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E2F Activation of S Phase Promoters via Association with HCF-1 and the MLL Family of Histone H3K4 Methyltransferases

Shweta Tyagi,[1,2] Anna Lena Chabes,[2,3] Joanna Wysocka,[2,4] and Winship Herr[1,2,*]
[1] Center for Integrative Genomics, University of Lausanne, Génopode, 1015 Lausanne, Switzerland
[2] Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
[3] Present address: Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87 Umeå, Sweden.
[4] Present address: Department of Chemical and Systems Biology, Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA.
*Correspondence: winship.herr@unil.ch
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SUMMARY

E2F transcriptional regulators control human-cell proliferation by repressing and activating the transcription of genes required for cell-cycle progression, particularly the S phase. E2F proteins repress transcription in association with retinoblastoma pocket proteins, but less is known about how they activate transcription. Here, we show that the human G1 phase regulator HCF-1 associates with both activator (E2F1 and E2F3a) and repressor (E2F4) E2F proteins, properties that are conserved in insect cells. Human HCF-1-E2F interactions are versatile: their associations and binding to E2F-responsive promoters are cell-cycle selective, and HCF-1 displays coactivator properties when bound to the E2F1 activator and corepressor properties when bound to the E2F4 repressor. During the G1-to-S phase transition, HCF-1 recruits the mixed-lineage leukemia (MLL) and Set-1 histone H3 lysine 4 methyltransferases to E2F-responsive promoters and induces histone methylation and transcriptional activation. These results suggest that HCF-1 induces cell-cycle-specific transcriptional activation by E2F proteins to promote cell proliferation.

INTRODUCTION

A central aspect of development and disease is the control of cell proliferation through regulation of the cell cycle. A key step in this regulation is the control of the passage from the G1 to S phases of the cycle. This critical passage is tightly coupled to the transcriptional control of genes involved in growth and DNA replication. In mammalian cells, this temporal control of gene expression is performed primarily by the E2F family of transcription factors (reviewed by Trimarchi and Lees [2002], Blais and Dynlacht [2004], and Dimova and Dyson [2005]).

E2F transcription factors, originally discovered as activators of adenovirus transcription, are heterodimeric transcription factors that consist of one member from the E2F protein family, called E2F1–E2F8, and another member from the DP protein family, called DP1 and DP2. Of the E2F proteins, E2F1–E2F5 represent a subfamily that shares the property of binding one or more members of the retinoblastoma (Rb) "pocket" protein family pRb, p107, and p130. The activation or repression specificity of E2F factors is largely conferred by the E2F protein subunit. Among the E2F1–E2F5 proteins, E2F1, E2F2, and E2F3a primarily activate transcription ("activator E2Fs"), and E2F3b, E2F4, and E2F5 primarily repress transcription ("repressor E2Fs").

The transcriptional activity of E2F transcription factors is modulated by multiple mechanisms. In quiescent or early G1 cells, repressor E2Fs bind p107 or p130 and inhibit transcription (reviewed by Dyson [1998] and Harbour and Dean [2000]). As cells progress through the G1 phase, activator E2Fs replace the repressor E2Fs on promoters but repression continues via association of activator E2Fs with the pRb pocket protein, which recruits the Sin3 histone deacetylase (HDAC), Su(Var) 39 histone methyltransferase, and SWI/SNF chromatin remodeler to promoters. Eventually, the activator E2Fs are freed of the repressive pRb complexes by cyclin-CDK-induced pRb phosphorylation (Dyson, 1998), allowing activation of transcription of genes required for S phase. Thus, E2F factors are important positive and negative regulators of the cell cycle. Although some E2F coactivators are known (e.g., histone acetyltransferases [HATs]; Hsu et al., 2001; Lang et al., 2001; Louie et al., 2004; El Messaoudi et al., 2006), the molecular details of how E2Fs effect passage into the S phase are relatively poorly understood. Here, we describe that the human G1 phase regulator HCF-1 is a key player in E2F-mediated transcriptional activation.

Human HCF-1, for herpes simplex virus (HSV) *h*ost c*e*ll *f*actor-1, is an important regulator of multiple phases of the cell cycle. It is conserved in animals and associates with

a variety of histone-modifying activities, including the trithorax-related mixed-lineage leukemia (MLL) and Set1 histone H3 lysine 4 methyltransferases (H3K4 HMTs), Sin3 HDAC, and MOF HAT (Wysocka et al., 2003; Yokoyama et al., 2004; Dou et al., 2005; Smith et al., 2005). Native HCF-1 is a heterodimeric complex of N- and C-terminal HCF-1_N and HCF-1_C subunits resulting from proteolytic maturation of a single precursor protein (Wilson et al., 1993, 1995; Kristie et al., 1995). The HCF-1_N subunit is responsible for promoting G1 phase progression, and the HCF-1_C subunit is involved in proper M phase progression (Goto et al., 1997; Julien and Herr, 2003). A molecular understanding of how HCF-1 supports cell-cycle progression is lacking, although in HSV-infected cells HCF-1 is known to stabilize a multiprotein-DNA transcriptional regulatory complex with the virion protein VP16 that activates the HSV immediate-early promoters (see Gerster and Roeder [1988]; reviewed in Wysocka and Herr [2003]).

Although HCF-1 is not known to bind DNA directly, each subunit displays chromatin association activity (Wysocka et al., 2001; Julien and Herr, 2004). For example, an N-terminal HCF-1_N "Kelch" domain can tether HCF-1 to chromatin by binding the tetrapeptide motif $^{D}/_{E}$HxY (where x denotes any residue), called the *H*CF-1-*b*inding *m*otif (HBM; Freiman and Herr, 1997; Lu et al., 1998), present in some DNA-binding proteins (see Luciano and Wilson [2003]). The biological importance of HCF-1_N-chromatin association through the Kelch domain is emphasized by the finding that a single point mutation in this domain (called P134S) prevents HBM binding and causes both a temperature-induced G1 phase cell-proliferation arrest and disruption of HCF-1 chromatin association in the temperature-sensitive baby hamster kidney cell line tsBN67 (Goto et al., 1997; Wysocka et al., 2001). Interestingly, inactivation of the pRb pocket-protein family can overcome the HCF-1_{P134S}-induced G1 phase arrest without restoring HCF-1 chromatin association, a result that has suggested that HCF-1 regulates G1 phase progression by opposing the function of one or more pocket proteins (Reilly et al., 2002).

A number of the aforementioned properties of HCF-1 make it an attractive candidate to be an E2F coregulator: the E2F1 and E2F4 proteins carry the HBM sequence responsible for mediating HCF-1 association (Luciano and Wilson, 2003; Knez et al., 2006; this study); pocket-protein inactivation can overcome the tsBN67 HCF-1_{P134S}-induced G1 phase arrest; and HCF-1 binds histone-modifying activities associated with activation of transcription (e.g., MLL family of H3K4 HMTs). Our present studies indicate that HCF-1 has multiple roles as a coregulator of E2F functions and show that it plays a direct role in the activation of E2F-responsive promoters through the cell-cycle-specific recruitment of the MLL family of H3K4 HMTs.

RESULTS

HeLa Cell E2F Protein Association with HCF-1

To initiate our study of E2F protein-HCF-1 association, we (1) prepared HeLa cell nuclear extracts, (2) immunoprecipitated HCF-1, and (3) probed the immunoprecipitates for E2F1, E2F2, E2F3 (a or b), and E2F4 proteins by immunoblot analysis. As shown in Figure 1A, E2F1, E2F3, and E2F4 proteins, but not E2F2, were recovered in the HCF-1, but not mock, immunoprecipitates (compare lanes 1–3), indicating that HCF-1 associates with multiple members of the E2F protein family. Related to these observations, Knez et al. (2006) have previously observed native HeLa cell E2F4-HCF-1 association as well as the association of ectopically overexpressed E2F1 and HCF-1 molecules in hamster cells. HCF-1 association with both activator and repressor E2Fs suggests that HCF-1 is involved in E2F-mediated transcriptional activation and repression.

