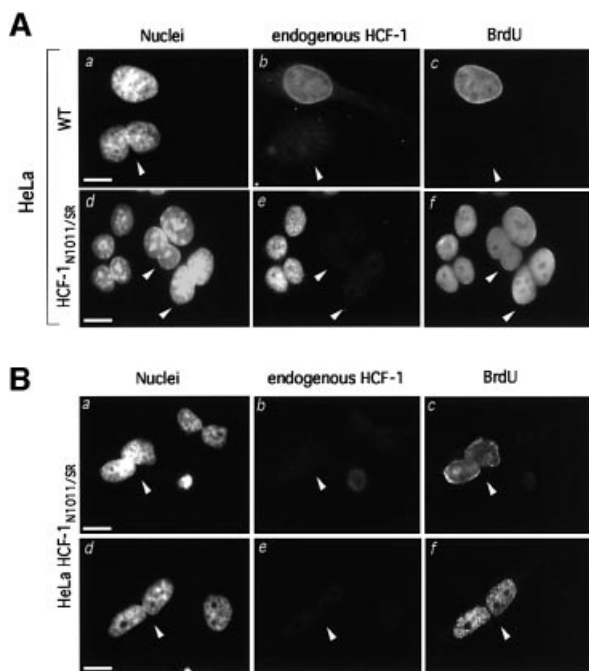


Fig. 8. The HCF-1_N subunit can promote G₁ phase progression and S phase entry after improper cytokinesis. (A) Immunofluorescence analysis of the long-term BrdU incorporation assay of multinucleated cells is shown for parental (a–c) and HCF-1_{N1011/SR} (d–f) HeLa cells. (a and d) DAPI staining; (b and e) αHCF-1_C staining for endogenous HCF-1; (c and f) αBrdU staining. White arrowheads identify HCF-1-depleted binucleated cells. (B) Immunofluorescence analysis of short-term BrdU incorporation in HCF-1 siRNA-treated HCF-1_{N1011/SR} cells. (a and d) DAPI staining; (b and e) αHCF-1_C staining for endogenous HCF-1; (c and f) αBrdU staining. Arrowheads identify BrdU-positive, HCF-1-depleted binucleated cells. Scale bar: 5 μm.

Before any siRNA treatment, the percentage of multinucleated cells present in the different populations of HeLa cells varied between 5 and 8%, and this variation was not affected by the transfection of control siRNA (Figure 7C, compare samples 4 and 5, 7 and 8, 10 and 11, and 13 and 14); this variability could result from the overexpression of the various recombinant HCF-1 proteins and represents the multinucleation background for each sample (see the hatched portion of the bar in samples 3, 6, 9, 12 and 15).

The HCF-1_{WT/SR} cells displayed a pronounced decrease in the level of HCF-1 siRNA-induced multinucleation: the HCF-1 siRNA treatment induced multinucleation in 6% of the HCF-1_{WT/SR} cells as opposed to 27% in the parental HeLa cells (compare the solid portions of samples 3 and 6). Thus, the wild-type HCF-1 siRNA-resistant construct can rescue much but not all of the multinucleation defect, indicating that the cytokinesis defect is indeed caused by loss of HCF-1 function.

HCF-1 siRNA treatment led to induction of multinucleation in 18% of the HCF-1_{N1011/SR} cells (Figure 7C, sample 9), indicating that the HCF-1_N subunit cannot independently rescue the cytokinesis defect effectively. In contrast, the HCF-1_C subunit alone suppressed the multinucleation phenotype (sample 12). These results suggest that the roles of HCF-1 in cell growth and cell division, rather than connected as previously suggested (Reilly and Herr, 2002; Reilly *et al.*, 2002), are largely segregated into

the different HCF-1 subunits: the HCF-1_N subunit promotes cell growth and the HCF-1_C subunit ensures proper cell division.

Proteolytic processing of HCF-1 is important for ensuring proper cytokinesis

To date, the role of HCF-1 processing has been enigmatic; it is not required to rescue tsBN67 (Wilson *et al.*, 1997) nor HeLa cell HCF-1 siRNA-induced (Figure 6) cell growth defects. Inactivation of HCF-1 processing in the HCF-1_{ΔREP/SR} construct results, however, in failure to suppress the HeLa cell HCF-1 siRNA-induced multinucleation phenotype (Figure 7C, sample 15). Thus, surprisingly, proteolytic processing of HCF-1 separates the cell growth and mitotic functions of the HCF-1_N and HCF-1_C subunits, respectively, and is required for the HCF-1_C subunit to function in cytokinesis.

