The chromatin remodeling factor CHD8 interacts with elongating RNA polymerase II and controls expression of the cyclin E2 gene

M. Rodríguez-Paredes¹, M. Ceballos-Chávez¹, M. Esteller², M. García-Domínguez¹ and J. C. Reyes¹,*

¹Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER), CSIC, Américo Vespucio s/n, E-41092 Sevilla and ²Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro 3, E-28029 Madrid, Spain

Received August 21, 2008; Revised January 29, 2009; Accepted February 4, 2009

ABSTRACT

CHD8 is a chromatin remodeling ATPase of the SNF2 family. We found that depletion of CHD8 impairs cell proliferation. In order to identify CHD8 target genes, we performed a transcriptomic analysis of CHD8-depleted cells, finding out that CHD8 controls the expression of cyclin E2 (CCNE2) and thymidylate synthetase (TYMS), two genes expressed in the G1/S transition of the cell cycle. CHD8 was also able to co-activate the CCNE2 promoter in transient transfection experiments. Chromatin immunoprecipitation experiments demonstrated that CHD8 binds directly to the 5' region of both CCNE2 and TYMS genes. Interestingly, both RNA polymerase II (RNAPII) and CHD8 bind constitutively to the 5' promoter-proximal region of CCNE2, regardless of the cell-cycle phase and, therefore, of the expression of CCNE2. The tandem chromodomains of CHD8 bind in vitro specifically to histone H3 di-methylated at lysine 4. However, CHD8 depletion does not affect the methylation levels of this residue. We also show that CHD8 associates with the elongating form of RNAPII, which is phosphorylated in its carboxy-terminal domain (CTD). Furthermore, CHD8-depleted cells are hypersensitive to drugs that inhibit RNAPII phosphorylation at serine 2, suggesting that CHD8 is required for an early step of the RNAPII transcription cycle.

INTRODUCTION

The packaging of eukaryotic DNA into nucleosomes and higher order structures represents an obstacle for transcription initiation and elongation (1,2). Different nuclear machineries are responsible for the reorganization of interactions within the chromatin which allows specific and general transcription factors to gain access to their targets in the DNA and to carry out gene transcription in a tightly regulated manner. A specific type of chromatin remodeling machine uses the energy of ATP hydrolysis to alter interactions between histones and DNA within the nucleosome (3). It is believed that ATP-dependent chromatin remodeling machines (CRMs) are able to move nucleosomes, reorganize their structure and composition or even remove them (4). All ATP-dependent CRMs contain a DNA-dependent ATPase of the SNF2 family, which is considered as the catalytic subunit responsible for the remodeling. According to the sequence homology of their conserved ATPase domains, SNF2 proteins have been classified into distinct subfamilies such as SWI2/SNF2, ISWI, CHD1, Mi-2, CHD7, INO80, etc. (5).

Many authors have shown how ATP-dependent CRMs positively or negatively affect the formation of the transcription pre-initiation complex (6–12) but, on the contrary, little is known about how these enzymes may affect subsequent steps of the transcription cycle such as elongation or termination. The RNA polymerase II (RNAPII) complex is very inefficient at transcribing chromatinized templates in vitro (1). In fact, biochemical studies have revealed that nucleosomes represent a strong barrier against elongation, so that RNAPII stops at certain arrest sites near them, becoming blocked in a catalytically inactive state (13,14). Two elongation complexes, FACT (facilitates chromatin transcription) (15) and CTEA (chromatin transcription enabling activity) (16), have been shown to stimulate RNAPII passage through a nucleosome. However, whether ATP-dependent chromatin remodeling enzymes are also required to remodel nucleosomes during RNAPII elongation is still unclear. In this respect, Kingston and colleagues reported that the SWI/SNF complex, one of the best characterized...
ATP-dependent CRMs, which contains an ATPase of the SWI2/SNF2 subfamily, enhances HSF1-mediated stimulation of promoter-proximal paused RNAPII in vitro (17). Furthermore, Carey et al. also demonstrated a role for SWI/SNF and the related RSC complex in elongation in vitro, as they are able to release RNAPII from the blockade caused by a positioned nucleosome (18). Despite these facts, so far there is no clear evidence in vivo for a role of SWI/SNF-type CRMs in elongation.

On the other hand, CHD1 (chromodomain-ATPase/helicase-DNA-binding domain 1), another SNF2-like ATPase belonging to the so called CHD1 subfamily, does appear to function in both elongation and termination. In yeast and flies CHD1 associates with highly active transcription sites (19,20). Furthermore, CHD1 mutant alleles in yeast are sensitive to 6-azauracil (21) and genetically interact with Set2 and Spt5, both involved in elongation (20,22). Finally, CHD1 physically associates with factors involved in elongation such as the polymerase II-associated complex (PAF), DSIF (DRB-sensitivity-inducing factor) and FACT (20,23,24). These results strongly indicate that CHD1 works as an elongation factor in vivo, although very little mechanistic data had been provided until recently, when Konev et al. (25) showed that Drosophila CHD1 is involved in histone H3.3 replacement in the transcriptionally silent male pronucleus. Moreover, the Schizosaccharomyces pombe homolog of CHD1, Hrp1, has been shown to function in loading of the centromere-specific H3 variant CENPA (26). Therefore, CHD1 also may have a general role in replication-independent nucleosome assembly. The connection between this CHD1 function and its role in elongation remains unknown.