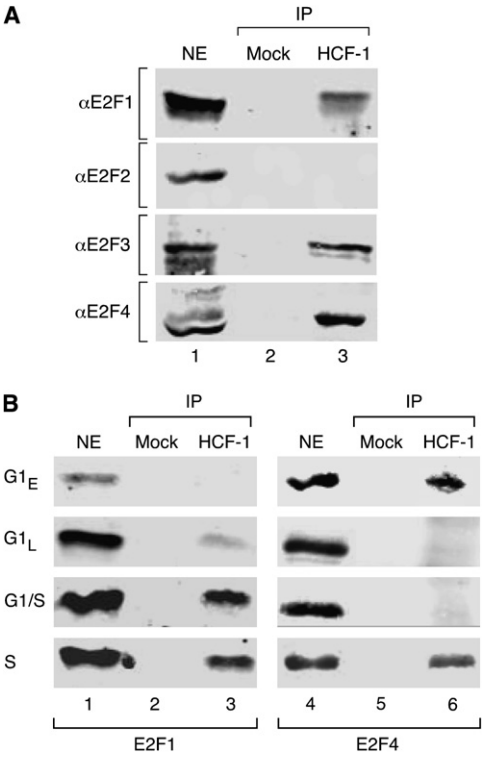

Figure 1. HCF-1 Associates with E2F Proteins

(A) Native HCF-1 association with endogenous HeLa cell E2F proteins. A nuclear extract was immunoprecipitated with anti-HCF-1 (lane 3) or nonimmune (lane 2) sera, and recovered E2F proteins were visualized by immunoblot with anti-E2F1, E2F2, E2F3, and E2F4 antisera. NE (lane 1), 30 μg HeLa cell nuclear extract (3% input).

(B) HCF-1 associates with activator and repressor E2Fs differentially during the cell cycle. Synchronized HeLa cell nuclear extracts (see the Supplemental Data) were immunoprecipitated with anti-HCF-1 (lanes 3 and 6) and nonimmune (lanes 2 and 5) sera and analyzed by immunoblot with anti-E2F1 (lanes 1–3) and E2F4 (lanes 4–6) antisera. NE (lanes 1 and 4), 30 μg HeLa cell nuclear extract (3% input).

HCF-1 Associates with Activator and Repressor E2Fs at Different Stages of the Cell Cycle

Although HCF-1 is required for cell-cycle progression (Goto et al., 1997), its levels and nuclear localization are not known to be cell-cycle regulated (see Figure S1Ba in

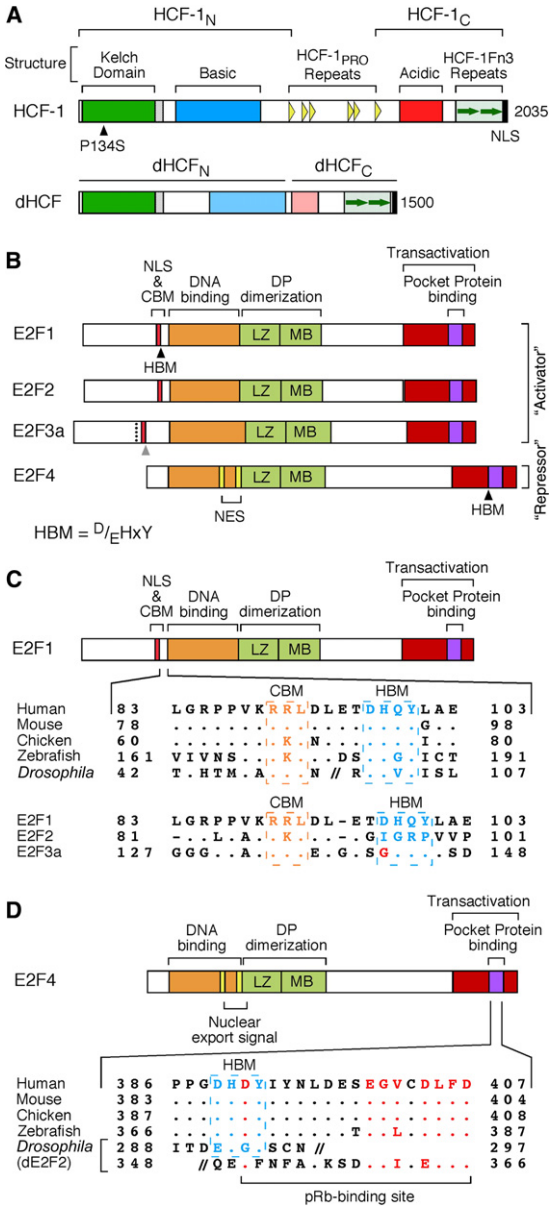

Figure 2. Conserved HBMs in E2F Transcription Factors

(A) Schematic structure of human HCF-1 and *Drosophila* dHCF. Structural elements are labeled above HCF-1, and related elements are shown similarly for dHCF. HCF-1_{PRO}, HCF-1 proteolytic processing repeats; HCF-1_{Fn3}, fibronectin type 3 repeats; NLS, nuclear localization signal. The position of the HCF-1 tsBN67 P134S mutation is indicated.

(B) Schematic structure of human activator E2F1–E2F3a and repressor E2F4 proteins. Functional elements are indicated. CBM, cyclin binding motif; LZ, leucine zipper; MB, marked box; NES, nuclear export signal; NLS, nuclear localization signal. Black and gray arrowheads, positions of perfect and imperfect *H*CF-1-binding *m*otifs (HBM), respectively. Dashed line in E2F3 indicates E2F3b N terminus.

(C) Conserved E2F1 HBM sequences. (Top) sequence alignment of E2F1 HBM region from human (NP_005216), mouse (AAL90789), chicken (NP_990550), zebrafish (XP_696201), and *Drosophila* (NP_524437) E2F1. CBM (orange) and HBM (blue) sequences are indicated. Dots indicate identity with the human sequence. // denotes missing *Drosophila* dE2F1 amino acids 53–99. (Bottom) sequence alignment of human E2F1, E2F2 (NP_004082), and E2F3a (NP_001940) proteins. – indicates absence of the corresponding amino acid.

(D) Conserved E2F4 HBM sequence. Sequence alignment of HBM (blue) and pRb-binding site (red, Lee et al., 2002) from human (NP_001941), mouse (NP_683754), chicken (CAG32002), and zebrafish (AAH56832) E2F4, and *Drosophila* dE2F2 (NP_477355). dE2F2 has nonoverlapping HBM and pRb-binding sites. // denotes missing dE2F2 amino acids 298–347.

the Supplemental Data available with this article online), suggesting that HCF-1 regulates the cell cycle through temporally regulated association with effector proteins. Thus, we asked whether nuclear HCF-1 association with the activator E2F1 and repressor E2F4 differs during the cell cycle. We synchronized cells in G1/S by double thymidine block treatment and isolated cells at additional cell-cycle stages by differential timed release in normal media. Given our interest in G1 and S phase progression, we used cell populations synchronized for the early G1 ($G1_E$), late G1 ($G1_L$), G1/S, and S phases (Figure S1).

As shown in Figure 1B, consistent with a G1-to-S expression pattern, E2F1 increased from the $G1_E$ to the G1/S fractions and then decreased from the S to $G1_E$ fractions (lane 1; see also Figure S1B). Nonetheless, HCF-1 association with E2F1 was delayed toward the G1/S and S phase fractions (compare lanes 1 and 3). In contrast, as expected (Lindeman et al., 1997), nuclear E2F4 protein was present throughout the cell cycle (lane 4) but only bound HCF-1 primarily in the $G1_E$ and S phase fractions (compare lanes 4 and 6). Thus, HCF-1 interacts with activator E2F1 and repressor E2F4 molecules during respective cell-cycle phases in which these E2F molecules regulate the cell cycle (e.g., E2F1 at G1/S and E2F4 at $G1_E$), suggesting an active role for HCF-1 in cell-cycle control by E2F proteins.

Some E2F Proteins Possess Conserved HBMs and HBM-like Sequences

The HCF and E2F proteins used in this study are shown schematically in Figure 2. Figure 2A illustrates human and *Drosophila melanogaster* HCF proteins, highlighting the conserved regions. Although *Drosophila* HCF (dHCF) lacks the HCF-1_{PRO} repeats responsible for HCF-1 proteolytic cleavage (Kristie et al., 1995; Wilson et al., 1995), through an unknown process it also undergoes proteolytic maturation into $dHCF_N$ and $dHCF_C$ subunits (Mahajan et al., 2003; Guelman et al., 2006).