The HCF-1_N subunit can promote G₁ phase progression and S phase entry after improper cytokinesis

The HCF-1 siRNA-induced cell proliferation arrest occurs in cells with either a single nucleus or multiple nuclei, as illustrated for a binucleated cell in Figure 8A (a–c). Because HCF-1_{N1011/SR} cells display wild-type HCF-1 siRNA-resistant cell growth (Figure 6) but defective cytokinesis (Figure 7), we asked whether these cells can enter S phase after improper cytokinesis. Figure 8A (d–f) shows that binucleated HCF-1_{N1011/SR} cells incorporate BrdU in a long-term labeling assay (see arrowheads).

To determine whether these labeled multinucleated HCF-1_{N1011/SR} cells indeed result from S phase entry (as opposed to improper mitosis after being labeled in S phase), we performed a short BrdU incorporation assay (1 h). As shown in Figure 8B, in this short-term assay, multinucleated HCF-1-depleted BrdU-positive cells appeared (arrowheads), suggesting that the HCF-1_N subunit can promote S phase entry even after improper cell division owing to the loss of HCF-1_C subunit function. The short-term BrdU staining patterns in Figure 8B (c and f) differ (diffuse and punctate, respectively), indicating labeling during different stages of S phase (O'Keefe *et al.*, 1992), but the patterns are similar in both nuclei of the individual cells. These results suggest that, within a binucleated HCF-1_{N1011/SR} cell, both nuclei appear to progress through S phase synchronously. These results illustrate how the role of the HCF-1_N subunit in cell growth is independent of HCF-1_C subunit function.

Discussion

We have used siRNA to inactivate HCF-1 in cells of multiple mammalian species, and in normal and transformed cells to unravel its cellular function. Our results show that HCF-1 is a broadly acting regulator of two stages of the mammalian cell cycle: passage through the G₁ phase and exit from mitosis. These two activities of HCF-1 are performed by separate HCF-1 subunits. The HCF-1_N subunit promotes cell growth and passage through the G₁ phase, whereas the HCF-1_C subunit ensures proper exit from mitosis. HCF-1 proteolytic processing is necessary for HCF-1_C subunit function in cytokinesis. These results reveal the varied and broad roles of HCF-1 in the

mammalian cell cycle and show that, in addition to its important role in HSV replication, HCF-1 possesses important cell proliferation regulatory functions in uninfected cells.

A limitation of the siRNA approach is that gene inactivation is restricted to the transfected cells and even all of the transfected cells do not respond to the siRNA treatment. We therefore used multiple single-cell assays to determine the effects of HCF-1 loss in individual cells, including (i) detection of HCF-1-depleted cells; (ii) long-term BrdU incorporation to assay entry and progression through S phase; (iii) Aurora-B localization combined with BrdU incorporation to differentiate G₁, S and G₂ phase cells; and (iv) α -tubulin and DAPI staining to detect multinucleated cells. These assays allowed us to study the role of HCF-1 in both cell growth and division. To show that the effect of the HCF-1 siRNA was due to the loss of HCF-1 function, we created siRNA-resistant wild-type HCF-1 molecules in HeLa cells and rescued the wild-type phenotype. Use of mutant siRNA-resistant HCF-1 molecules resulted in the identification of the different roles of the HCF-1_N and HCF-1_C subunits, and HCF-1 processing in human cell cycle progression.

