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The chromodomains of CHD1 are critical for enzymatic activity but less important for chromatin localization

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ABSTRACT

The molecular motor protein CHD1 has been implicated in the regulation of transcription and in the transcription-independent genome-wide incorporation of H3.3 into paternal chromatin in *Drosophila melanogaster*. A key feature of CHD1 is the presence of two chromodomains, which can bind to histone H3 methylated at lysine 4 and thus might serve to recruit and/or maintain CHD1 at the chromatin. Here, we describe genetic and biochemical approaches to the study of the *Drosophila* CHD1 chromodomains. We found that overall localization of CHD1 on polytene chromosomes does not appreciably change in chromodomain-mutant flies. In contrast, the chromodomains are important for transcription-independent activities of CHD1 during early embryonic development as well as for transcriptional regulation of several heat shock genes. However, neither CHD1 nor its chromodomains are needed for RNA polymerase II localization and H3K4 methylation but loss of CHD1 decreases transcription-induced histone eviction at the *Hsp70* gene *in vivo.* Chromodomain mutations negatively affect the chromatin assembly activities of CHD1 *in vitro,* and they appear to be involved in linking the ATP-dependent motor to the chromatin assembly function of CHD1.

INTRODUCTION

Chromo helicase DNA-binding protein 1 (CHD1) belongs to the SNF2 family of ATPases (1,2), a group of enzymes that have been intimately linked to the modification of chromatin structure [e.g. (3–11)]. The CHD subgroup of this family is evolutionarily conserved between *Saccharomyces cerevisiae* and humans with budding yeast possessing one CHD protein and mammals having nine family members (12,13). Structural signature motifs of CHD proteins are a central ATPase/helicase domain and N-terminally located tandem chromodomains (14). Within the CHD-family, three subgroups can be distinguished according to the presence of additional sequence motifs: CHD1-CHD2 subfamily members, which have a DNA-binding domain in the C-terminal part that preferentially binds to A/T-rich sequences; the CHD3-CHD4 subfamily proteins, which contain paired PHD (plant homeodomain) fingers N-terminal of the chromodomains; and the CHD5-CHD9 subfamily, whose members harbor different additional protein motifs, such as SANT-like (SWI3, ADA2, N-CoR, TFIIB-like) domain, BRK (Brahma and Kismet) domain, CR domain and DNA-binding domains (13).

CHD1-type proteins have been implicated in transcription-related and transcription-independent processes. Our previous studies revealed that CHD1 is necessary for the incorporation of the variant histone H3.3 in the absence of transcription into paternal pronuclear chromatin at fertilization in *Drosophila* embryos (15). Without maternally provided CHD1, H3.3 is not correctly assembled into the paternal pronucleus resulting in immediate cessation of development or in the development of haploid embryos that only contain the maternal chromosome complements (15). These results corroborated data from earlier *in vitro* studies demonstrating that CHD1 acts as a chromatin assembly factor (16). Studies in chicken and human cells and in fission yeast revealed that the respective CHD1 homologs are required for the assembly of the

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centromere-specific histone H3 variant CenH3 into centromeric chromatin (17,18). Notably, however, no such function was observed for *Drosophila* CHD1 (19).

CHD1-type proteins have also been implicated in transcription control, particularly at the level of elongation and termination. Several studies in *S. cerevisiae* demonstrated the interaction of Chd1p with transcription elongation factors, including FACT, the Paf complex and Spt5 (20,21), decreased sensitivity of *chd1* mutants to the transcription-interfering drug 6-azauracil (14), association of Chd1p with genic regions (21) and termination defects at some genes in *chd1* mutant yeast (22). In *Drosophila,* CHD1 also interacts with the FACT subunits SSRP1 (23) and DRE4 (our unpublished data) and co-localizes with elongating RNA polymerase II (Pol II) to transcriptionally active interbands and puffs on salivary gland polytene chromosomes (23–25). Moreover, transcription-related functions of mouse CHD1 appear to be responsible for maintaining ES cell pluripotency (26). Mammalian CHD1 has further been found to interact with splicing factors (23,27,28) and to modulate pre-mRNA splicing *in vitro* (27,28).

Chromodomains, such as those of CHD-type proteins, are short (approximately 50 amino acids) structural motifs found in a variety of proteins across all eukaryotic species (29). Some chromodomains can bind to DNA and RNA (30,31), whereas others recognize and bind methylated lysine residues in the N-terminal tails of histones (12,32). The double chromodomains of CHD1 cooperate to generate an interaction surface for methylated H3 tails (33). Biophysical and structural studies have shown that human CHD1 specifically interacts with H3K4me3 and H3K4me2 but not with unmodified H3K4 or other methylated lysines in H3 tails (33–35).

Based on the specificity of the chromodomains of human CHD1 for H3K4me3, which accumulates in the promoter regions of active genes, it was suggested that they may mediate the recruitment and/or maintenance of CHD1 at sites of active transcription (20,28,34,36–38). In this work, we have studied the role of the chromodomains in the biological functions and enzymatic properties of CHD1 in *Drosophila*. We show that the presence of intact chromodomains is not required for overall recruitment of CHD1 to chromatin *in vivo*. Nevertheless, the transcription-stimulatory effects of CHD1 are compromised upon mutation of the chromodomains. We investigated the mechanisms that could be responsible for these defects and found that chromodomain mutations impair chromatin assembly activities of CHD1.

MATERIALS AND METHODS

Drosophila stocks

The $Df(2L)Chd1^{1}$ and $Df(2L)Chd1^{2}$ stocks have been described before (15). The $Df(2L)Exel7014$ and w^{1118} (which served as wild-type control) stocks were obtained from the Bloomington stock center. Flies were kept on standard cornmeal media at 25°C.