Figure 2B illustrates E2F1–E2F4, with HBMs indicated by arrowheads. Note that E2F3a and E2F3b differ in the N-terminal region—here we only used the E2F3a "activator" form. Consistent with their HCF-1 association, E2F1 and E2F4 have HBMs but in distinctly different positions: in E2F1, it lies in the N-terminal region adjacent to the cyclin A binding motif (CBM) RxL (Krek et al., 1994; Xu et al., 1994; Lowe et al., 2002), and in E2F4 it overlaps the pocket protein-binding site near the C terminus (see Lee et al. [2002]). Interestingly, the E2F1 (Figure 2C) and E2F4 (Figure 2D) HBMs are highly conserved in vertebrates and even insects (e.g., *Drosophila*).

Consistent with the lack of association, the E2F2 protein, although very similar to E2F1, does not possess an HBM (Figure 2C, bottom). To our surprise, although E2F3a and/or E2F3b associates with HCF-1, neither has a canonical HBM. Instead, they have a degenerate albeit conserved (data not shown) HBM-like sequence (GHQY, variant position underlined) at a position corresponding with that of the E2F1 HBM (Figure 2C, bottom).

The HCF-1 Kelch Domain Interacts with E2F3a and E2F4, but Not E2F2, in a Yeast Two-Hybrid Assay

To dissect HCF-1-E2F interactions, we used the yeast two-hybrid assay. The human HCF-1 Kelch domain was fused to the GAL4 DNA-binding domain (DBD) as "bait." Full-length E2F1–E2F4 sequences were fused to the GAL4 transcriptional activation domain (AD) as "prey." Two-hybrid specificity controls showed that each individual bait and prey molecule did not sustain histidine-independent yeast growth (Figure S2a), but all four E2F-prey proteins were expressed as they scored positive with a DP1 bait protein (Bandara et al., 1993) as shown in Figure 3Aa.

Consistent with the HeLa cell association results (Figure 1A), E2F3a and E2F4 scored positive and E2F2 scored negative for HCF-1 Kelch-domain interaction in the two-hybrid assay (Figure 3Ab). And consistent with a biologically relevant Kelch-domain interaction, the tsBN67 P134S Kelch-domain mutation (Figure 3Ac) and the E2F3a G142A and E2F4 D389A HBM mutations (Figure 3Ad) interfered with the interaction. The HCF-1 Kelch-domain interaction with E2F4 is consistent with that described by Knez et al. (2006); in contrast, the yeast two-hybrid assay did not detect any significant E2F4 interaction with the HCF-1 Basic region (Figure S2b). The unexpected result with E2F3a, in which a divergent HBM appears to direct E2F3a association with the Kelch domain, suggests that E2F3 proteins possess an atypical albeit functional HBM.

Evidence for an E2F1 Autoinhibitory Association with HCF-1

Although E2F1 associated with HCF-1 in HeLa cells (Figure 1A), the full-length HBM-containing E2F1 protein did not interact with the HCF-1 Kelch domain in the two-hybrid assay (Figure 3Ab), even though it interacts with DP1 (Figure 3Aa) and contained no point mutations (data not shown). In contrast, a 153 amino-acid C-terminal E2F1 deletion activated HBM-dependent HCF-1 Kelch domain interaction (compare E2F1[1–284] with the HBM mutant E2F1[1–284, D97A]) (Figure 3Ba) that is sensitive to the P134S Kelch-domain mutation (Figure 3Bb). This result suggested that full-length E2F1 contains inhibitory sequences that prevent HBM recognition. Indeed, a series of E2F1 N- and C-terminal truncations, with even one as small as seven amino acids (E2F1[1–430]), interacted with the HCF-1 Kelch domain as long as they retained the HBM (Figure 3B, bottom). The unexpected inactivity of the full-length E2F1 prey construct was verified by showing that a de novo full-length E2F1 prey construct from two active N- and C-terminal truncations (E2F1[94–437] and E2F1 [1–402]) was inactive (Figure 3B, construct 1–281 + 282–437). We conclude that full-length E2F1 has regions that can inhibit its own interaction with the HCF-1 Kelch domain.

This proposed autoinhibition depends on the nature of the E2F1 HBM, because when the E2F1 HBM and surrounding sequence are replaced with the corresponding prototypical VP16 HBM sequence (LETDHQYLAE to VMREHAYSRA, HBM underlined), the resulting full-length $E2F1_{VP16HBM}$ molecule interacts with the HCF-1 Kelch domain (Figure 3Bc). Together with the observed E2F1-HCF-1 association in HeLa cells, these two-hybrid results suggest that E2F1 has a latent ability to associate with HCF-1.

HCF-1-E2F Interactions Have Been Conserved during Evolution

As the HCF-1 Kelch domain and the E2F1 and E2F4 HBMs are conserved in *Drosophila* (see Figure 2), we tested whether there is an HCF-1-E2F interaction in insect cells. Indeed, dHCF is recovered in endogenous dE2F1 and dE2F2 (the ortholog of human E2F4) immunoprecipitates of a *Drosophila* SL2 extract (Figure 3C). (Recovery of dE2F1 and dE2F2 in dHCF immunoprecipitates is shown in Figure S3.) Furthermore, dE2F1 and dE2F2 interact with both the dHCF and human HCF-1 Kelch domains and human E2F1(1–284) interacts with the dHCF Kelch domain in the two-hybrid assay (Figure 3D). These cross-species interactions suggest that HCF-1 Kelch domain-E2F HBM interactions have been highly conserved; in contrast, the human E2F1 autoinhibition has not been conserved in *Drosophila*.

Selective Association of Ash2-Containing H3K4 HMTs and Sin3 HDAC with HCF-1-E2F1 and HCF-1-E2F4 Complexes

As aforementioned, HCF-1 associates with chromatin-modifying activities associated with both activation (e.g., MLL and Set1 H3K4 HMTs) and repression (i.e., Sin3 HDAC) of transcription (Wysocka et al., 2003; Yokoyama et al., 2004; Dou et al., 2005). The activator MLL and Set1 H3K4 HMTs are protein complexes that each possess three shared subunits, Ash2, WDR5, and RBP5, and a unique SET-domain catalytic subunit. Interestingly, HCF-1 can associate with the "activator" Set1 H3K4 HMT complex and "repressor" Sin3 HDAC complex simultaneously, but, when bound to the viral transcriptional activator VP16, HCF-1 associates with the Set1 H3K4 HMT but not the Sin3 HDAC, suggesting preferential association of an activator with HCF-1 complexes containing activating histone-modifying activities (Wysocka et al., 2003). The finding here that HCF-1 can bind to cellular transcriptional regulators associated with either activation (i.e., E2F1) or repression (i.e., E2F4) led us to ask whether HCF-1-E2F1 and HCF-1-E2F4 complexes might display selective association with the HMT or HDAC activities in HeLa cells.