Role of HCF-1 proteolysis in promoting proper cytokinesis

An unusual feature of HCF-1 is its proteolytic processing, which occurs stochastically at six centrally located 26 amino acid repeats, resulting in stable N- and C-terminal subunits, the large majority of which remain associated with one another after proteolysis (Wilson *et al.*, 1995). Although HCF-1 proteolytic processing has been conserved in *Drosophila* (Mahajan *et al.*, 2003), albeit not *Caenorhabditis elegans* (Wysocka *et al.*, 2001b), its role in HCF-1 function has been a mystery because of the absence of any cellular assay to study this process. The only previous cellular model for HCF-1 function has been the tsBN67 hamster cell line in which processing of the mutant HCF-1 occurs normally (Goto *et al.*, 1997) and processing of wild-type HCF-1 is not required to rescue HCF-1 function (Wilson *et al.*, 1997). The inability of the HCF-1 Δ REP molecule to rescue the multinucleated phenotype indicates that HCF-1 processing is important for the cytokinesis functions of the HCF-1_C subunit. Alternatively, the HCF-1_{PRO} repeat or flanking sequences that are deleted in the HCF-1 Δ REP construct play a role in cytokinesis. We do not favor this hypothesis because, in contrast to the rest of the protein, the sequences flanking the HCF-1_{PRO} repeats are not evolutionarily conserved (Goto *et al.*, 1997; Kristie, 1997) and therefore probably do not possess important functional activities. We suggest that the proteolytic processing is important to permit the dissociation of the HCF-1 subunits at a specific phase of the cell cycle, perhaps mitosis, as a part of performing their function in the cell. In this manner, HCF-1 proteolytic processing separates cell growth and mitotic functions of HCF-1 and ensures their proper execution.

The proteolytic processing properties of HCF-1 are similar to those recently described for the mixed lineage leukemia (MLL, also known as acute lymphoblastic leukemia or ALL) oncoprotein (Nakamura *et al.*, 2002; Yokoyama *et al.*, 2002; Hsieh *et al.*, 2003). MLL is a 3968 amino acid protein that is processed at two sites to create

stable subunits that remain associated with one another. Interestingly, much like the results described here, the MLL N- and C-terminal subunits possess separate negative and positive transcriptional activities, respectively. In this regard, we note that the HCF-1_C subunit possesses a well-defined transcriptional activation domain (Luciano and Wilson, 2002) and the HCF-1_N subunit possesses both repressive and activating properties (Vogel and Kristie, 2000b; Piluso *et al.*, 2002).

HCF-1_N functions to promote G₁ phase progression

The siRNA-induced inactivation of HCF-1 causes a G₁ phase cell cycle arrest in human cells that is reminiscent of the HCF-1 P134S temperature-induced G₁/G₀ arrest in hamster tsBN67 cells (Goto *et al.*, 1997). Owing, however, to the stability of the P134S HCF-1 protein in tsBN67 cells, it was not possible to determine all the regions of HCF-1 that are necessary and sufficient to promote cell proliferation (Wilson *et al.*, 1997). By using siRNA, we depleted both of the HCF-1_N and HCF-1_C subunits simultaneously and showed, by use of siRNA-resistant molecules, that the HCF-1_N subunit alone is sufficient to promote G₁ phase progression as assayed by S phase entry. Thus, the HCF-1_N subunit apparently is sufficient to rescue cell growth.

Because the HCF-1_N subunit interacts with and modulates the transcriptional activity of many transcription factors such as GABP, LZIP and Miz-1 (Vogel and Kristie, 2000b; Luciano and Wilson, 2002; Piluso *et al.*, 2002) and associates selectively with the Sin3 histone deacetylase and Set1/Ash2 histone methyltransferase (Wysocka *et al.*, 2003), HCF-1 probably promotes progression through G₁ by regulating gene transcription. Although the relative significance of these various interactions remains to be unraveled, the role of HCF-1 in Miz-1 function is particularly interesting. Miz-1 can inhibit G₁ phase progression, possibly by activating the promoter for the cyclin-dependent kinase inhibitor p15^{INK4b}, which in turn inhibits pRb inactivation by phosphorylation and thus S phase entry. HCF-1 can interfere with Miz-1 p15^{INK4b} activation, suggesting that it can indirectly promote S phase entry by permitting phosphorylation and inactivation of pRb (Piluso *et al.*, 2002). Consistent with this model, inactivation of pRb family members by the SV40 large T antigen and adenovirus oncoproteins can rescue the cell cycle defect in tsBN67 cells (Reilly *et al.*, 2002).

This model, however, probably does not explain all of HCF-1's roles in promoting G₁ progression because, although pRb is inactivated in HeLa, tsBN67_{SV40e} and MEF_{E1A/Ras} cells, our results show that the depletion of HCF-1 in these cells still causes a defect in cell growth, albeit less severe than in normal cells (Figures 2–4). These results indicate that HCF-1 is probably involved in multiple regulatory pathways. Consistent with this hypothesis, in addition to a role in transcription, HCF-1 recently has been implicated in pre-mRNA spliceosome assembly by interacting with snRNPs (Ajuh *et al.*, 2002).