Generation of transgenes

Point mutations in the chromodomains of CHD1 were generated by site-directed mutagenesis using the pFastBacI-Flag-CHD1 vector (see below) as a template and verified by sequencing. The altered CHD1 sequences were subcloned into the P element transformation vector pCaSpeR4 as described before (15). Transgenic animals were produced by embryo injections of wild-type *Chd1* ($P\{Chd1^{WT}\}$) and chromodomain-mutated *Chd1* constructs ($P\{Chd1^{CD}\}$), respectively, performed by BestGene Inc. Second chromosome insertions were mapped by inverse PCR to cytological positions 55E1 ($Chd1^{WT}$) and 38D2 ($Chd1^{CD}$), respectively. The transgenes were recombined with the chromosomes bearing the $Df(2L)Chd1^{1}$, $Df(2L)Chd1^{2}$ and $Df(2L)Exel7014$ deficiencies, respectively, by standard procedures. Successful recombination events were identified by PCR. Primer sequences are available upon request.

Viability and sterility tests

To assess viability of $Chd1^{CD}$-rescued flies heterozygous $Df(2L)Chd1^{1},P\{Chd1^{CD}\}/CyO$ or $Df(2L)Chd1^{2},P\{Chd1^{CD}\}/CyO$ flies were crossed to $Df(2L)Exel7014,P\{Chd1^{CD}\}/CyO$ flies and progeny were counted. To analyze embryonic development, virgin $Df(2L)Chd1^{1},P\{Chd1^{CD}\}/Df(2L)Exel7014,\ P\{Chd1^{CD}\}$ or $Df(2L)Chd1^{2},P\{Chd1^{CD}\}/Df(2L)Exel7014,\ P\{Chd1^{CD}\}$ females were mated to wild-type (w^{1118}) males. About 100 embryos derived from each cross were transferred to apple juice agar plates and hatching of first instar larvae was monitored after 24 h at 25°C. The same procedure was used for the analysis of the $Chd1^{WT}$-rescued lines.

Analysis of embryo development

For cytological analysis of the embryos, flies were allowed to lay embryos onto apple juice/agar plates for 2 h. Plates were removed and embryos aged for another 2 h at 25°C before they were collected, fixed in methanol and stained with DAPI or propidium iodide exactly as described before (15). The following numbers of embryos were examined: $Chd1^{WT}$, $n = 410$; $Chd1^{CD}$, $n = 306$; $Chd1^{-}$, $n = 545$.

Immunostaining of polytene chromosomes

Salivary gland polytene chromosomes were prepared from wild-type and GFP-negative third instar larvae obtained from crosses of heterozygous mutant and transgenic flies balanced with *CyO-GFP* as described above. Salivary glands were fixed for 5 min in 45% acetic acid/1.85% formaldehyde, squashed and stained with antibodies against Pol II_{ser2} (Covance) at 1:50 and/or CHD1 (24) at 1:75. For stainings with antibodies against H3K4me3 (Abcam; 1:50), salivary glands were fixed in a solution containing 6 mM $MgCl_2$, 1% citric acid and 1% Triton X-100 for 2 min. Staining procedures were performed according to (25). Samples were mounted in Vectashield (Vector Laboratories) and images were taken on an Axioplan (Carl Zeiss) microscope equipped with a MRc camera (Carl Zeiss). When comparing signals on chromosomes

from *Chd1*-deficient, $Chd1^{WT}$- and $Chd1^{CD}$-rescued larvae photographs were taken with the same exposure times. Pictures were processed with Axiovision (version 4.8) and Adobe Photoshop CS3 software.

Western blot

Extracts were prepared from GFP-negative third instar larvae obtained from crosses of heterozygous mutant and transgenic flies balanced with *CyO-GFP* as well as from wild-type larvae as described above by homogenizing larvae in 1× SDS sample buffer and boiling for 5 min. Extracts were cleared by centrifugation and aliquots were subjected to SDS–PAGE and western blotting. Blots were incubated with antibodies against CHD1 or H3K4me3 and actin as a loading control.

Real-time PCR analysis

Total RNA was extracted by the TriBase (Sigma) method from larvae that had been subjected to various times (0, 5, 10, 15, 30 and 60 min) of heat treatment at 37°C and subsequently been frozen in liquid nitrogen. RNA was further purified using the RNeasy kit (Qiagen), digested with DNase I and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. Real-time PCR was carried out with primers specific for the *Hsp70* gene cluster, the *Hsp83* and *Hsp22* genes and GAPDH as internal reference, respectively, in a StepONE Instrument (Applied Biosystems) with POWER SYBR Green PCR mastermix (Applied Biosystems). Primer sequences are available upon request.

Micrococcal nuclease digestion of larval chromatin

For micrococcal nuclease digestion 200 mg larvae of each line were collected and frozen in liquid nitrogen. Chromatin preparation and digestion was performed exactly as previously described (39).

ChIP analysis

Chromatin preparation from L3 larvae, isolation of nuclei, cross-linking, sonication and immunoprecipitation was performed according to (40), except that cross-linked nuclei were sonicated in a buffer containing 50 mM Tris–HCl pH 8.0, 0.8% SDS, 10 mM EDTA, 10 mM Na-butyrate in an ice-water bath using the Bioruptor sonifier (Diagenode) for 13 cycles of alternating ON/OFF periods of 30 s each on 'high' setting. Prior to immunoprecipitation chromatin was diluted to reach a final SDS concentration of 0.1%, and NaCl was added to 100 mM. About 50 µg of chromatin was incubated in a 1:200 dilution of anti histone H3, CT, pan antibody (Millipore). Washing of the beads, elution and DNA purification was performed essentially as described in (41). Real-time PCR was performed using a StepONE Instrument (Applied Biosystems) and POWER SYBR Green PCR mastermix (Applied Biosystems) with primer pairs spanning the *Hsp70* gene from position +334 to +423 and an intergenic region, respectively, between genes CG3558 and CG17265 on chromosome 2L (primer sequences available upon request). The amount of DNA immunoprecipitated is shown as percent of input DNA. A standard curve was derived from the analysis of serially diluted input material. H3 occupancy differences between the different lines were evaluated by unpaired *t*-test analysis.