In addition to CHD1, there are other eight CHD proteins in mammalian cells, which are classified into three subfamilies called CHD1 (CHD1 and CHD2), Mi-2 (CHD3 to CHD5) and CHD7 (CHD6 to CHD9), subfamilies (5,27). Here we have investigated the role of CHD8 in transcription. In addition to the typical helicase-like domain that characterizes the SNF2 family, CHD8 contains two tandem chromodomains within the amino-terminal half of the protein. Chromodomains are evolutionary conserved motifs involved in interactions with DNA, RNA or methylated lysines (28). Moreover, CHD8 also contains a SANT domain and two BRK domains with unclear functions. CHD8 has been shown to interact with CTCF and is required for CTCF-dependent insulator activity (29). Recently, it has also been shown that CHD8 interacts with the human proteins Staf and β-catenin and contributes to regulate U6 and β-catenin-dependent gene transcription (30,31). We found that CHD8 is required for cell proliferation. CHD8 positively controls the expression of cyclin E2 (CCNE2) and thymidylate synthetase (TYMS), two genes specifically expressed in the G1/S transition (32). Furthermore, CHD8 associates constitutively with the promoter and the transcribed 5′ region of its target genes and interacts with elongating RNAPII, suggesting a role for this protein in transcription elongation.

**MATERIALS AND METHODS**

**Cell culture, growth curves and cell-cycle analyses**

All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, and were cultivated in a 37°C incubator with 5% CO2. For cell growth analysis, 2 × 10^5 C33KD1, C33KD2 and HeLaKD1 cells were seeded in 10 cm plates, with or without 2 μg/ml doxycycline (Sigma). At the indicated times cells were collected and counted using a Neubauer haemocytometer. Cell-cycle analysis was carried out using BD FACSCalibur flow cytometer (BD Biosciences), as previously described (32). When required cultures were treated with Flavopiridol (Sanofi-Aventis) or DRB (Sigma) at the indicated final concentration. Stock solution of Flavopiridol 10 mM was prepared in DMSO. For BrdU cell proliferation assays, cells were labeled with 10 mM BrdU during 15 min. BrdU incorporation was detected using the 5-bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche).

Cells were synchronized by double thymidine blocking as previously described (33), except that samples were taken at the following times after blocking release: t = 0 h (G1/S phase), t = 4 h (S phase), t = 14 h (early G1 phase) and t = 18 h (late G1 phase). The cell-cycle stage of each population was determined by flow cytometry analysis.

**Plasmid constructs**

To make pTER-CHD8-1 and pTER-CHD8-2, annealed gene-specific oligonucleotides encoding shRNA1 or shRNA2 (Supplementary Table S2) were ligated into pTER (34) and pSUPER vectors (35) previously digested with Bgl II and HindIII.

The pRSV-CHD8 expression vector containing the CHD8 protein (2581 amino acids) was constructed as follows: first, a BpiI-NotI fragment from pBC SK (+) KIAA1564 (provided by the Kazusa DNA Research Institute, Japan) was cloned into a pTriplEx2 (BD Biosciences) containing the EST AV762074 (provided by the Chinese National Human Genome Center, China) to constitute pTriplEx2-CHD8. After that, a PmiI restriction site was inserted before the CHD8 sequence using standard PCR techniques and, finally, a PmiI-NotI fragment from the former construct was cloned into a pAdRSV-Sp (36) derivative containing an in-frame FLAG epitope, and previously linearized with PmiI and NotI.

For Luciferase reporter assays, pCycE2 was constructed by inserting a PCR fragment ranging from nucleotide –960 to nucleotide +60 of CCNE2 gene into pCycE (17) previously digested with HindIII and NotI.

To make pGEX-CD1, pGEX-CD2, pGEX-CD1+CD2, and pGEX-CD1+mutCD2, pGEX-mutCD1+CD2 fragments encoding CHD8 CD1 (residues 629–715), CD2 (residues 710–785), CD1+CD2 (residues 629–785), CD1+mutCD2 (residues 629–785, W751L, Y756L) and mutCD1+CD2 (residues 629–785, Y672L, Y675L, Y677L), were cloned into a pGEX-4T-2 vector.
clones were selected in the presence of 100 μg/ml Zeocin and 5 μg/ml Blasticidin, and tested for their ability to down-regulate CHD8 by immunoblotting, at different times after the addition of 2 μg/ml Dox.

**Generation of inducible knockdown cell lines**

To generate inducible shRNA CHD8 knockdown cell lines, C33A and HeLa cells were co-transfected with pCDNA6-TR (Invitrogen) and pTER-CHD8-1. Resistant clones were selected in the presence of 100 μg/ml Zeocin and 5 μg/ml Blasticidin, and tested for their ability to down-regulate CHD8 by immunoblotting, at different times after the addition of 2 μg/ml Dox.

**ChIP assays**

ChIP assays were basically performed as described by Upstate/Millipore (http://www.millipore.com/techpublications/tech1/mcproto407). ChIP data are the average of 12–18 real-time PCR quantifications from two to three independent experiments. Real-time PCRs were performed in an Applied Biosystems 7500 FAST Real-Time PCR System, using the Applied Biosystems Power SYBR Green Master Mix and protocols. ChIP was quantified relative to inputs using the method described in (38).

Specific primers for CCNE2, TYMS and β-Actin (ACTB) were designed using the Primer Express 3.0 software (Applied Biosystems) and are described in the Supplementary Table S2.

Antibodies used in ChIP assays include polyclonal α-CHD8 (30 μl per sample), α-H3K4me3, α-H3K4me2 (Upstate/Millipore) (5 μl per sample) and α-RNAPII N-20 (Santa Cruz Biotechnologies) (5 μl per sample).

**Antibodies, immunoblotting and immunoprecipitations**

Immunoblotting experiments were carried out as previously described (39). Co-immunoprecipitations were carried out as described (40). To detect CHD8, a rabbit polyclonal serum raised against a GST fusion protein containing human CHD8 residues 16–227 was used. Commercial antibodies used for immunoblotting include α-CCNE2 (Cell Signalling Technology), α-E2F1 (KH95), α-SPT5 (H-300), α-BRAF35 (4.21), α-RNAPII (N-20) (Santa Cruz Biotechnology), α-Ser5-P-RNAPII (CTD4H8, Upstate/Millipore), α-RNAPIIIa (8WG16), α-Ser2-P-RNAPII (H5) and α-Ser5-P-RNAPII (H14) (Covance).