HCF-1_C functions to ensure proper cytokinesis

An important finding of our studies is that the role of HCF-1 in mitosis, specifically cytokinesis, is played by the HCF-1_C subunit (Figure 7). How might the HCF-1_C

subunit ensure proper exit from mitosis? Interestingly, the HCF-1_C subunit associates with protein phosphatase 1 (PP1; Ajuh *et al.*, 2000). There are three closely related PP1 isoforms, α , β and γ , encoded by three different genes (for a review, see Cohen, 2002). PP1 γ , at least, plays a role in cytokinesis (Cheng *et al.*, 2000). Although it is not known which PP1 proteins associate with HCF-1 and which PP1 targets are specified by HCF-1 *in vivo*, it is tempting to imagine that the HCF-1_C subunit modulates the activity of one or more PP1 isoforms, especially PP1 γ , during mitosis to ensure proper cytokinesis.

If the HCF-1_C subunit is responsible for ensuring proper cytokinesis, then why do tsBN67 cells, which contain wild-type HCF-1_C subunits, display this mitotic defect (Reilly and Herr, 2002)? Perhaps, the temperature-sensitive P134S HCF-1_N subunit has a dominant inhibitory effect on HCF-1_C function in tsBN67 cells, resulting in the multinucleation phenotype even in the presence of the wild-type HCF-1_C subunit. Additionally, the HCF-1_N subunit may play a role in cytokinesis as evidenced by the partial rescue of the multinucleation phenotype by the HCF-1_{N1011/SR} construct (Figure 7C). We note that even our wild-type HCF-1_{WT/SR} cDNA construct does not completely rescue the multinucleation defect; this may be due to the absence of an HCF-1 variant (called HCF-1 Δ 382–450) produced by alternative pre-mRNA splicing in which the resulting HCF-1_{N Δ 382–450} and HCF-1_C subunits fail to associate (Wilson *et al.*, 1995).

Does HCF-1 regulate the transition from mitosis to the G₁ or G₀ phase of the cell cycle?

Of the many steps in the orderly progression through the cell cycle, the regulation of the M/G₁ phase transition is perhaps the least well understood. Many very important events that regulate cell fate occur at this point in the cell cycle: it is here that the gene expression program of the cell is re-established following the dramatic chromosomal condensation events of mitosis. Furthermore, it is at this stage that cells generally make the decision to exit from the cell cycle into the G₀ phase and differentiate. Our current understanding of the roles of HCF-1 in cell proliferation suggests that it plays multiple roles in the M/G₁ phase transition, specifically cytokinesis and early G₁ cell cycle progression. Through proteolytic processing and the generation of a heterodimeric complex of HCF-1_N and HCF-1_C subunits with distinct functions, HCF-1 is apparently able to link the regulation of exit from mitosis and the G₁ phase of cell growth. If true, HCF-1 could also play important roles in the cellular decision to enter the G₀ phase and initiate specific gene expression cascades involved in cell differentiation. Such activities of HCF-1 in this critical transition of the cell cycle could explain why HCF-1 is an attractive target of the HSV VP16 regulator to control the lytic or latent outcome of HSV infection in proliferating and non-proliferating cells.

Materials and methods

Cell culture

HeLa cells, MEFs and tsBN67 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). MEFs (Serrano *et al.*, 1997) were provided by E.De Stanchina and S.Lowe (Cold Spring Harbor Laboratory).

Immunoblot analysis

For immunoblot analysis, cells washed with phosphate-buffered saline (PBS) were lysed in SDS Laemmli sample buffer and, after clarification by centrifugation, equal amounts of protein were resolved by SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell) and probed with either polyclonal rabbit antisera to the HCF-1_N (α N18; Goto *et al.* 1997) or HCF-1_C (α H12; Wilson *et al.*, 1993) subunits, or monoclonal antibodies to the HA tag (12CA5; see Wilson *et al.*, 1993) or β -actin (Sigma), followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The immunoreactive bands were detected by chemiluminescence as described (Pierce).