Cloning, synthesis and purification of recombinant CHD1 mutant proteins

Constructs for the expression of *Drosophila* and human wild-type CHD1 chromodomains were a kind gift of S. Khorasanizadeh. The fly construct encodes a polypeptide spanning amino acids 314–492 of *Drosophila* CHD1 in pET11a vector to give an N-terminal fusion with 6xHis tag. Alanine substitutions of tryptophans 372, 375 and 462 were introduced by site-directed mutagenesis. *Drosophila* and human constructs were transformed into *Escherichia coli* BL21pLysS (Stratagene) and purified by Ni-NTA affinity chromatography (Qiagen) under non-denaturing conditions according to manufacturer's instructions. In the case of the *Drosophila* proteins (wtCD1/2 and mutCD1/2), 20 mM β-mercaptoethanol and 0.5 U/µl benzonase were added. Following purification, all proteins were dialyzed against buffer D (50 mM Hepes-KOH pH 7.6, 150 mM NaCl, 0.1 % Tween-20, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM benzamidine and 1 mM PMSF).

Chromodomain deletions and point mutations in full-length CHD1 were generated by PCR using appropriate primers (primer sequences available upon request) and cloned into pFastBac1 (Invitrogen) to give an in-frame fusion with a C-terminal Flag-tag. The amino acid sequences that were deleted in the different mutant proteins are as follows: ΔCD1: 316–404, ΔCD2: 336–492, ΔCD1+2: 316–494. Site directed mutagenesis was used to introduce tryptophan to alanine substitutions at positions 372 and 375 (CD1m) and 372, 375 and 462 (CD1+2m), respectively. Wild-type and recombinant proteins were synthesized in *Sf9* cells and purified as described previously (16). In order to use equal amounts of the purified proteins for subsequent assays, protein concentrations were determined by Bradford assay followed by electrophoresis on 6% SDS–PAGE. Gels were stained with Coommassie Brilliant Blue and band intensities were requantified with Adobe Photoshop CS3.

Peptide binding assays

The binding of purified chromodomains to peptides corresponding to the N-terminal 21 amino acids of histone H3 (Upstate) was assayed as described before (42). Briefly, 500 ng of each CD polypeptide were incubated with 2 µg biotinylated H3 peptides unmodified, di- or trimethylated at lysine 4 and 30 µl streptavidin magnetic beads in binding buffer [50 mM Hepes-KOH pH 7.6, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, complete protease inhibitor cocktail (Roche), 0.5 mg/ml insulin, 20 mM β-mercaptoethanol, 250 U/µl Benzonase], for 2 h at 4°C with rotation. Subsequently the beads were washed twice with binding buffer and bound proteins were eluted in 2× SDS loading buffer. Aliquots of input, supernatant and

eluate were subjected to 15% SDS–PAGE and immunoblotting using an anti-His antibody (GenScript). Alternatively, gels were stained with silver.

Chromatin assembly assay

Chromatin assembly assays and DNA supercoiling analyses were performed as described (16). Forty nano molar wild-type or mutant CHD1 proteins were used in the assembly reactions and the reactions were stopped after 90 min incubation. Purified DNA was separated on a 0.8% agarose gel and stained with ethidium bromide. Gel pictures were quantified using ImageQuant software (Molecular Dynamics). To this end, signal intensities were determined of an area containing topoisomeres with the highest numbers of supercoils (indicated by a bracket in Figure 5C) as well as of the whole lane, and the ratio of supercoiled to total DNA was determined. These values were normalized against the value obtained with wild-type CHD1. Average values and standard deviations of three independent experiments were calculated.

ATPase assay

ATPase assays were performed for 60 min at 27°C in a 10 µl reaction mixture containing 10 mM potassium HEPES (pH 7.6), 5 mM $MgCl_2$, 100 µg/ml of BSA, 0.3 mM ATP, 0.1 µCi of [γ-^{32}P] ATP and 40 nM of recombinant wild-type or mutant CHD1 protein or 10 nM of recombinant ACF. Where indicated, reaction mixtures additionally contained 400 ng of a supercoiled 3 kb plasmid or 400 ng (DNA) of salt-dialyzed chromatin (16). Aliquots of 0.5 µl were spotted onto PEI-cellulose TLC plates (Merck), which were then developed with 0.8 M acetic acid/0.8 M LiCl in a TLC chamber. Resolved ATP-hydrolysis products were visualized by exposition to a phosphoimager screen and the percentage of hydrolyzed ATP was determined using ImageQuant software (Molecular Dynamics).

RESULTS

Generation of transgenic flies bearing a chromodomain-defective *Chd1* gene

In human CHD1, the highly conserved tryptophans 322 and 325 in chromodomain 1 (CD1) and 413 in chromodomain 2 (CD2) were found to be critically involved in the binding of methylated H3K4 *in vitro* (33,34). To examine the contribution of the chromodomains of *Drosophila* CHD1 to its functions *in vivo*, we generated fly lines expressing a transgene ($Chd1^{CD}$) that encodes Flag-tagged CHD1 with mutations in the corresponding tryptophan residues (W372A, W375A, W462A; Supplementary Figure S1A and B). To avoid interference of wild-type CHD1, we analyzed the effects of the mutated transgene in a *Chd1* double null genetic background ($Df(2L)Chd1^{1}$, $P\{Chd1^{CD}\}/Df(2L)Exel7014$, $P\{Chd1^{CD}\}$ or $Df(2L)Chd1^{2}$, $P\{Chd1^{CD}\}/Df(2L)Exel7014$, $P\{Chd1^{CD}\}$). To control for background differences, we also generated double null *Chd1* strains that carry a Flag-tagged wild-type *Chd1* rescue transgene ($Chd1^{WT}$; Supplementary Figure S1B). Western blot analysis of extracts from wild-type and transgenic third instar larvae confirmed that both transgenes were expressed and gave rise to comparable amounts of protein (Supplementary Figure S1C).

Overall CHD1 localization on polytene chromosomes appears to be independent of intact chromodomains

It was suggested that the ability of CHD1 to interact with methylated H3K4 *via* its chromodomains might serve to recruit and/or stabilize the association of CHD1 with chromatin (20,28,34,36–38). Mutation of the chromodomains of CHD1, such as in the $Chd1^{CD}$ transgene, then should result in its mislocalization or dissociation from chromatin.