To answer this question, we used an HeLa cell line expressing a Flag-epitope-tagged $HCF\text{-}1_N$ subunit (called

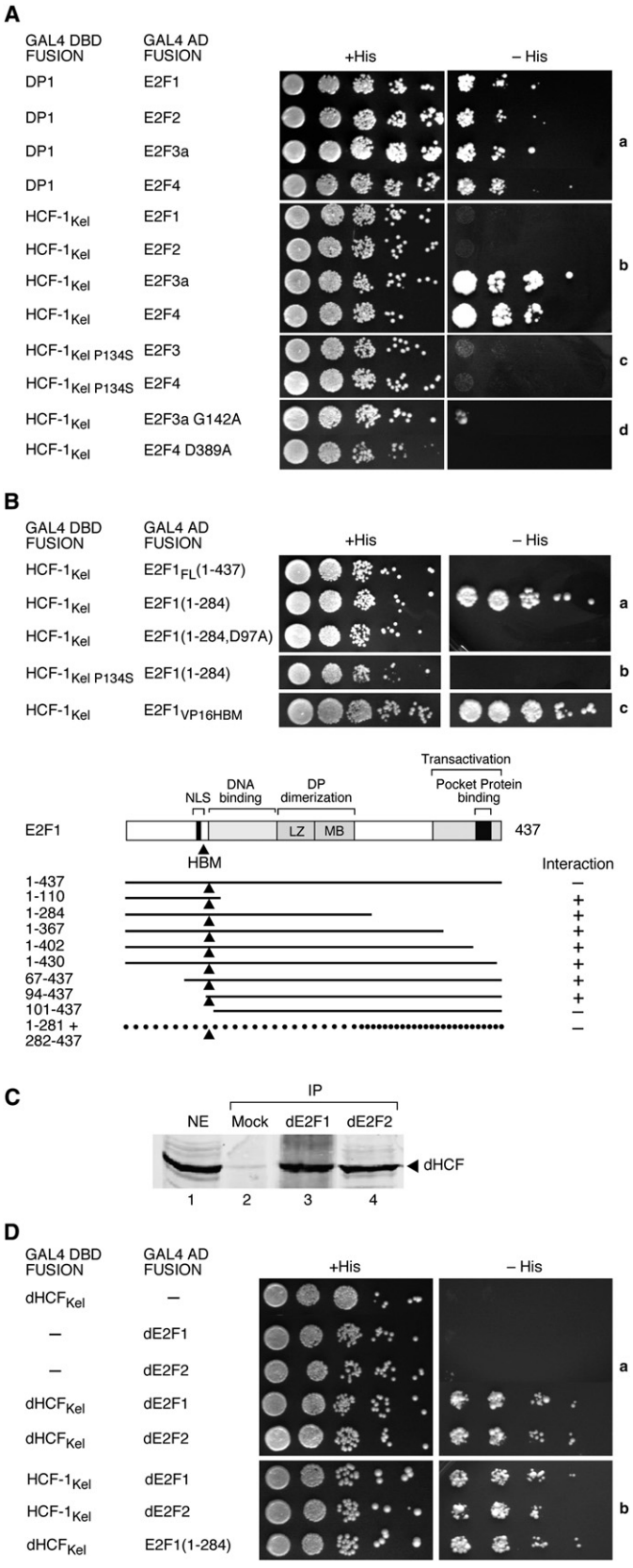

Figure 3. HCF-1 Interacts with E2F1, E2F3, and E2F4, but Not E2F2

(A) E2F-HCF-1 yeast two-hybrid assay. Yeast strain PJ69 cotransformed with GAL4-DBD bait and GAL4-AD prey fusion expression plasmids, as indicated, was assayed for growth with and without histidine (see Experimental Procedures).

(B) Mutant E2F1-HCF-1 yeast two-hybrid assay. (Top) assay as in (A). $E2F1_{VP16HBM}$, replacement of E2F1 HBM by VP16 HBM (see text). (Bottom) interaction of N- and C-terminal E2F1 deletion mutants with the HCF-1 Kelch domain. Positive (+) or negative (–) interaction is indicated. The position of the HBM is indicated (▲). The full-length E2F1(1–281 + 282–437) construct (shown by the differentially dotted line) was created by replacing the pGADGH-E2F1(94–437) BamHI-BglII fragment with that from pGADGH-E2F1(1–402).

(C) *Drosophila* dE2F-dHCF association. An SL2 nuclear extract was immunoprecipitated with anti-dE2F1 (lane 3), anti-dE2F2 (lane 4), or nonimmune (lane 2) sera, and recovered dHCF was analyzed by immunoblot with rabbit anti-dHCF antibody. NE (lane 1), 30 μg SL2 nuclear extract (5% input).

(D) *Drosophila* dE2F-dHCF (Da) and human and *Drosophila* E2F-HCF (Db) yeast two-hybrid assay. Assay as in (A). – indicates empty GAL4-DBD or GAL4-AD vectors.

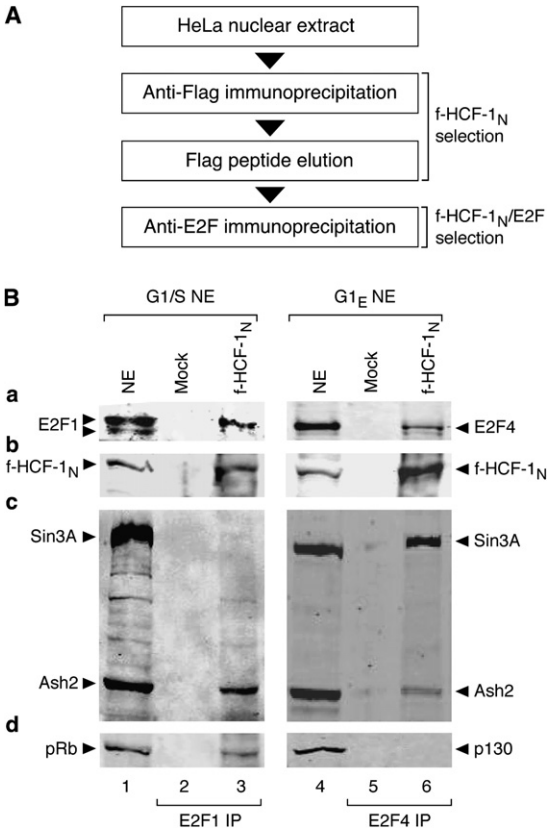

Figure 4. Selective Association of Ash2-Containing H3K4 HMT and Sin3 HDAC with HCF-1-E2F1 and HCF-1-E2F4 Complexes

(A) Schematic diagram showing double-immunoprecipitation procedure to isolate f-HCF-1_N-E2F complexes. f-HCF-1_N, Flag-epitope tagged HCF-1_N subunit.

(B) f-HCF-1_N-E2F1 (lanes 2 and 3) and f-HCF-1_N-E2F4 (lanes 5 and 6) immunoprecipitates from f-HCF-1_N (lanes 3 and 6) and normal (lanes 2 and 5) HeLa cell G1/S (lanes 2 and 3) and $G1_E$ (lanes 5 and 6) phase extracts were analyzed by immunoblot with anti-E2F1 ([Ba], lanes 1–3), E2F4 ([Ba], lanes 4–6), Flag for f-HCF-1_N (Bb), Sin3A and Ash2 (Bc), pRb ([Bd], lanes 1–3), and p130 ([Bd], lanes 4–6) antisera. NE, HeLa cell nuclear extract from G1/S (lane 1) and $G1_E$ (lane 4) phases corresponding to 0.5% (Ba–Bc) or 0.17% (Bd) of input.

f-HCF-1_N; Wysocka et al., 2001) and performed the double immunoprecipitation protocol outlined in Figure 4A—first isolating f-HCF-1_N complexes and subsequently f-HCF-1_N-containing E2F1 or E2F4 complexes—followed by immunoblot analysis for coimmunoprecipitating components. To maximize HCF-1-E2F1 and HCF-1-E2F4 complex isolation (see Figure 1B) and focus our attention on where E2F1 and E2F4 are active, we employed nuclear extracts from the G1/S phase for HCF-1-E2F1 analysis and the $G1_E$ phase for HCF-1-E2F4 analysis.