**Gene-expression analysis**

RNA was isolated using the RNeasy Mini kit (Qiagen). cDNA was synthesized from 3 μg of total RNA with the SuperScript First-Strand Synthesis system for RT–PCR (Invitrogen). One-twentieth of the reaction was used for PCR amplification with specific primers spanning an intron as a control for DNA contamination. To keep the amplification within the quantitative range, the number of cycles, usually 12–25, was set up for each experiment. Products were detected by Southern blot, using an InstantImager Electronic Autoradiography device (Packard Instrument) for quantification of radio-active areas. Gene-specific primers used in RT–PCR experiments are described in the Supplementary Table S2.

**Microarray gene-expression analysis**

Total RNA samples coming from C33KD2 cells cultured for 48 h either with or without 2 μg/ml doxycycline were used to generate complementary RNA labeled either with Cyanine 3 or Cyanine 5 with the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Dye incorporation was determined with a NanoDrop spectrophotometer. Once labeling was completed, both samples were combined and hybridized to a whole human genome Agilent 44K 60-mer Oligo Microarray (Agilent Technologies) according to manufacturer’s instructions. The experiment was performed in triplicate in order to achieve statistical significance. The microarrays were scanned on an Agilent G2565 scanner (Agilent Technologies) and images were extracted and quality assessed with the Agilent Feature Extraction software (Agilent Technologies). Data from the three individual hybridizations were normalized using the RMA algorithm, and the data were then further analyzed using the microarray software package Genespring GX 7.3 software (Agilent Technologies). Microarray data have been deposited in the GEO (Gene Expression Omnibus) database with the accession number GSE11422.

**Luciferase reporter assays**

For luciferase reporter assays, 2 x 10^5 COS-7 cells per well were seeded in six-well plates and co-transfected with 200 ng of the luciferase reporter vectors, pCycE2, or pMMTV-Luc, and increasing quantities of pRSV-CHD8, by using Fugene-6 (Roche). Luciferase activity was determined 36 h after transfection with Lysis Solution 1× (Promega), Luciferase Substrate (Promega) and a GloMax 20/20 luminometer (Promega), following the manufacturer’s instructions. All transfections were normalized by measuring β-galactosidase activity of the samples. Data are the average of at least three independent experiments and error bars indicate standard deviation.

**Peptide binding assays**

Sequences encoding CHD8 CD1 (residues 629–715), CD2 (residues 710–785) CD1 + CD2 (residues 629–785), CD1+mutCD2 (residues 629–785, W751L, Y756L) and mutCD1+CD2 (residues 629–785, Y672L, Y675L, Y677L) were expressed as GST fusion proteins in BL21 (DE3) bacterial cells and purified using standard procedures. For binding assays, 0.5 μg of each GST fusion protein were incubated with 2 μg biotinylated di-methylated Lys 4, tri-methylated Lys 4, di-methylated Lys 9 or unmodified histone H3 peptides (Upstate), in a 200 μl reaction using 156 μl of interaction buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.2% (v/v) NP-40, 0.25% BSA and protease inhibitors), for 2 h at 4°C with rotation. Then, 15 μl Dynabeads M-280 Streptavidin (Dynal Biotech) were added to the samples for an additional hour and, after three washes in interaction buffer, each one was eluted with 20 μl Laemmli buffer 2× and subjected to western blot analysis using an α-GST antibody.
RESULTS

Depletion of CHD8 impairs cell proliferation

In order to identify CHD8 functions we decided to decrease the expression of CHD8 by a doxycycline-regulated inducible shRNA expression vector (34). For that, two knockdown stable cell lines (C33KD1 and C33KD2) were derived from human C33A cervical carcinoma cells. Both lines showed a significant reduction in the level of CHD8 expression upon doxycycline (Dox) addition (85% reduction after 72 h), as determined by immunoblotting (Figure 1A). RT–PCR experiments demonstrated that expression of the closely related paralogous genes CHD6, CHD7 and CHD9 was unaffected, indicating that the knockdown was specific (Supplementary Figure S1). To determine whether reduced CHD8 levels affect cell proliferation, the growth rate of C33KD1 and C33KD2 cell lines was studied in the presence or the absence of Dox. Both cell lines exhibited a slower growth rate under conditions of CHD8 depletion, indicating that CHD8 is required for C33A cell proliferation (Figure 1B). As a control, we verified that Dox had no effect on the growth rate of C33A cells (data not shown).

To extend our results to another cell line we generated knockdown HeLa clones that showed identical defects in proliferation under conditions of CHD8 depletion (Supplementary Figures S2A and B). FACS analysis of proliferating C33KD1 and C33KD2 cells demonstrated that knockdown of CHD8 reduced the S-phase cell population from ~40% to 32%, with a concomitant increase in the G1 cell population from about 40–50%. Meanwhile, the G2/M cell population remained essentially unchanged (Figure 1C). Consistently, BrdU incorporation experiments revealed that upon a 15-min pulse of BrdU

Figure 1. Depletion of CHD8 impairs cell proliferation. CHD8 shRNA knockdown cell lines (C33KD1 and C33KD2) were generated by stable transfection of C33A cells. (A) Immunoblot analysis of Dox-treated (+) and non-treated (−) cells after 72 h, using α-CHD8 antibody. α-Ser2-P RNAPII antibody (H5) was used as a loading control. (B) Growth curve of CHD8 knockdown cell lines in the presence or absence of Dox. Data are the average of three independent experiments. Error bars represent standard deviation. (C) Flow cytometry analysis of asynchronous C33KD1 and C33KD2 cells treated with or without Dox for 72 h. Representative experiments are shown. Numbers are the average of three independent experiments. (D) BrdU incorporation analysis of cycling cells. C33KD1 and C33KD2 cells treated or non-treated with Dox for 48 and 72 h were labeled with BrdU as described in Methods. Percentage of cells that had incorporated BrdU was determined by immunofluorescence using α-BrdU antibodies. Numbers are the average of two independent experiments.
around 50% of the cells were able to incorporate BrdU in the absence of Dox but only about 35% of the CHD8-depleted cells were BrdU-positive (Figure 1D). These data suggest that CHD8 positively regulates the cell cycle by facilitating the G1/S transition.