To examine this hypothesis, we performed co-staining of polytene chromosomes prepared from wild-type and transgenic larvae with antibodies against CHD1 and the elongating form of RNA polymerase II (Pol II_{ser2}). We detected colocalization of CHD1 and Pol II_{ser2} throughout chromosomes isolated from wild-type and $Chd1^{WT}$ transgenic larvae (Figure 1A, C, D and F), which is consistent with previous observations (24,25,43). When we analyzed chromosomes from $Chd1^{CD}$ expressing larvae, we observed CHD1 signals that colocalized with Pol II_{ser2} in a pattern that was highly similar to that seen with wild-type chromosomes (Figure 1B and E). Hence, mutating the chromodomains of CHD1 does not preclude its ability to bind to chromatin or result in strong defects in its localization on polytene chromosomes. However, we cannot rule out the possibility that the resolution of the polytene-staining method is not sufficient to detect defects in specific positioning and/or levels of mutant CHD1 recruitment/maintenance on chromatin.

To confirm that the mutations that were shown to eliminate H3K4me3 binding in human CHD1 produced the same effect in fly CHD1, we used bacterially expressed His-tagged polypeptides spanning both CHD1 chromodomains to perform pull-down assays with unmodified, di- and trimethylated H3K4 peptides. In contrast to a corresponding polypeptide encoding the human CHD1 chromodomains (33), however, and despite the use of several different expression constructs, the fly chromodomain polypeptides displayed poor solubility. When the soluble fraction of the *Drosophila* proteins was tested in peptide binding assays, we observed that the wild-type chromodomains interacted with all H3 peptides irrespectively of the K4 methylation state (Supplementary Figure S2A). In contrast, the human chromodomains showed selective binding to H3K4me2 and H3K4me3 as demonstrated before [Supplementary Figure S2B, (33,34)]. The analysis of the mutated chromodomains of *Drosophila* CHD1 revealed a selective and strong decrease of binding to H3K4me3, while binding to H3K4me2 and the unmodified H3 peptide was retained. These results suggest that the conserved tryptophane residues 372, 375 and 462 in the chromodomains of fly CHD1 contribute to the interaction with H3 trimethylated at K4, but are less critical for binding to di- and unmethylated H3K4. However, due

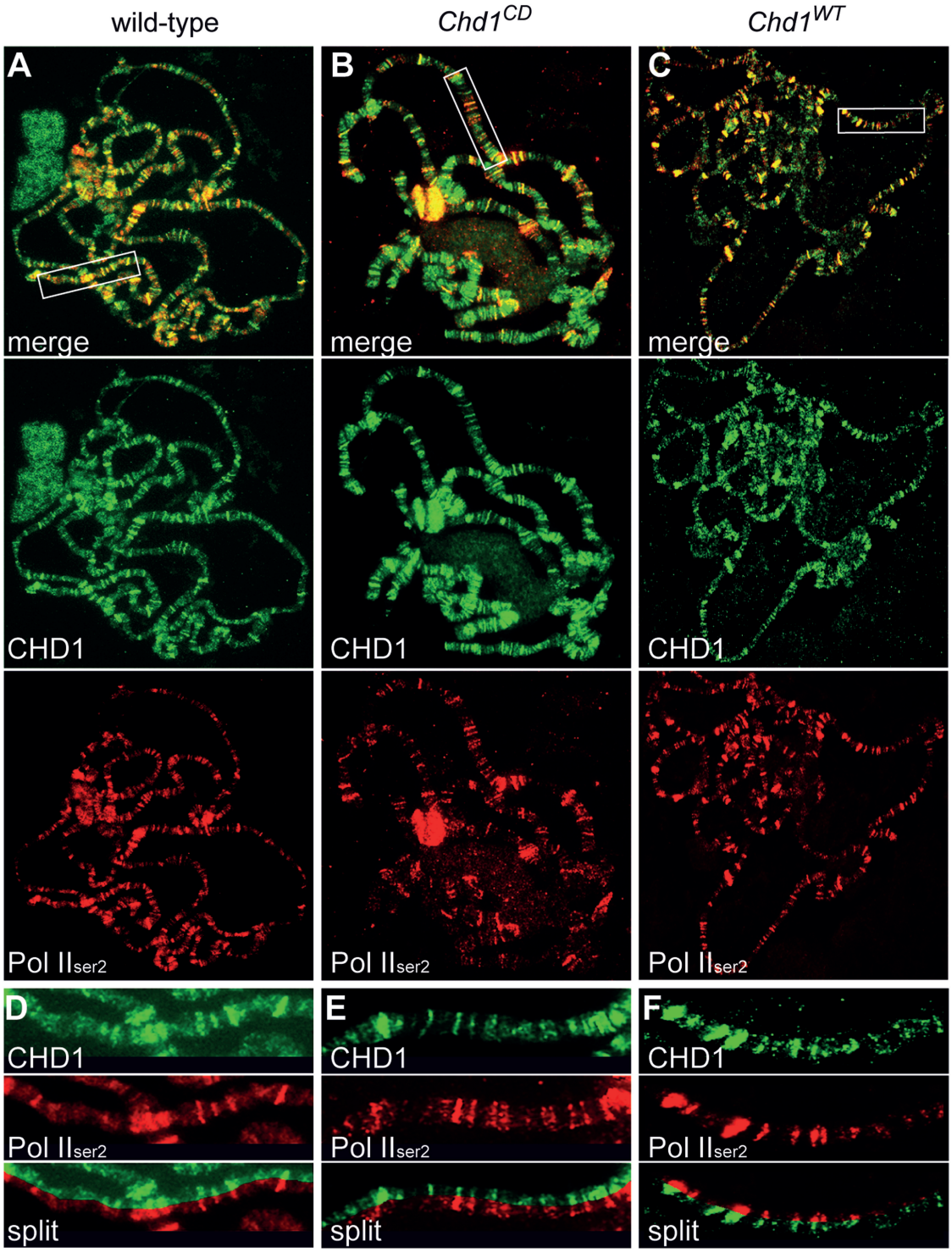

Figure 1. Chromatin localization of CHD1 appears similar in wild-type and chromodomain-mutant larvae. Polytene chromosomes were isolated from wild-type (**A** and **D**), *Chd1*^{CD} (**B** and **E**) and *Chd1*^{WT} larvae (**C** and **F**) and stained with antibodies against CHD1 (green) and the elongating form of RNA polymerase II (Pol II$_{ser2}$; red). Merge (**A–C**) and split (**D–F**) images indicate extensive colocalization between CHD1 and Pol II$_{ser2}$ in both wild-type and chromodomain mutant chromosomes.