As expected, the G1/S f-HCF-1_N/E2F1 and $G1_E$ f-HCF-1_N/E2F4 immunoprecipitates both contained f-HCF-1_N, and E2F1 and E2F4, respectively (Figures 4Ba and 4Bb, compare lanes 2 and 3, and 5 and 6). To reveal coassociated MLL family H3K4 HMTs as a whole, we probed for the shared Ash2 subunit, and, to reveal coassociated Sin3 HDAC, we simultaneously probed for the Sin3A subunit (Figure 4Bc). Compared to one another, the HCF-1-E2F1 complexes bound the Ash2 polypeptide preferentially and the HCF-1-E2F4 complexes bound the Sin3A polypeptide preferentially (compare lanes 3 and 6). These differences were not reflected in the general levels of Ash2 or Sin3A association with HCF-1 in $G1_E$ and G1/S fractions (Figure S4), indicating that the differences in HCF-1-E2F1 and HCF-1-E2F4 complexes are specific to these different complexes. These results suggest that, as a coregulator, HCF-1 is able to selectively associate with activating activities (e.g., one or more MLL family H3K4 HMTs) with E2F1 in the G1/S phase and repressive activities (e.g., Sin3 HDAC) with E2F4 in the $G1_E$ phase.

pRb Can Associate with HCF-1-E2F1 Complexes in the G1/S Fraction, but p130 Fails to Associate with HCF-1-E2F4 Complexes in the $G1_E$ Fraction

As aforementioned, owing to the positions of the HBM (see Figures 2B–2D), HCF-1 and pocket-protein association with E2F4, but not E2F1, is likely to be mutually exclusive. Thus, we determined whether the G0/G1 pocket protein p130 is present in the $G1_E$ HCF-1-E2F4 complexes and pRb in the G1/S HCF-1-E2F1 complexes. Consistent with an interference between pocket-protein and HCF-1 interaction with E2F4, no p130 was detected in the isolated $G1_E$ HCF-1-E2F4 complexes (Figure 4Bd, lane 6) even though p130 associates with HCF-1-free E2F4 in these extracts (data not shown). Interestingly, however, pRb was detected in the G1/S HCF-1-E2F1 complexes (Figure 4Bd, lane 3), indicating that HCF-1 and pRb can bind simultaneously to E2F1. The electrophoretic mobility of the HCF-1-E2F1-associated pRb was phosphatase sensitive (data not shown). We conclude that at some point(s) in the G1-to-S phase transition of the HeLa cell cycle, phosphorylated pRb and HCF-1 are bound together to E2F1, perhaps a transition state between a repressive E2F1 complex and activating E2F1 complex. Consistent with it being a G1-to-S phase transition state, the levels of pRb associated with the HCF-1-E2F1 complex are significantly reduced in S phase cells (Figure S5).

HCF-1 Binds to E2F-Responsive Promoters in a Cell-Cycle-Regulated Manner

To extend the biological significance of the HCF-1-E2F protein association, we asked whether HCF-1 associates with E2F-responsive promoters by chromatin immunoprecipitation (ChIP) as shown in Figure 5A. For this analysis, we selected three E2F-regulated promoters previously analyzed in detail by ChIP—*p107*, *E2F1*, and *cyclin A* (Takahashi et al., 2000; a fourth promoter *cdc25* was also analyzed and gave analogous results, data not shown)—and a negative control from the *U2* snRNA gene, called $U2_C$ (N. Hernandez, personal communication). Consistent with previous studies (Takahashi et al., 2000; Wells et al., 2000), in asynchronous HeLa cells, E2F1 and E2F4 proteins were detected on all three promoters, but not on

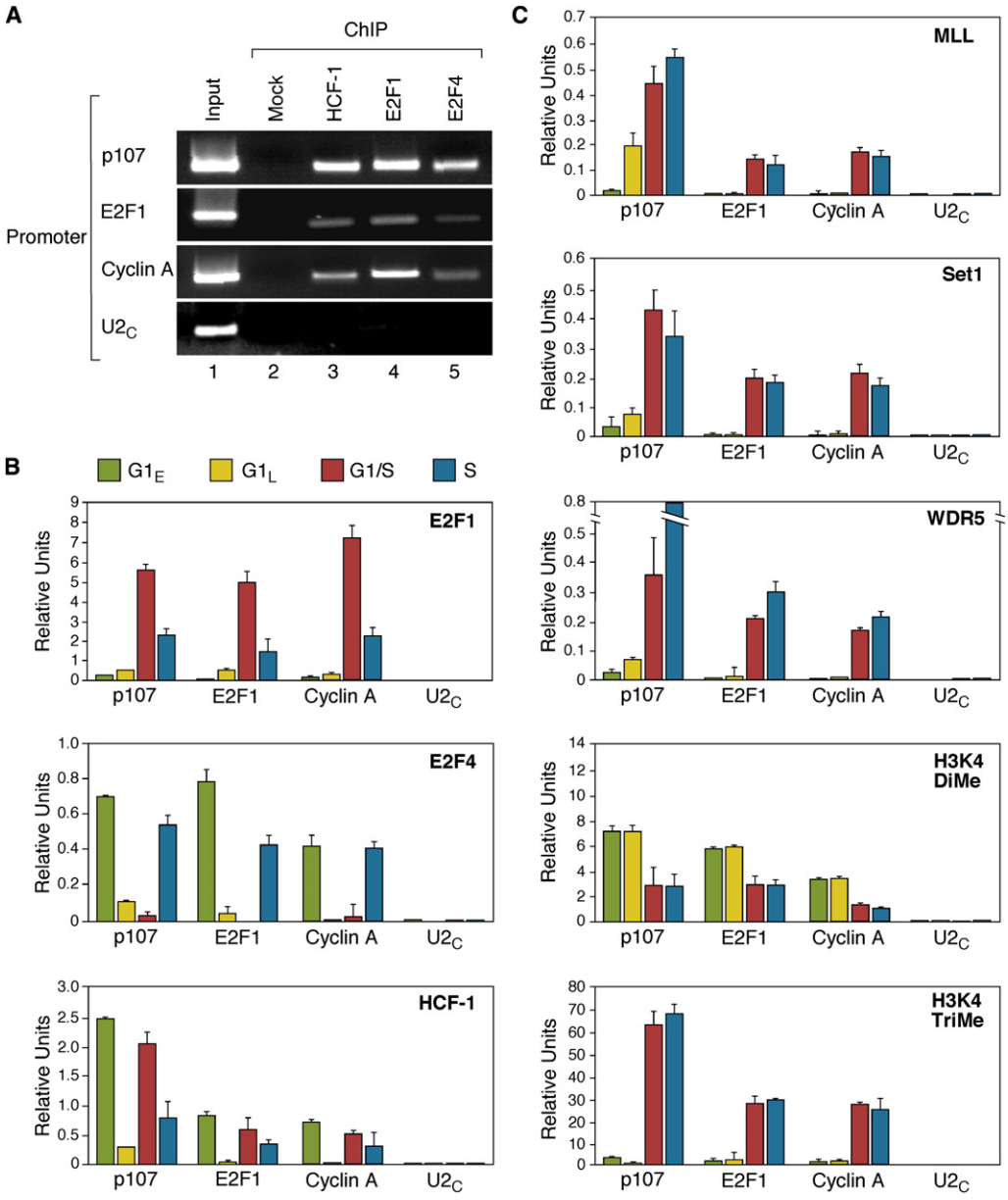

Figure 5. Cell-Cycle-Regulated HCF-1 and MLL/Set1 H3K4 HMT Association with E2F-Responsive Promoters

(A) HCF-1 binds to E2F-responsive promoters. PCR products of ChIP analyses of asynchronous HeLa cells with nonimmune (lane 2), HCF-1_N (lane 3), E2F1 (lane 4), and E2F4 (lane 5) antisera are shown. Input (lane 1) corresponds to 0.3% of ChIP input.

(B) Cell-cycle regulation of HCF-1 association with E2F-responsive promoters. Quantitation of E2F1, E2F2, and HCF-1_N ChIP analyses of $G1_E$ (green), $G1_L$ (yellow), G1/S (red), and S (blue) phase-synchronized HeLa cells by triplicate real-time PCR of the indicated promoters is shown.

(C) Cell-cycle regulation of MLL family H3K4 HMT association with and H3K4 methylation status of E2F-responsive promoters. Quantitation of MLL_C, Set1, WDR5, and H3K4 di- and trimethylation ChIP analyses of synchronized HeLa cells as in (B). MLL_N antiserum generated results analogous to MLL_C (data not shown). All ChIP experiments in (B) and (C) were performed at least three and usually six times with similar results; the results shown in (B) and (C) are from a single representative experiment. Data are represented as mean ± SD. The relatively high levels of p107 signal seen for MLL_C in the $G1_L$ phase were not reproduced in other experiments.

the $U2_C$ region (Figure 5A, lanes 4 and 5). HCF-1 was also detected on these three promoters, but not the $U2_C$ region (lane 3; note that although negative in the ChIP results shown here, $U2_C$ was positive for histone H3 ChIP, Figure S6). Thus, during at least some point of the cell cycle, HCF-1 is likely present on these E2F-responsive promoters.