CHD8 contributes to the expression of CCNE2 and TYMS genes

To identify CHD8 target genes we compared the genome-wide expression profiling of C33KD2 cells after Dox addition with that of non-treated cells. For this purpose, cells were collected after only 48 h of Dox treatment, when CHD8 levels were about 40% of its original quantity (data not shown). This was thought to avoid secondary effects produced by a pronounced growth arrest and to increase the probability of identifying direct target genes. Three independent biological replicates were performed. Taking into consideration only transcripts with a log₂(+/Dox−/−Dox expression ratio) >0.5 or <−0.5, with statistically significant P values ≤0.01, 10 and 31 genes were found to be up-regulated and down-regulated, respectively, in CHD8-depleted cells compared to non-depleted cells (Supplementary Table S1). As expected, levels of the CHD8 transcript were down-regulated in the CHD8-depleted cells. Efficiency of the microarray-based identification of CHD8-dependent genes was confirmed by RT–PCR analyses of a subset of misregulated genes (Supplementary Figure S3). Given the cell-cycle progression defects observed in the CHD8-depleted cells, we specifically focused on the cell-cycle-related genes among the CHD8-dependent ones. Interestingly, cyclin E2 (CCNE2) and thymidylate synthetase (TYMS), two E2F1-dependent genes expressed during the G1/S transition (41), were found to be down-regulated in CHD8-depleted cells (Figure 2A, B and C and Table 1). As a control, we verified that the expression of these two genes was not affected by Dox treatment (Figure 2A, B and C and Table 1).

Figure 2. Depletion of CHD8 leads to down-regulation of TYMS and CCNE2 genes. (A) Transcript levels of genes induced at the G1/S transition (TYMS, CCNE2, CCNE1, CCNA2, TK1) were analyzed by RT–PCR in Dox-treated (+) or non-treated (−) C33KD2 cells, at the indicated times. β-Actin (ACTB) transcript levels were also determined as a control for the amount of input cDNA. (B) Quantification of signals shown in (A). Values were normalized to the ACTB gene transcript level. The level of cDNA amplified in Dox non-treated cells (−) was considered 100%. Values are the average of three independent experiments. Bars indicate standard error of the mean. (C) Levels of CHD8, CCNE2, E2F1 and PCNA proteins were analyzed by immunoblot in C33KD2 cells treated (+) or non-treated (−) with Dox during the indicated time. α-Ser2-P-RNAPII antibody (H5) was used as a loading control. (D) The CCNE2 promoter is activated by CHD8. COS-7 cells were transfected with 0.2 μg of pCycE2-LUC plasmid along with 0.3 or 0.7 μg of pRSV-CHD8 plasmid. 0.2 μg of the pMMTV-LUC plasmid were co-transfected with a glucocorticoid receptor expression vector (0.2 μg) and with or without 0.7 μg of pRSV-CHD8. When indicated, the glucocorticoid receptor ligand dexamethasone (DEX) was added at 1 μg/ml for 12 h. Values are the average of three independent experiments ± SD.
genes was not affected by Dox in C33A cells (data not shown). These results led us to explore the possibility that other genes specifically expressed in the G1/S cell-cycle transition were also misregulated in CHD8-depleted cells. Expression of none of the tested genes (CCNE1, cyclin E1; CCNA2, cyclin A; TK1, thymidine kinase; E2F1, or PCNA) was found to be significantly altered upon Dox addition (Figure 2A, B and C) indicating that the reduction in the amount of CCNE2 and TYMS transcripts is not a consequence of the G1 arrest provoked by the depletion of CHD8. Down-regulation of cyclin E2 was also verified using two independent shRNAs targeting two different regions of the CHD8 mRNA (Supplementary Figure S4), as well as in CHD8-depleted HeLa cells (Supplementary Figure S2C).

To verify whether CCNE2 is a direct target of CHD8, a promoter-reporter construct was co-transfected with increasing quantities of a CHD8 expression vector and the expression of the luc reporter gene was determined. As shown in Figure 2D, CHD8 stimulated about 4-fold the activity of the promoter. As a negative control, CHD8 was not able to activate the MMTV promoter either in the absence or in the presence of dexamethasone, its inductor. We further investigated whether CCNE2 and TYMS are direct targets of CHD8 in vivo, we performed chromatin immunoprecipitation (ChIP) assays in C33A cells. Figure 3A shows that CHD8 binds to the 5' region of these genes. Depletion of CHD8 upon Dox addition to C33KD2 cells led to a significant reduction in the amount of immunoprecipitated CCNE2 and TYMS chromatin, which confirms the specificity of the α-CHD8 antibodies in our ChIP assays (Figure 3B and C). Next, we decided to investigate the precise location of CHD8 within the CCNE2 gene. As shown in Figure 3D, CHD8 was enriched in the promoter and the transcribed 5' region of the gene.

CCNE2 is an E2F-dependent gene transcriptionally active only during the G1/S and S phase fractions of the cell cycle (41,42) (Figure 4B and C). Thus, we wondered whether the association of CHD8 with the CCNE2 gene was cell-cycle-regulated. For that, we synchronized HeLa cells in G1/S by double thymidine blocking and performed ChIP experiments at different cell-cycle stages obtained by differential timed release in normal media. Given our interest in the G1/S transition, we used cell populations synchronized at early G1, late G1, G1/S and S (Figure 4A). As shown in Figure 4B and C, while the levels of CHD8 were not affected by the cell cycle, CCNE2 was only expressed at the G1/S and S fractions. ChIP experiments evidenced that CHD8 remains bound to the CCNE2 gene during the entire cell cycle (Figure 4D). Interestingly, a constitutive RNAPII occupancy at the CCNE2 promoter-proximal region was also observed (Figure 4E), suggesting that RNAPII is paused at the CCNE2 gene during both early and late G1 fractions, when the gene is not transcriptionally active (Figure 4B and C). However, a significant increase of RNAPII occupancy in the promoter and in body of the gene was observed at the G1/S transition coincident with the activation of the gene (Figure 4E).