to the tendency of the *Drosophila* chromodomain polypeptides to aggregate, which might cause unspecific interactions with dimethylated and unmodified H3 peptides in this assay, and given the high degree of conservation of critical sequences within the chromodomains between human and *Drosophila* proteins, it is possible that *Drosophila* CHD1 *in vivo* exhibits binding properties that are similar to those of human CHD1.

Distinct impact of CHD1 chromodomains on viability and fertility

We then determined viability and fertility of transgenic $Chd1^{CD}$ flies in comparison to *Chd1* deficient and $Chd1^{WT}$ rescued flies. *Chd1*-deficient flies displayed reduced viability, which is illustrated by a decrease of double null progeny to 45% ($Df(2L)Chd1^{1}/Df(2L)Exel7014$) and 29% ($Df(2L)Chd1^{2}/Df(2L)Exel7014$), respectively, of the expected numbers [Figure 2A and (15)]. While the $Chd1^{WT}$ transgene completely rescued this phenotype, only partial restoration (56% and 81%, respectively) of viability was observed with the $Chd1^{CD}$ transgene (Figure 2A). These findings show that the chromodomains are necessary for full activity of CHD1 in processes that affect fly viability.

We have previously reported that *Chd1* deficiency causes sterility in both females and males (15). To examine whether the CHD1 chromodomains contribute to female fertility, *Chd1*-deficient females carrying the $Chd1^{CD}$ transgene were mated to wild-type males, and progeny was scored by counting the number of embryos that hatched into larvae. As expected, the $Chd1^{WT}$ transgene completely rescued the fertility of *Chd1*-deficient females while none of the embryos laid by *Chd1*-deficient females developed into larvae (Figure 2B). In contrast, 17% and 27.5%, respectively, of the embryos laid by females carrying the $Chd1^{CD}$ transgene were able to hatch (Figure 2B). Since, we have shown previously that the inability of *Chd1*-deficient embryos to complete normal development is due to a defect in the incorporation of histone H3.3 into paternal pronuclear chromatin, these results suggest that the chromodomains enhance the ability of CHD1 to assemble H3.3 into chromatin.

To further investigate this issue, we assayed the distribution of developmental stages in 2–4 h embryos. Embryos from *Chd1*-deficient females ($Df(2L)Chd1^{2}/Df(2L)Exel7014$) accumulated predominantly at the very early stages of embryonic development around or shortly after the first two nuclear divisions [Figure 2C and (15)].

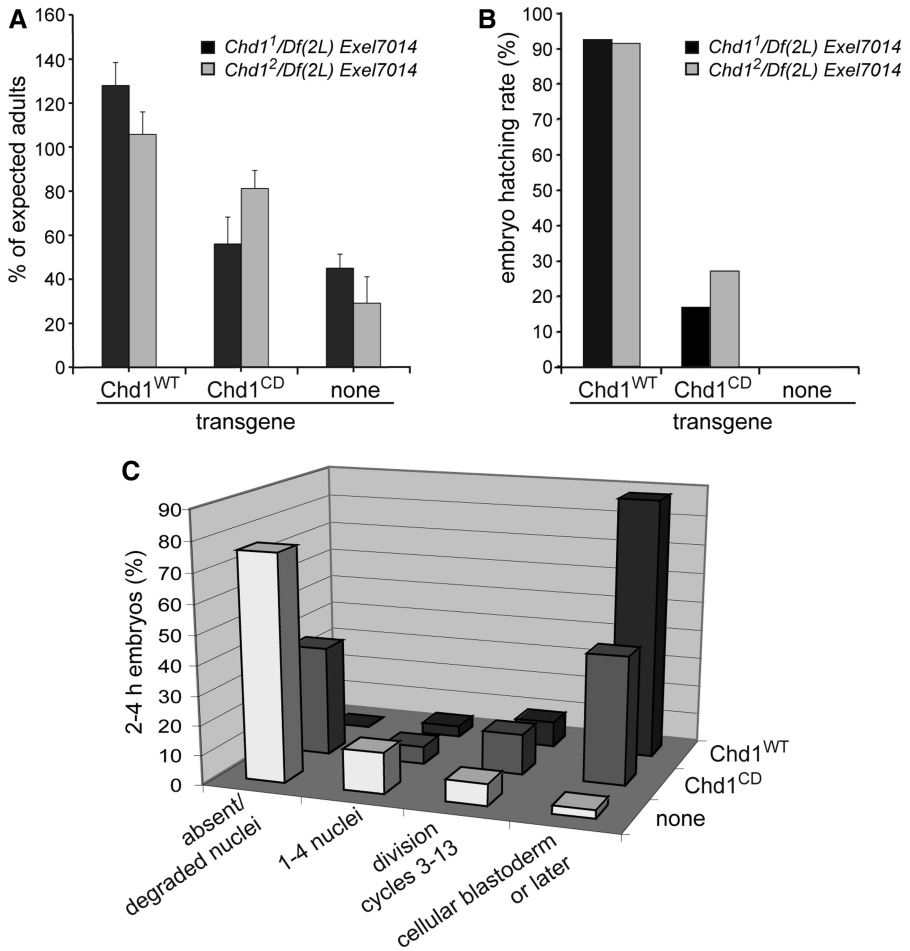

Figure 2. The $Chd1^{CD}$ transgene partially rescues viability and embryonic development of *Chd1*-deficient flies. (**A**) Transgenes were recombined onto the chromosomes bearing either the $Chd1^{1}$ or the $Chd1^{2}$ mutant allele or the deficiency *Exel7014*, respectively. Flies were mated and heterozygous combinations of either mutant allele with the deficiency chromosome were counted and normalized against the expected number of progeny. Column color indicates the genetic background. (**B**) Embryos ($n = 100$) laid by flies expressing the indicated transgene in a *Chd1*-deficient genetic background (indicated by column color) were transferred to apple juice agar plates and hatching was monitored after 24 h incubation. (**C**) Embryos laid by *Chd1*-deficient females ($Chd1^{2}/Exel7014$) bearing the indicated transgenes were collected at 0–2 h after egg deposition and aged at 25°C for another 2 h ($Chd1^{WT}$, $n = 410$; $Chd1^{CD}$, $n = 306$; $Chd1^{-}$, $n = 545$). Developmental distribution of the embryos was monitored by DAPI staining.