Mutagenesis and generation of cell lines

A human neomycin-resistant expression vector with the genomic β -actin promoter and exon 1, intron 1 and the β -actin ATG initiation codon in exon 2 (pHBA-BGH; Miller and Rizzino, 1996) was modified to encode the HA epitope tag (SYPYDVPDYASLGGPS) downstream of the ATG in a *SalI* site, creating pAPN. The HA epitope tag sequence is followed by a *SpeI*-*BamHI*-*HindIII* polylinker. *XbaI*-*BamHI* cDNA fragments encoding the wild-type human HCF-1, the HCF-1_{N1011} subunit (residues 2–1011), the HCF-1_{C600} subunit (residues 1436–2035) or HCF-1 Δ REP (with residues 1011–1435 deleted) were cloned between the *SpeI* and *BamHI* sites of pAPN. The HCF-1_{SR} constructs were prepared by introducing nine silent mutations between *HCF-1* cDNA nucleotides 124–144 (AAAGAATTAATTGTAGTITTC; silent mutations underlined) into each pAPN-HCF-1 construct. For stable recombinant HCF-1 protein synthesis, HeLa cells were transfected by electroporation with 5 μ g of the plasmid DNAs and transfected cells were selected in G418-containing medium (500 μ g/ml) for 3 weeks. Drug-resistant colonies were tested for recombinant HCF-1 expression by HA tag immunoblot and immunofluorescence analyses. For each HCF-1 recombinant protein, three or more positive clones were selected and pooled.

siRNA preparation and transfection

A human HCF-1 siRNA duplex directed against the *HCF-1* cDNA sequence AAGGAGCTCATCGTGGTGTTC (nucleotides 124–144) was checked against the human genome sequence to ensure that only the *HCF-1* mRNA would be targeted. For the mouse (AAGGAGCTTATAGTGGTGTTC) and hamster (AAGGAGCTCATAGTGGTGTTC) HCF-1 siRNA duplexes, the respective 124–144 residues were used. Complementary RNA oligomers (Dharmacon) were annealed at 20 μ M as described by the manufacturer. The firefly luciferase non-specific control siRNA duplex (Elbashir *et al.*, 2001) was used. For siRNA transfection, 10⁵ cells were seeded onto each well of 6-well plates in 2 ml of DMEM with 10% FBS, and transfected the following day with 10 μ l of siRNA duplex and 5 μ l of Oligofectamine in Opti-MEM medium as described (Invitrogen). At 12 h, cells were rinsed with PBS and then transfected again following the same protocol.

Semi-quantitative RT-PCR

To assay mRNA transcript levels, total RNAs were extracted with the Ultraspec-II RNA system (Biotecx). cDNA synthesis and PCR amplification were performed with Superscript one-step RT-PCR (Invitrogen), using 100 ng of total RNA. HCF-1 cDNA was amplified with sense (GTGGGAGTGGGAAGAGACTCA) and antisense (GTGGGAGTGGGAAGAGACTCA) primers for 30 cycles; exponential amplification was verified. β -actin cDNA was amplified for 24 cycles with the primers GGAGCACCCCGTCTGCTGA and CCAGCCAGGTCCAGACGCA.

Immunofluorescence

For immunofluorescence, siRNA-transfected cells grown on glass coverslips were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and then blocked for 60 min with 1% bovine serum albumin (BSA) at room temperature. Incubations with primary antibodies against HCF-1 (α N18, 1:800 dilution; α H12, 1:1000 dilution), HA tag [12CA5, 1:500 dilution; or polyclonal (Babco) 1:250 dilution], Aurora-B (a kind gift of P.Sassone-Corsi, IGBMC, France; 1:200 dilution) and β -tubulin (Sigma; 1:500 dilution) were conducted at room temperature for 60 min. After washing, cells were incubated with Alexa 568- or Alexa 488-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Molecular Probes) for 60 min. Cells were finally stained with DAPI and coverslips were mounted using Prolong Antifade (Molecular Probes). Samples were examined and pictures acquired on a ZeissAxioPhot microscope equipped with a Photometrics Sensys (Oberkochen) cooled CDD camera using Image 2.0.5 software (ONCOR).

BrdU incorporation assays

For S phase analysis, siRNA-transfected cells grown on coverslips were incubated with BrdU (10 nM) and BrdU incorporation determined by immunofluorescence, using the BrdU labeling and detection kit I (Roche Molecular Biochemicals), which involves ethanol fixation. Subsequently, cells were co-stained with DAPI and α HCF-1 or α HA antibodies as described above.

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