The tandem chromodomains of CHD8 bind H3K4me2
in vitro

CHD8 contains two chromodomains (CDs) within its amino-terminal half. Chromodomains have been shown to mediate interactions of chromatin factors with methylated lysines of the histone tails. In fact, the similar CHD1
tandem chromodomains bind histone H3 tri-methylated in lysine 4 (43,44). Therefore, we decided to investigate whether the tandem CDs of CHD8 directly bind methylated H3K4, in vitro. For that, its first (CD1), second (CD2) or tandem chromodomains (CD1+CD2) (Figure 5A) were expressed in *Escherichia coli* as GST-fusion proteins. Recombinant purified proteins were incubated with biotinylated peptides corresponding to the first 21 amino acids of histone H3, either unmodified or modified by methylation in lysine 4 (di-methylated or tri-methylated) or in lysine 9 (di-methylated). Figure 5B shows that while CD1 was unable to interact with any histone H3 peptide, CD2 bound the four peptides without significant preference. More importantly, the tandem chromodomains mostly interacted with the H3K4me2 peptide. Single chromodomain proteins bind to methylated lysines by interaction with several aromatic residues (aromatic cage) (28). Thus, mutation of the conserved aromatic residues W751 and Y756 of CD2 completely abolished the binding activity of the tandem chromodomains. However, a triple mutation of the conserved aromatic residues (Y672L, Y675L, Y677L) of CD1 does not affect the binding. Therefore, our data suggest that CD1 contribution to the binding specificity does not require its aromatic cage. This is consistent with data from the human CHD1 protein, where the H3-methylated peptide interacts with an acidic surface bridging chromodomains 1 and 2 (44). These data suggest a possible role of H3K4me2 in recruiting CHD8 to the chromatin. However, further in vivo experiments are required to confirm this hypothesis. Then we decided to investigate whether the level of CHD8 affects the degree of methylation of histone H3 in the *CCNE2* gene. ChIP experiments demonstrated that histone H3 is di- and tri-methylated in lysine 4 in the *CCNE2* promoter and 5′ transcribed region, irrespectively of the levels of CHD8 (Figure 5C), indicating that CHD8 is not required for H3K4 methylation at the *CCNE2* gene.

**CHD8 associates with phosphorylated RNAPII**

As a first step in the characterization of the mechanism by which CHD8 controls gene expression we investigated whether CHD8 interacts with RNAPII. When recruited to promoters, the CTD of the largest subunit of RNAPII is unphosphorylated (RNAPIIa).
After promoter clearance and during early stages of elongation, firstly serine 5 (Ser5) and subsequently serine 2 (Ser2) are phosphorylated (Ser5-P and Ser2-P) (RNAPIIo) (45). The 8WG16 antibody recognizes mostly unmodified CTD repeats and some intermediate phosphorylated forms (46), while antibodies H14 and CTD4H8 recognize Ser5-P CTD and antibody H5 recognizes Ser2-P CTD repeats. In contrast, antibody N20 recognizes all RNAPII forms. Interestingly, CHD8 co-immunoprecipitated with hyperphosphorylated RNAPIIo complexes enriched for Ser5-P and Ser2-P (Figure 6A). However, CHD8 did not co-immunoprecipitate with the transcription elongation factor SPT5 or with the HMG protein BRAF35 (Figure 6A).

The fact that CHD8 associates with the elongating forms of RNAPII suggested that CHD8 might have a role in elongation. To support this hypothesis we asked whether CHD8-depleted cells were hypersensitive to...
inhibitors of transcription elongation. We reasoned that if elongation of some genes is altered in CHD8-depleted cells, a partial inhibition of elongation should enhance the effect of CHD8 depletion provoking a synthetic phenotype. Indeed, CHD8-depleted cells were hypersensitive to low concentrations of flavopiridol or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), two drugs that impair elongation by inhibiting CDK9, a component of the P-TEFb complex (47–49) (Figure 6B and C).

**DISCUSSION**

Here we show that CCNE2 is a direct target of CHD8. A number of results support this conclusion. First, CHD8-depleted cells present decreased levels of the CCNE2 transcript and its protein. Second, CHD8 transactivates the CCNE2 promoter in transient transfection experiments. And third, CHD8 binds directly to the CCNE2 gene in vivo. We also provide several lines of evidence suggesting that CHD8 plays a positive role in the first steps of RNAPII elongation. First, CHD8 co-immunoprecipitates with RNAPII phosphorylated in serines 5 and 2 of its CTD, but not with non-phosphorylated RNAPII. Similarly to CHD8, other typical elongating factors such as Spt6, DSIF and the PAF complex associate with elongating RNAPII (50–52). Second, ChIP experiments demonstrate that CHD8 binds not only to the promoter but also to the promoter-proximal transcribed region of the CCNE2 gene. In the same way, but unlike initiation factors, elongation factors such as DSIF, Spt6, TREX and PAF complexes associate with the coding region of active genes (53–55). Third, CHD8-depleted cells are hypersensitive to flavopiridol and DRB, drugs that provoke RNAPII arrest by inhibiting RNAPII CTD phosphorylation at serine 2 (56,57). The fact that CHD8-depleted cells are hypersensitive to both inhibitors suggests that CHD8 acts in the same process that is being inhibited by the drugs.
Although these experiments suggest a role for CHD8 during the first steps of elongation, we can not rule out an additional role for this factor in the formation of the pre-initiation complex.