In contrast, most embryos from females carrying the *Chd1*^{WT} transgene had completed syncytial nuclear divisions and had entered cellular blastoderm or later stages at the assayed time point (Figure 2C). Embryos laid by females carrying the *Chd1*^{CD} transgene, however, displayed a biphasic distribution of developmental stages. About 57% of the embryos were found in syncytial and cellular blastoderm or later stages, thus resembling the distribution of wild-type embryos (Figure 2C). On the other hand, we observed that 37% of *Chd1*^{CD} embryos, similar to *Chd1*-deficient embryos, had abolished development at a very early developmental stage prior to pronuclear fusion (Figure 2C). These results suggest that the chromodomains are not absolutely required for the ability of CHD1 to reorganize paternal pronuclear chromatin and for progression of embryonic development. Nevertheless, since a considerable fraction of the *Chd1*^{CD} embryos behaves like *Chd1*-deficient embryos, the chromodomains appear to contribute to this early development-specific activity of CHD1, and defective chromodomains compromise the activity of CHD1.

Maximal induction of heat shock gene transcription is dependent on the CHD1 chromodomains

CHD1 has been suggested to function at different stages of transcription, such as elongation, termination and transcript splicing, mostly on the basis of its interaction with factors involved in these processes (20–25). To examine direct effects of CHD1 on transcription and to investigate the role of the chromodomains in this process, we monitored transcriptional activity of the *Hsp70* genes upon induction by heat shock (HS) in third instar larvae. Exposure to HS conditions (37°C) resulted in rapid activation of *Hsp70* transcription in wild-type larvae and in larvae carrying the *Chd1*^{WT} rescue transgene, which continued to strongly increase until 60 min of HS (Figure 3A). In contrast, in *Chd1*-deficient larvae, we found that transcription increased until 30 min of HS, when it reached about 30% of the levels observed with the *Chd1*^{WT} line. We did not detect any further increase of transcripts upon prolonged heat treatment (60 min; Figure 3A). In larvae carrying the *Chd1*^{CD} transgene transcription activation was low at the earliest time point assayed (5 min) but eventually increased to levels about 3-fold higher than in the deletion mutant. However, at 60 min of HS transcript levels reached only 22% of the activation observed in the *Chd1*^{WT}-expressing larvae (Figure 3A). To examine, if similar transcriptional deficits were detectable at other HS induced genes, we also analyzed mRNA levels of the *Hsp83* and *Hsp22* genes. The results shown in Figure 3B and C demonstrate that the transcription activation defects of *Chd1*-deficient and *Chd1*^{CD}-rescued larvae were even more pronounced in those genes. *Hsp83*, which displays relatively high transcript levels also under NHS conditions (44), failed to become further activated upon HS in the absence of CHD1 (Figure 3B). Although in the *Chd1*^{CD}-rescued larvae transcripts at 60 min of HS had accumulated 3-fold relative to steady-state levels, they still reached only ~6% of the HS induced transcripts in the isogenic

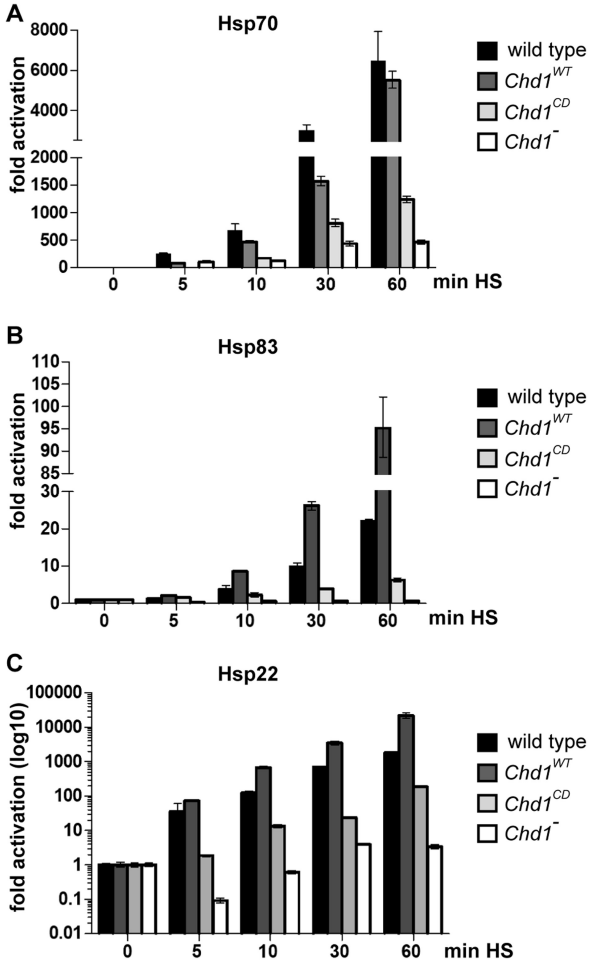

Figure 3. HS-induced transcription of *Hsp70, Hsp83* and *Hsp22* genes is decreased in *Chd1*-deficient and *Chd1*^{CD}-rescued larvae. Larvae bearing the indicated transgenes in a *Chd1*^{2}/*Exel7014* genetic background and wild-type larvae were subjected to the indicated periods of HS before RNA was prepared and expression of the HS genes was analyzed by real-time PCR. All values were normalized against the non-HS (0) sample of the respective fly strain. Error bars indicate standard deviations of three independent experiments. (**A**) *Hsp70,* (**B**) *Hsp83* and (**C**) *Hsp22.*

Chd1^{WT}-rescued larvae and ~30% of mRNA levels of w^{1118} wild-type larvae (Figure 3B). A similar pattern was observed with the *Hsp22* gene that exhibited very strong activation (several 1000-fold) at 60 min HS in the wild-type and in the *Chd1*^{WT}-rescued larvae, whereas only 3- and 185-fold activation was detected in the *Chd1*-deficient and the *Chd1*^{CD}, respectively, larvae (Figure 3C).