Because HCF-1 interacts with E2F1 and E2F4 at different stages of the cell cycle, we reasoned that HCF-1 might occupy these E2F-responsive promoters in a

cell-cycle-dependent manner. We therefore performed ChIP analysis with HeLa cells synchronized as in Figure 1B. Consistent with differential association of E2F1 and E2F4 with E2F-responsive promoters through the cell cycle, E2F1 bound the three promoters more prevalently in the G1/S and S phase fractions and E2F4 bound more prevalently in the S and early $G1_E$ fractions (Figure 5B). (We note that the cell-cycle phase-specific E2F1 promoter occupancy we observe is delayed compared to Takahashi et al. [2000], which could result from the different cells [HeLa versus T98G] or cell-synchronization strategies [thymidine block versus serum starvation] used.) Interestingly, HCF-1 displayed an E2F-responsive promoter occupancy that resembles the sum of the E2F1 and E2F4 protein-promoter occupancy, precisely the phases during which E2F1- or E2F4-HCF-1 association is observed (Figure 1B). These results suggest HCF-1 involvement in both E2F1 and E2F4 transcriptional regulation of E2F-responsive promoters.

Cell-Cycle-Specific Recruitment of MLL and Set1 H3K4 HMT Complexes to E2F-Responsive Promoters

The selective association of Ash2 with HCF-1-E2F1 protein complexes (Figure 4) prompted us to ask whether MLL H3K4 HMT family members occupy the E2F-responsive promoters in a cell-cycle phase-specific manner. We therefore performed ChIP analysis for the MLL and Set1 H3K4 HMT catalytic subunits and their shared WDR5 subunit (Figure 5C). Remarkably, all three proteins showed similar cell-cycle-dependent promoter binding patterns to E2F1, consistent with the preferential association of H3K4 HMT complexes with E2F1 as opposed to E2F4 (Figure 4). These results suggest that E2F1 recruits H3K4 HMT activities to E2F-responsive promoters around the transition from the G1 to S phases.

Because H3K4 HMTs methylate H3K4, we tested for di- and trimethylation of H3K4 at these promoters (Figure 5C). H3K4 dimethylation was relatively constant in the four samples. In contrast, the H3K4 trimethylation was enriched in the G1/S and S samples coinciding with E2F1 and H3K4 HMT occupancy, suggesting that trimethylation more closely tracks the transcriptional activation status of these promoters than does dimethylation.

HCF-1 Recruits MLL and Set1 H3K4 HMT Complexes to E2F-Responsive Promoters and Participates in E2F-Responsive Promoter Activation

The results in Figures 4 and 5 led us to hypothesize that HCF-1 recruits H3K4 HMT complexes to E2F-responsive promoters during the G1-to-S phase transition. If true, HCF-1 depletion should lead to loss of H3K4 HMT recruitment to E2F-responsive promoters at the G1-to-S phase transition. We therefore performed siRNA knockdown of HCF-1 in HeLa cells (Julien and Herr, 2003) and ChIP analysis of G1/S-synchronized HCF-1-depleted cells. To minimize long-term effects of loss of HCF-1, we analyzed cells 2 days after initial siRNA treatment, a time point at which HCF-1 is depleted but the cells have not stopped proliferating (Julien and Herr, 2004; see Figure S7A), thus permitting G1/S phase cell isolation.

HCF-1 protein levels (Figure 6Ba) and hence E2F-responsive promoter binding (Figure 6A) were successfully knocked down by the 2 day siRNA treatment. In contrast, E2F1 binding was largely unaffected (Figure 6A). Significantly, however, there was a dramatic decrease in H3K4 HMT-component (i.e., MLL, Set1, and WDR5) occupancy on these promoters (Figure 6A). In an independent replicate experiment where the HCF-1 siRNA-induced depletion was only about 50%, the decrease in MLL, Set1, and WDR5 signal was correspondingly attenuated (see Figures S7C and S7D). To show that the H3K4 HMT-component decreases reflected promoter occupancy specifically and not just protein depletion, we probed for WDR5 in the HCF-1 siRNA-treated cells and did not detect any change in its levels (Figure S7B). These results suggest that HCF-1 serves as an intermediary to recruit MLL family H3K4 HMT activities to E2F-responsive promoters. Consistent with this conclusion, in contrast to minimal effects on global H3K4 trimethylation by HCF-1 siRNA treatment (Figure 6Bb), the E2F-responsive promoter H3K4 trimethylation levels were significantly reduced (Figure 6A).

These results suggest that during the transition from the G1 to the S phase, HCF-1 directs the recruitment of MLL family H3K4 HMT activity to E2F-responsive promoters, which results in H3K4 trimethylation and transcriptional activation. To test this hypothesis, we performed real time RT-PCR analysis of *p107*, *E2F1*, and *cyclin A* transcripts and compared their levels to those of *GAPDH* transcripts. Consistent with the requirement of HCF-1 for activation of these promoters, 72 hr HCF-1 siRNA treatment leads to their inactivity (Figure 6C). Furthermore, in the G1/S phase cells after 48 hr siRNA treatment where E2F1 is still bound, HCF-1 depletion leads to a marked reduction in the levels of *p107*, *E2F1*, and *cyclin A* transcripts (Figure 6C). The continued presence of promoter-bound E2F1 but loss of transcripts suggests that HCF-1 is an important effector of E2F1 transcriptional activation during the G1-to-S phase. These results suggest that a major element of how E2F1 brings about the activation of E2F-responsive promoters is by recruiting HCF-1 and its associated MLL family H3K4 HMT complexes to these promoters.

DISCUSSION

Our studies have revealed mechanisms by which E2F cell-proliferation transcription factors function through conserved associations with the G1 phase regulator HCF-1. Both activator and repressor E2F proteins associate with HCF-1 and, depending on the E2F factor, HCF-1 associates with "activating" H3K4 HMT complexes (e.g., with the activator E2F1) or with the "repressive" Sin3 HDAC complex (e.g., with the repressor E2F4). These selective associations are cell cycle dependent on E2F-responsive promoters in concert with the expected activity of E2F1 and

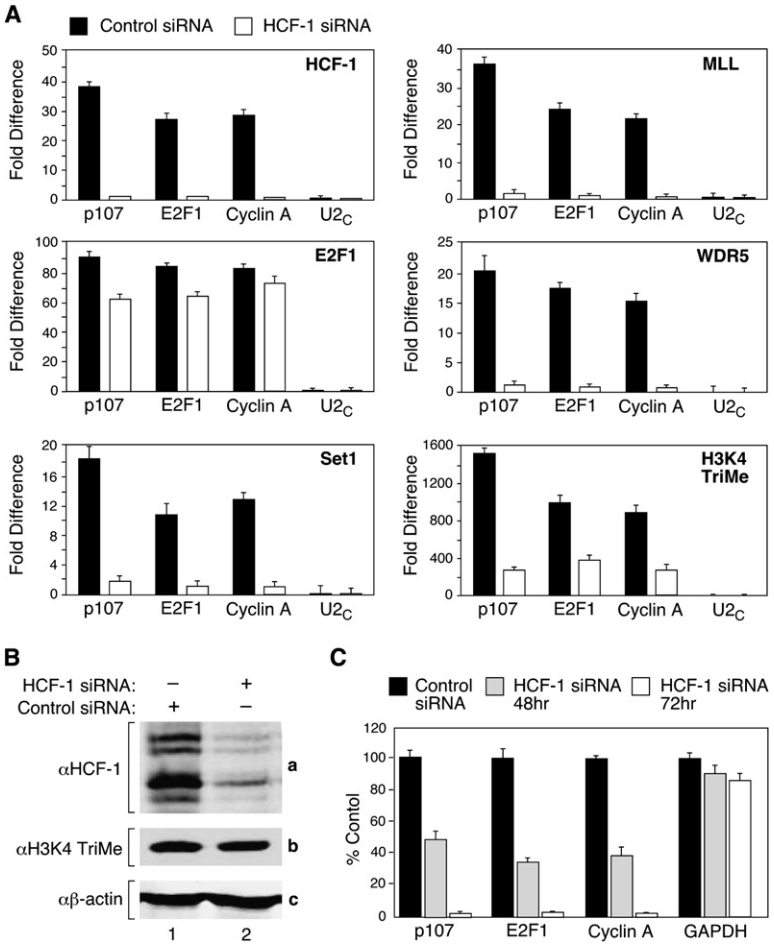

Figure 6. HCF-1 Recruits MLL/Set1 H3K4 HMT Complexes to E2F-Responsive Promoters and Participates in Their Transcriptional Activation

(A) HCF-1 recruitment of MLL/Set1 H3K4 HMT complexes. ChIP analyses of G1/S-synchronized control (black) and HCF-1 (white) siRNA-treated HeLa cells using the indicated antisera. The signals for HCF-1 siRNA-treated cells immunoprecipitated with the anti-HCF-1 antiserum are set to 1, with all other signals indicated as relative-fold differences.