As defined by Flaus et al., the ATPase CHD8 belongs to the CHD7 subfamily of the SNF2 family (5). In addition to CHD8, other three proteins belong to this subfamily in mammals: CHD6, CHD7 and CHD9. In contrast, only one protein called Kismet belongs to this subfamily in Drosophila. The kismet gene was originally identified as a suppressor of Polycomb mutations and, therefore, belongs to the trithorax group of genes (58). Kismet has been shown to play important roles in both segmentation and determination of body segment identity (59), and was found to co-localize with almost all RNAPII sites (60). kis mutants display a dramatic reduction in the levels of both CTD-Ser2-phosphorylated RNAPII and the elongation factors dSPT6 and dCHD1 on polytene chromosomes, suggesting that Kismet is required for transcription elongation (60). In contrast to kis mutations, CHD8 depletion does not generally reduce the levels of Ser2-phosphorylated RNAPII (Figure 1). One possibility is that the function that Kismet performs in Drosophila is carried out in mammals by several members of the subfamily (CHD6 to CHD9). This would explain why the depletion of only one member of the subfamily does not have a general effect, but a gene-specific effect. This is consistent with our whole genome expression analysis, as only a small number of genes were found to be misregulated upon knocking down CHD8.

While preparing the revised version of this manuscript it has been reported that loss of Kismet function leads to a reduction in the level of the H3K4 methyl transferases TRX and ASH1 on polytene chromosomes (61). In agreement with the hypothesis that CHD8 and Kismet are functional orthologs, it has been shown that CHD8 co-purifies with components of the MLL1 (mixed-lineage leukemia) complex (31,62), the mammalian TRX homolog. Moreover, and consistently with what we describe for CHD8, MLL1 has been found to interact with elongating RNAPII and bind to the promoters and 5′ transcribed regions of the genes. However, while MLL1 mostly associates with transcriptionally active genes, CHD8 binds to the CCNE2 gene under conditions of both activation and repression. Furthermore, depletion of CHD8 does not affect the levels of H3K4me2 or H3K4me3, suggesting that CHD8 does not cooperate with the MLL1 complex for histone H3K4 methylation. Interestingly, Srinivasan et al. (61) have also shown that, despite the reduced recruitment of TRX and ASH1, the loss of Kismet has no effect on the level of H3K4 methylation on polytene chromosomes or over the fkh gene. The connection between H3K4 histone methyl transferases and Kismet/CHD8 proteins requires further investigation.

The way CHD8 is recruited to its target genes remains unknown. One possibility is that some specific histone modifications contribute to the recruitment of CHD8. Here we demonstrate that, in contrast to Drosophila Kismet (61), CHD8 directly binds in vitro to H3K4me2 through its tandem chromodomains. Consistently with our results, Yuan et al. recently used fractions enriched for CHD8 to demonstrate its direct or indirect association with di- and tri-methylated lysine 4 of histone H3 amino-terminal peptides (63). We also found that CHD8 is constitutively bound to the CCNE2 gene, even during G1, when the gene is not active. This is also consistent with the fact that CHD8 associates with histone H3K4me2 in vitro, since H3K4me3 correlates with actively transcribed genes while H3K4me2 can be present on poised, inactive genes (64,65). Interestingly we also found significant levels of RNAPII associated to the CCNE2 gene under conditions of repression. Promoter-proximal pausing (1) is a widespread phenomenon that takes place in many genes, where RNAPII pauses in the 5′ region of transcription units and only progresses into productive elongation upon stimulation by appropriate signals.

Figure 6. CHD8 interacts with elongating RNAPII. (A) CHD8 associates with RNAPII in vivo. Immunoblot analysis of C33A total extract (input) and fractions immunoprecipitated with α-CHD8 or nonspecific antiserum (mock). Antibodies used for immunoblot are indicated on the left in each panel. (B and C) CHD8-depleted cells are hypersensitive to transcription elongation inhibitors. C33K2 cells were grown with or without flavopiridol (B) or DRB (C) at the indicated concentrations. Total cell numbers were determined after 5 days under the indicated conditions. Percentage of growth inhibition in the presence of either of flavopiridol (B) or DRB (C) is represented.
Paused RNAPII is phosphorylated at serine 5 of its CTD. The association of CHD8 with the repressed CCNE2 gene and the fact that it interacts with Ser5-P-RNAPII suggest that CHD8 might be recruited to genes having paused RNAPII.

CHD8 may also be recruited to its targets by interaction with specific transcription factors. In fact, it has been shown that CHD8 associates with human staf/ZNF143, a zinc finger protein that controls the expression of the U6 gene (63), and with β-catenin (31). In this respect we found that, although CHD8 is required for normal expression of CCNE2 and TYSM, two G1/S-specific genes which are targets of the E2F1 transcription factor (41), the expression of other typical E2F1-dependent genes such as CCNE1, CCNA2, TK1, PCNA or E2F1 itself did not change significantly under conditions of CHD8 depletion. Nonetheless and interestingly, we have verified the presence of CHD8 in the 5' region of CCNE1 and TK1 by ChIP experiments (data not shown). Furthermore, in transient transfection experiments CHD8 was also able to activate the expression of a minimal promoter containing an E2F1 synthetic binding site (data not shown). One possibility is that E2F1 could recruit CHD8 to all E2F1-dependent genes but that only those genes with a specific chromatin structure at the 5' region require CHD8 for normal elongation. Therefore, we suggest that the specificity of CHD8 for a subset of genes may involve its interaction with specific transcription factors, specific histone modifications and the phosphorylation status of RNAPII.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank Kazusa DNA Research Institute and Chinese National Human Genome Center for providing partial CHD8 cDNA clones. We thank Drs C. Muchardt, J.R. Nevins, H. Clevers and M. van de Wetering for providing different plasmids and cell lines, A. Aguilera for critical reading of the manuscript and Sanofi-Aventis for the gift of flavopiridol.