These results demonstrate that CHD1 is necessary for maximal transcriptional induction of HS genes. Moreover, the chromodomains appear to play a critical role for this function of CHD1, since the chromodomain mutant protein only moderately improves HS gene activation relative to the lack of endogenous CHD1.

Recruitment of Pol II and H3K4 methylation levels are not affected upon mutation of the CHD1 chromodomains

To further investigate the mechanisms by which the chromodomains contribute to the activity of CHD1

during transcription, we analyzed the effect of mutation of the CHD1 chromodomains on the recruitment of Pol II. Although, we have demonstrated that the overall distribution of Pol II_{ser2} on *Chd1*^{CD} polytene chromosomes appears unaltered when compared to wild-type chromosomes (Figure 1), it was still possible that the reduced transcription activity at the *Hsp70* locus in mutant larvae resulted from inefficient recruitment of Pol II_{ser2} to HS puffs upon induction. Thus, we more closely inspected the localization of Pol II_{ser2} at the HS puffs at cytological locations *87A*/C. This region contains the *Hsp70* gene clusters and displays prominent chromatin decondensation upon activation (45). Staining of polytene chromosomes from wild-type, *Chd1*-deficient, *Chd1*^{WT}-rescued and *Chd1*^{CD}-rescued larvae after HS with antibodies against Pol II_{ser2} and CHD1 revealed strong Pol II_{ser2} signals at the 87A/C locus upon HS in all strains (Figure 4A). Moreover, the CHD1^{CD} protein was still able to accumulate at this locus to an extent that appeared similar to that seen with wild-type CHD1 (Figure 4A). Hence, we conclude that the reduced transcriptional activity at the *Hsp70* genes does not appear to be due to defects in Pol II_{ser2} recruitment in the absence of CHD1 or in the presence of chromodomain-mutated CHD1. Although, we cannot rule out any subtle changes in the distribution or amount of mutant CHD1 at the *Hsp70* locus, these results strongly suggest that the observed transcriptional defects are not due to aberrant localization of CHD1 due to defective chromodomains.

To investigate whether CHD1 affects H3K4 methylation, we stained salivary gland polytene chromosomes of wild-type, *Chd1*-deficient larvae as well as larvae carrying the *Chd1*^{CD} or *Chd1*^{WT} transgenes with antibodies against H3K4me3. These experiments showed that neither the loss of CHD1 protein nor the mutation of the CHD1 chromodomains led to noticeable changes in the level or distribution of H3K4me3 (Figure 4B and Supplementary Figure S3). Similar results were obtained with immunoblot analysis of bulk histones extracted from third instar larvae (Figure 4C). Thus, neither CHD1 nor its chromodomains contribute to the bulk of histone H3K4 methylation.

Chromodomain mutations impair the chromatin assembly activity of CHD1

Next, we examined whether the chromodomains affect the biochemical activities of CHD1, which might result in decreased transcriptional activity. To this end, we generated a panel of recombinant CHD1 proteins bearing either complete deletions of one or both chromodomains (ΔCD1, ΔCD2, ΔCD1+2) or single amino acid substitutions in one or both chromodomains (CD1m, CD1+2m; Figure 5A). [Note that the CD1+2m protein corresponds to the protein that is encoded by the *Chd1*^{CD} transgene.] The proteins were synthesized using the baculovirus system and purified by Flag-affinity chromatography (Figure 5B). We then determined the effect of the mutations upon the ability of these proteins to mediate the deposition of histones onto DNA. To this end, we performed *in vitro* nucleosome assembly reactions with