(B) HCF-1 and trimethylated H3K4 levels in siRNA-treated cells. Immunoblot analysis of HCF-1 (Ba), trimethylated H3K4 (Bb), and β-actin (Bc) proteins from control (lane 1) and HCF-1 (lane 2) siRNA-treated G1/S-synchronized cells used in (A) is shown.

(C) HCF-1 depletion is accompanied by reduced expression of E2F-regulated genes. Total RNA isolated from HCF-1 and control siRNA-treated cells was amplified using real-time RT-PCR for the indicated mRNAs. Signals from 48 hr control siRNA-treated cells (black) are set at 100% for each gene, and signals in 48 hr (gray) and 72 hr (white) HCF-1 siRNA-treated cells are indicated as the percentage of control. All p107, E2F1, and cyclin A signals were normalized to the GAPDH mRNA signals from the respective siRNA treatment. GAPDH signals shown are not normalized. Data are represented as mean ± SD.

E2F4. Significantly, during G1-to-S phase progression, HCF-1 recruits MLL family H3K4 HMT complexes to E2F-responsive promoters and stimulates both H3K4 trimethylation at these promoters and the levels of the associated transcripts. We propose that HCF-1 promotes passage from the G1 phase to the S phase of the cell cycle by supporting transcriptional activation by E2F1.

A Diversity of E2F Factor-HCF-1 Molecular Interactions

This study has revealed diverse interactions between E2F1–E2F4 and the HCF-1 Kelch domain. Thus, E2F4 displays direct interaction with HCF-1 through a canonical HBM whereas E2F2, which lacks an HBM, does not associate with HCF-1. In contrast, E2F1, while possessing a canonical HBM, inhibits its own association with HCF-1 via a form of autoinhibition that is exquisitely sensitive to the integrity of the E2F1 molecule: small deletions of E2F1 or changes to its HBM can activate E2F1 HBM association with HCF-1 (Figure 3). This autoinhibition is overcome in HeLa cells by an unknown mechanism. We suggest that posttranslational modification (e.g., phosphorylation) is a likely mechanism. Lastly, E2F3a associates with HCF-1 without a canonical HBM, suggesting additional diversity in HBM sequences.

There is also the diversity in the temporal association of HCF-1 with the activator E2F1 and the repressor E2F4, revealed by the association of HCF-1 with E2F1 in the G1/S and S phase samples and with E2F4 in the S and $G1_E$ samples. And, in these different points of the cell cycle, HCF-1 is recruiting either (1) a "repressive" chromatin modifier—the Sin3 HDAC—to E2F4 when E2F-responsive promoters are repressed or (2) "activating" chromatin modifiers—the MLL family of H3K4 HMTs—to E2F1 when E2F-responsive promoters are active as illustrated in Figure 7A. Thus, HCF-1 plays a directive role in the nature of transcriptional regulatory complexes formed by sequence-specific transcriptional regulators.

Multiple Mechanisms for Sin3 HDAC Promoter Recruitment by E2F4

The repressor E2F4 associates with pocket proteins, in particular p107 and p130, which themselves associate with the Sin3 HDAC corepressor (Lai et al., 1999). Interestingly, the E2F4 HBM overlaps the pocket protein-binding site (Figure 2) and p130 does not associate with

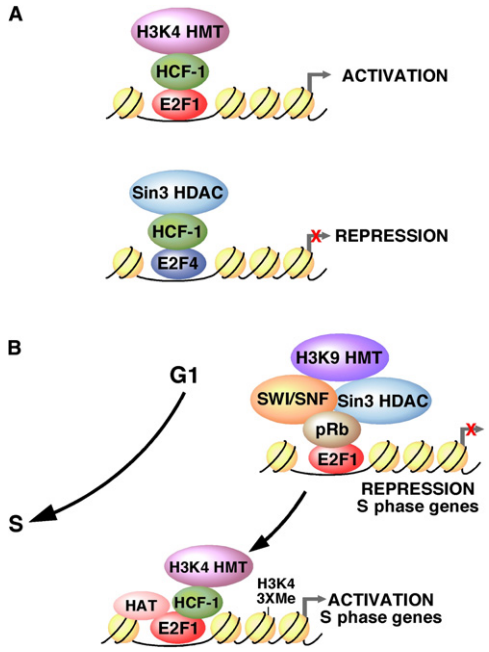

Figure 7. HCF-1, a Coregulator of E2F-Regulated Transcription

(A) Model of HCF-1 as a coregulator of both E2F-mediated activation and repression of transcription. The model proposes that HCF-1 selectively associates with "activator" H3K4 HMT complexes, when bound to E2F1, to activate transcription and with "repressor" Sin3 HDAC complex, when bound to E2F4, to repress transcription.

(B) Model for E2F1-repressive to E2F1-activating complexes during the G1-to-S phase transition. See text for details.

HCF-1-bound E2F4 (Figure 4), suggesting that pocket-protein and HCF-1 association with E2F4 is mutually exclusive. Nevertheless, when HCF-1 is bound to E2F4, the Sin3 HDAC is also present. Thus, E2F4 may repress transcription of E2F-responsive promoters by recruiting the Sin3 HDAC either through association with pocket proteins or HCF-1. Interestingly, p107 or p130 is not present on about 75% of E2F4 target genes in early G1 phase (Balciunaite et al., 2005) and Sin3 recruitment to some E2F-responsive promoters is unaffected in $p107^{-/-}p130^{-/-}$ MEFs (Rayman et al., 2002). These results suggest that pocket proteins are not the only proteins involved in E2F4-mediated repression, and our results suggest that HCF-1 is an important player in recruiting Sin3 HDAC complexes to E2F4-regulated promoters.

A Model for Cell-Cycle-Regulated Transcriptional Activation by E2F1

Figure 7B presents a simple view of how we imagine that E2F1 effects G1-to-S phase activation of E2F-responsive promoters. It involves a transition from E2F1-bound pRb-mediated repressive complexes to E2F1-bound HCF-1-mediated MLL family H3K4 HMT complexes that lead to H3K4 methylation and transcriptional activation. The MLL family of H3K4 HMTs is a particularly attractive mediator of E2F transcriptional activation, because H3K4 trimethylation is known to directly recruit the NURF chromatin remodeler to support gene expression (Wysocka et al., 2006).

Gene-Specific Recruitment of MLL

Our study provides insights into how MLL, the best studied of MLL family H3K4 HMTs (see Ruthenburg et al. [2007]), is recruited to chromatin in a gene- and cell-cycle-specific manner. Despite considerable progress in understanding the molecular function of MLL, largely precipitated by its involvement in human leukemias (reviewed by Hess [2004]), mechanisms of MLL recruitment to target genes remain poorly understood. A conserved mechanism of recruitment observed with both the yeast MLL family homolog Set1/COMPASS (Shilatifard, 2006) and human MLL is direct association with RNA polymerase II and components of the basal machinery (Dou et al., 2005; Milne et al., 2005a; Guenther et al., 2005). Mounting evidence, however, suggests that in humans MLL plays gene-specific regulatory roles through promoter-specific recruitment (Milne et al., 2005b; Sierra et al., 2006; Dreijerink et al., 2006). Our work provides a direct mechanism for promoter-specific MLL recruitment for transcriptional activation—HCF-1-mediated recruitment to E2F-responsive promoters by E2F1. There may also be direct mechanisms of E2F recruitment of MLL proteins to E2F-responsive promoters because Takeda et al. (2006) have shown that MLL and/or MLL2 can interact with E2F2–E2F6. Given that HCF-1 associates with the MLL family of H3K4 HMTs in multiple phases of the cell cycle (e.g., Figures S4 and S5), we predict that HCF-1-mediated recruitment of the MLL family of H3K4 HMTs to specific promoters will occur in other regulatory pathways involving HCF-1-interacting regulators.