FUNDING
Ministerio de Educación y Ciencia [BFU2005-01047, CSD2006-00049]; Junta de Andalucia [P06-CVI-01400]. Funding for open access charge: Spanish Ministerio de Educación y Ciencia.

Conflict of interest statement. None declared.

REFERENCES
1. Saunders,A., Core,L.J. and Lis,J.T. (2006) Breaking barriers to transcription elongation. Nat. Rev. Mol. Cell Biol., 7, 557–567.
2. Li,B., Carey,M. and Workman,J.L. (2007) The role of chromatin during transcription. Cell, 128, 707–719.
3. Smith,C.L. and Peterson,C.L. (2005) ATP-dependent chromatin remodeling. Curr. Top. Dev. Biol., 65, 115–148.
4. Flaus,A. and Owen-Hughes,T. (2004) Mechanisms for ATP-dependent chromatin remodeling: farewell to the tuna-can octamer? Curr. Opin. Genet. Dev., 14, 165–173.
5. Flaus,A., Martin,D.M., Barton,G.J. and Owen-Hughes,T. (2006) Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. Nucleic Acids Res., 34, 2887–2905.
6. Mizuguchi,G., Tsukiyama,T., Wisniewski,J. and Wu,C. (1997) Role of nucleosome remodeling factor NURF in transcriptional activation of chromatin. Mol. Cell, 1, 141–150.
7. Gregory,P.D., Schmid,A., Zavari,M., Munsterkotter,M. and Horz,W. (1999) Chromatin remodeling at the PHO8 promoter requires SWI-SNF and SAGA at a step subsequent to activator binding. EMBO J., 18, 6407–6414.
8. Zhang,Z. and Reese,J.C. (2004) Snf6-Tup1 requires the ISW2 complex to position nucleosomes in Saccharomyces cerevisiae. EMBO J., 23, 2246–2257.
9. de la Serna,I.L., Carlson,K.A. and Imbalzano,A.N. (2001) Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. Nat. Genet., 27, 187–190.
10. Salma,N., Xiao,H., Mueller,E. and Imbalzano,A.N. (2004) Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adiogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. Mol. Cell Biol., 24, 4651–4663.
11. Soutoglou,E. and Talianidis,I. (2002) Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. Science, 295, 1901–1904.
12. Agalioti,T., Lomvardas,S., Parekh,B., Yie,J., Maniatis,T. and Thanos,D. (2000) Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell, 103, 667–678.
13. Studitsky,V.M., Walter,W., Kireeva,M., Kashlev,M. and Felsenfeld,G. (2004) Chromatin remodeling by RNA polymerases. Trends Biochem. Sci., 29, 127–135.
14. Kireeva,M.L., Hancock,B., Cremona,G.H., Walter,W., Studitsky,V.M. and Kashlev,M. (2005) Nature of the nucleosomal barrier to RNA polymerase II. Mol. Cell, 18, 97–108.
15. Reinberg,D. and Sims,R.J. 3rd. (2006) de FACTo nucleosome dynamics. J. Biol. Chem., 281, 23297–23301.
16. Guernah,M., Pallhan,V.B., Tackett,A.J., Chait,B.T. and Roeder,R.G. (2006) Synergistic functions of SII and p300 in productive activator-dependent transcription of chromatin templates. Cell, 125, 275–286.
17. Brown,S.A., Imbalzano,A.N. and Kingston,R.E. (1996) Activator-dependent regulation of transcriptional pausing on nucleosomal templates. Genes Dev., 10, 1479–1491.
18. Carey,M., Li,B. and Workman,J.L. (2006) RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. Mol. Cell, 24, 481–487.
19. Stokes,D.G., Tartof,K.D. and Perry,R.P. (1996) CHD1 is concentrated in interbands and pulled regions of Drosophila polytene chromosomes. Proc. Natl Acad. Sci. USA, 93, 7137–7142.
20. Simic,R., Lindstrom,D.L., Tran,H.G., Roinick,K.L., Costa,P.J., Johnson,A.D., Hartzog,G.A. and Arndt,K.M. (2003) Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. EMBO J., 22, 1846–1856.
21. Woodage,T., Basrai,M.A., Baxevanis,A.D., Hieter,P. and Collins,F.S. (1997) Characterization of the CHD family of proteins. Proc. Natl Acad. Sci. USA, 94, 11472–11477.
22. Krogan,N.J., Kim,M., Tong,A., Golshani,A., Cagney,G., Canadien,V., Richards,D.P., Beattie,B.K., Emili,A., Boone,C. et al. (2003) Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. Mol. Cell Biol., 23, 4207–4218.
23. Kelley,D.E., Stokes,D.G. and Perry,R.P. (1999) CHD1 interacts with SSRP1 and depends on both its chromodomain and its ATPase/helicase-like domain for proper association with chromatin. Chromosoma, 108, 10–25.
24. Krogan,N.J., Kim,M., Ahn,S.H., Zhong,G., Kobor,M.S., Cagney,G., Emili,A., Shlaitfard,A., Buratowski,S. and Greenblatt,J.F. (2002) RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol. Cell Biol., 22, 6979–6992.
25. Konev,A.Y., Tribus,M., Park,S.Y., Podhrasky,V., Lim,C.Y., Emelyanov,A.V., Vershilo,E., Perrotta,V., Kadonaga,J.T., Lusser,A. et al. (2007) CHD1 motor protein is required for deposition of histone variant H3.3 into chromatin in vivo. Science, 317, 1087–1090.

26. Walfridsson,J., Bjerging,P., Thalen,M., Yoo,E.J., Park,S.D. and Ekkvall,K. (2009) The CHD remodeling factor Hrpi stimulates CENP-A loading to centromeres. Nucleic Acids Res., 37, 2868–2879.

27. Marrella,C.G. and Imbalzano,A.N. (2007) The Chd family of chromatin remodelers. Mutat. Res., 618, 30–40.

28. Brehm,A., Tufteland,K.R., Aasland,R. and Becker,P.B. (2004) The many colours of chromodomains. Bioessays, 26, 133–140.

29. Ishihara,K., Oshimura,M. and Nakao,M. (2006) CTCF-dependent chromatin insulator is linked to epigenetic remodeling. Mol. Cell, 23, 733–742.

30. Yuan,C.C., Zhao,X., Floresn,L., Swanson,S.K., Washburn,M.P. and Hernandez,N. (2007) CHD8 associates with human Stats and contributes to efficient U6 RNA polymerase III transcription. Mol. Cell Biol., 27, 8729–8738.

31. Thompson,B.A., Tremblay,V., Lin,G. and Bochar,D.A. (2008) CHD8 is an AT-rich-dependent chromatin remodeling factor that regulates beta-catemin target genes. Mol. Cell Biol., 28, 3894–3904.

32. Reyes,J.C., Barra,J., Muchardt,C., Camus,A., Babinet,C. and Yaniv,M. (1998) Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpma). EMBO J., 17, 4979–6991.

33. Tyagi,S., Chabes,AL., Wysocka,J. and Herr,W. (2007) E2F activation of S phase promoters via association with HIC-1 and the MLL family of histone H3K4 methyltransferases. Mol. Cell, 27, 107–119.

34. van de Wetering,M., Oving,I., Muncan,V., Pon Fong,M.T., Brehm,A., Tufteland,K.R., Aasland,R. and Becker,P.B. (2004) The Spid S12 domain of P-TEFb and direct Iws1-dependent mRNA splicing and export. Genes Dev., 21, 160–174.

35. Kim,M., Ahn,S.H., Krogan,N.J., Greenblatt,J.F. and Buratowski,S. (2004) Transitions in RNA polymerase II elongation complexes at the 3’ ends of genes. EMBO J., 23, 354–364.

36. Saunders,A., Werner,J., Andrusis,E.D., Nakayama,T., Hirose,S., Reinberg,D. and Lis,T.J. (2003) Tracking FACT and the RNA polymerase II elongation complex through chromatin in vivo. Science, 301, 1094–1096.

37. Kaplan,C.D., Laprade,L. and Winston,F. (2003) Transcription elongation factors repress transcription initiation from cryptic sites. Science, 301, 1096–1099.

38. Glover-Cutter,K., Kim,S., Espinosa,J. and Bentley,D.L. (2008) RNA polymerase II pauses and associates with pre-mRNA processing factors at both ends of genes. Nat. Struct. Mol. Biol., 15, 71–78.

39. Yoh,S.M., Cho,H., Pickle,L., Evans,R.M. and Jones,K.A. (2007) The Spt6 S12 domain of P-TEFb binds in a 1:1 stoichiometry and regulates elongation in a cell cycle specific manner. J. Cell. Biol., 176, 273–284.

40. Cran,KE., DeGregori,J. and Nevins,J.R. (1995) Regulation of the G1 cyclin and activating partner of CDK2 and CDK3, is induced by viral oncoproteins. J. Biol. Chem., 270, 12542–12552.

41. Marfella,C.G. and Imbalzano,A.N. (2007) The Chd family of chromatin remodelers. Mutat. Res., 618, 30–40.

42. Reyes,J.C., Barra,J., Muchardt,C., Camus,A., Babinet,C. and Yaniv,M. (1998) Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpma). EMBO J., 17, 4979–6991.

43. Tyagi,S., Chabes,AL., Wysocka,J. and Herr,W. (2007) E2F activation of S phase promoters via association with HIC-1 and the MLL family of histone H3K4 methyltransferases. Mol. Cell, 27, 107–119.

44. van de Wetering,M., Oving,I., Muncan,V., Pon Fong,M.T., Brehm,A., Tufteland,K.R., Aasland,R. and Becker,P.B. (2004) The Spid S12 domain of P-TEFb and direct Iws1-dependent mRNA splicing and export. Genes Dev., 21, 160–174.

45. Kim,M., Ahn,S.H., Krogan,N.J., Greenblatt,J.F. and Buratowski,S. (2004) Transitions in RNA polymerase II elongation complexes at the 3’ ends of genes. EMBO J., 23, 354–364.

46. Saunders,A., Werner,J., Andrusis,E.D., Nakayama,T., Hirose,S., Reinberg,D. and Lis,T.J. (2003) Tracking FACT and the RNA polymerase II elongation complex through chromatin in vivo. Science, 301, 1094–1096.

47. Kaplan,C.D., Laprade,L. and Winston,F. (2003) Transcription elongation factors repress transcription initiation from cryptic sites. Science, 301, 1096–1099.

48. Glover-Cutter,K., Kim,S., Espinosa,J. and Bentley,D.L. (2008) RNA polymerase II pauses and associates with pre-mRNA processing factors at both ends of genes. Nat. Struct. Mol. Biol., 15, 71–78.

49. Stock,J.K., Giadrosi,S., Casanova,M., Brooks,E., Vidal,M., Koski,H., Brockdorff,N., Fisher,A.G. and Pombo,A. (2007) Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat. Cell. Biol., 9, 1428–1435.

50. Kennison,J.A. and Tamkun,J.W. (1998) Dosage-dependent modifiers of polycomb and antennapedia mutations in Drosophila. Proc. Natl Acad. Sci. USA, 95, 12146–12150.

51. Shi,X., Finkelstein,A., Wolf,A.J., Wade,P.A., Burton,Z.F. and Winston,F. (2003) Transcription factor in Saccharomyces cerevisiae, may have both positive and negative roles in transcription. Science, 301, 1096–1099.

52. Glover-Cutter,K., Kim,S., Espinosa,J. and Bentley,D.L. (2008) RNA polymerase II pauses and associates with pre-mRNA processing factors at both ends of genes. Nat. Struct. Mol. Biol., 15, 71–78.