Interestingly, MLL proteins are now implicated in both promoting cell-cycle progression through activation of E2F-dependent genes (Takeda et al., 2006; this study) and in inhibiting cell-cycle progression through activation of cyclin-dependent kinase inhibitor genes (Milne et al., 2005b). Thus, MLL has a varied role in regulating cell proliferation.

In Their Association with Activator E2Fs, HCF-1 and pRb May Have Opposing Roles

Inactivation of pocket proteins by the SV40 large T antigen and adenovirus E1A oncoprotein can rescue the temperature-sensitive HCF-1-induced G1 phase arrest of tsBN67 cells (Reilly et al., 2002). These observations have suggested that HCF-1 opposes pocket-protein function, in particular pRb. The results presented here indicate that this opposition is quite direct as pRb and HCF-1 bind the same molecule—E2F1—and indeed can bind simultaneously to E2F1 (Figure 4) in what we suggest is a transition complex from the repressive pRb-E2F1 to activating HCF-1-E2F1 complexes.

Recent studies have suggested a two-step cyclin-CDK phosphorylation-induced pRb and E2F1 dissociation

pathway in which pRb interactions with the Marked box domain of the E2F1-DP1 heterodimer and the classical pRb-binding site are sequentially disrupted (Rubin et al., 2005). Because pRb is already phosphorylated but still associated with E2F1 in the pRb-E2F1-HCF-1 complex (Figure 4), we propose that HCF-1 binds to an intermediate pRb-E2F1 complex in which pRb has undergone early phosphorylation by cyclin D/CDK4; subsequent cyclin-CDK phosphorylation events would then release the HCF-1-E2F1 complex from pRb.

Although we show that HCF-1 and pRb both associate with E2F1, this result per se does not explain why pocket-protein inactivation by T antigen and E1A can overcome the loss of HCF-1 association with E2F1 (Reilly et al., 2002) as in this study HCF-1 is required for activation of transcription by E2F1 (Figure 6). We suggest that HCF-1-mediated recruitment of MLL family H3K4 HMTs is not required for transcriptional activation when the pocket proteins are inactivated because E2F complexes no longer mark promoters with repressive modifications (i.e., H3K9 methylation and deacetylation).

Differential Targeting of E2F-Associated Molecules by DNA Viruses

The DNA tumor viruses (e.g., adenoviruses and papova viruses) and HSV modulate the cell cycle of infected cells but in opposite ways. The DNA tumor viruses induce S phase entry to create an infected-cell environment supportive of viral replication. In contrast, HSV, which encodes its own proteins for viral DNA replication, inhibits infected-cell entry into the S phase (de Bruyn Kops and Knipe, 1988). It is interesting, therefore, that the DNA tumor viruses and HSV target apparently opposing coregulators of E2F function. The DNA tumor viruses target the E2F1-associated "repressor" pRb (e.g., with E1A, T antigen, and E7), and HSV targets the E2F1-associated "activator" HCF-1 (i.e., with VP16). Indeed, HSV might inhibit G1 phase progression, at least in part, through competitive VP16 association with HCF-1, thus preventing its normal association with molecules such as E2F1. Whichever the case, the studies described here illustrate how, like DNA tumor viruses, HSV targets the heart of the cell-cycle regulatory machinery through the interaction of VP16 with the E2F-associated protein HCF-1.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa cells were grown in Dulbecco's modified Eagles's medium (DMEM) with 10% fetal bovine serum (FBS). HeLa cells stably expressing N-terminally Flag-epitope-tagged HCF-1 residues 2–1011 (f-HCF-1_N) were described previously (Wysocka et al., 2001). Cells were synchronized using double thymidine block as described (see the Supplemental Data). *Drosophila* SL2 cells were cultured at 25°C in Schneider's (GIBCO) media containing 10% heat-inactivated FBS.

Coimmunoprecipitation and Immunoblot Analysis

Nuclear extracts were prepared (Dignam et al., 1983) and coimmunoprecipitations performed (Wysocka et al., 2003) as described. Sequential anti-Flag and either E2F1 or E2F4 immunoprecipitation of f-HCF-1_N-containing complexes was performed as described (Wysocka et al., 2003). For immunoblot analysis, nitrocellulose membranes were incubated for 1 hr with 10 ml of LI-COR blocking buffer, then with relevant antibodies in 50% LI-COR blocking buffer and 50% PBST (PBS containing 0.2% Triton X-100) at 4°C overnight. The membranes were washed at least three times in PBST followed by incubation with the appropriate secondary antibodies (Alexa Fluor or IRE Dye) at 1:15,000 dilution in 50% LI-COR blocking buffer and 50% PBST at room temperature for 1 hr. The membranes were washed at least three times in PBST and scanned with an Odyssey infrared imager (LI-COR). The antibodies used in this study are described in the Supplemental Data.

Yeast Two-Hybrid Assay

The yeast *Saccharomyces cerevisiae* GAL4-based two-hybrid system was essentially as described in Freiman and Herr (1997). The GAL4-DBD fusion expression plasmids pGBT9-HCF-1_{N380} (HCF-1_{KEL}) and pGBT9-$HCF_{N380/P134S}$ (HCF-$1_{KELP134S}$), and GAL4-AD fusion expression plasmid pGADGH-VP16ΔC, have been described previously (Freiman and Herr, 1997). The E2F proteins and mutant derivatives were expressed as fusion proteins with GAL4-AD. dHCF Kelch domain (amino acids 51–420, cDNA generously provided by A. Wilson) and dE2F1 and dE2F2 (cDNAs generously provided by N. Dyson) were expressed as fusion proteins with GAL4-DBD and GAL4-AD, respectively. Protein interaction was tested by screening for growth on SD plates without Leu, Trp, and His containing 10 mM of 3-amino-1,2,3-triazole for 5 days at 30°C in a serial 10-fold dilution spot test.

siRNA Transfections

Human HCF-1 and control siRNAs and their double serial transfection into HeLa cells were done as described (Julien and Herr, 2003). Cells were harvested 48 hr after the first transfection and either lysed in SDS Laemmli buffer, subjected to RNA preparation, or fixed with formaldehyde for ChIP analysis. To obtain G1/S phase siRNA-treated cells, the first thymidine block was initiated 12 hr after the first siRNA transfection.

Chromatin Immunoprecipitation and Real-Time PCR Quantification

HeLa cells were formaldehyde crosslinked, DNAs were isolated and sonicated, and samples were immunoprecipitated, washed, and reverse crosslinked as described (Wells et al., 2000), except that, instead of RSB buffer, the cells were lysed in 5 mM PIPES (pH 8.0), 85 mM KCl, and 0.5% NP40, and the DNA was sonicated for 16 cycles of 30 s pulse at maximum power using a Bioruptor (Diagenode). ChIP DNA was detected by ethidium bromide staining of PCR products after gel electrophoresis or by real-time PCR.

Real-time PCR of ChIP DNAs was performed in triplicate using a SYBER green quantitative PCR kit (Applied Biosystems) and a Rotorgene RG300A sequence detector (Corbett Research) under conditions standardized for each primer set described in the Supplemental Data. PCR quantification was done with (1) the two standard curve analysis program in Rotor-gene 6.0 software or (2) delta relative C_T quantification, in which the values are calculated relative to input as follows: delta $C_T = C_T$ (input) − C_T (sample); relative unit = $2^{\text{delta CT}}$. Inputs correspond to 0.3% of total ChIP input DNA.

Quantitative Reverse-Transcriptase PCR

To assay mRNA transcript levels, total RNA was extracted with the RNeasy system (QIAGEN), treated with DNase, and reverse transcribed with ImProm II reverse transcription system (Promega). Real-time PCR was as above and quantitated using the comparative analysis program in Rotor-gene 6.0 software.

Supplemental Data

Supplemental Data include seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this

article online at http://www.molecule.org/cgi/content/full/27/1/107/DC1/.

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