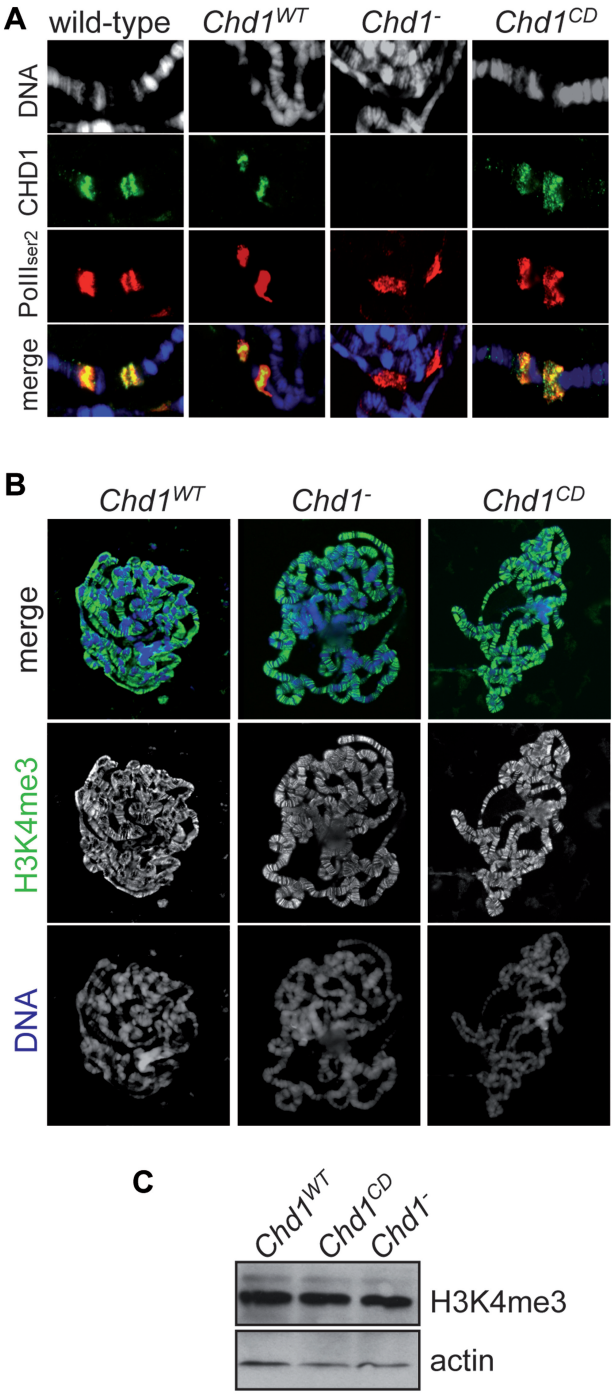

Figure 4. Pol II_{ser2} levels at HS puffs 87A/C and overall H3K4 methylation appear to be unaltered in *Chd1* mutant larvae. (**A**) Polytene chromosomes were isolated from wild-type, *Chd1*^{WT}, *Chd1*-deficient and *Chd1*^{CD}-rescued larvae after 20 min of heat stress and stained with antibodies against CHD1 (green) and Pol II_{ser2} (red). DNA is visualized in blue. Pol II_{ser2} is readily redistributed to the *Hsp70* puffs in the *Chd1*-deficient and *Chd1*^{CD}-rescued larvae. Also, there are no apparent differences between the recruitment of chromodomain-mutated CHD1 and wild-type CHD1. (**B**) H3K4 methylation is not affected in *Chd1*-mutant larvae. Polytene chromosomes were isolated from *Chd1*^{WT}-rescued (left), *Chd1*-deficient (middle) and *Chd1*^{CD}-rescued (right) larvae and stained with antibodies against H3K4me3 (green). DNA is visualized in blue. (**C**) Western blot analysis of total protein extracts from *Chd1*^{WT}, *Chd1*^{CD} and *Chd1*-deficient third instar larvae reveals similar levels of H3K4me3 in all three strains. Actin was probed to control for equal loading.

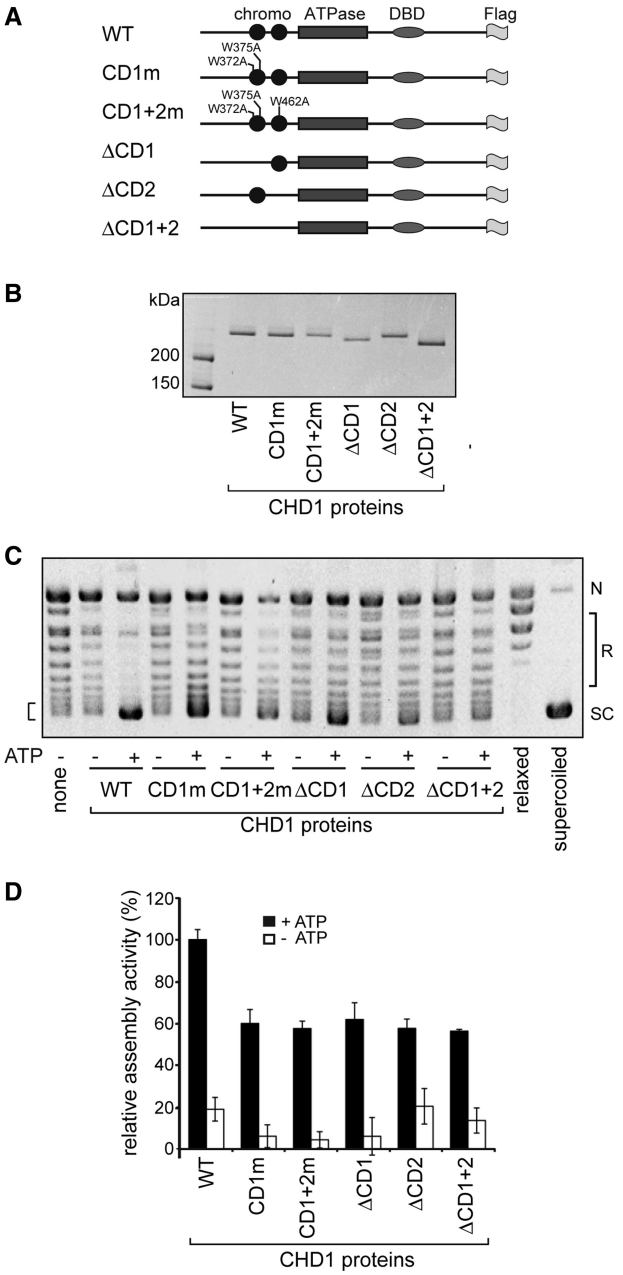

Figure 5. Mutations in the chromodomains affect nucleosome assembly activities of CHD1. (**A**) Diagram of CHD1 mutant proteins. ATPase domain (boxes), DNA binding domain (ovals), chromodomains (circles). The positions of tryptophan to alanine mutations are indicated. (**B**) Mutant proteins were synthesized in *Sf9* cells via the baculovirus expression system. Flag-tagged proteins were purified by Flag-affinity chromatography, equal amounts were separated on a 6% SDS–PAGE and visualized by Coommassie staining. (**C**) DNA supercoiling analysis of nucleosome assembly products. Chromatin assembly reactions were carried out with the indicated mutant proteins or without CHD1 in the absence or presence of ATP. Reactions were terminated after 90 min and reaction products were analyzed by agarose gelelectrophoresis of deproteinized samples. N, nicked circular DNA; R, relaxed circular DNA; SC, supercoiled DNA. (**D**) Quantification of nucleosome assembly activity. The percentage of DNA supercoiling was determined as described in 'Materials and Methods' section. The amount of signal obtained in the absence of CHD1 (none in **C**) was subtracted as background. The resulting data were normalized against those obtained with wild-type CHD1. Error bars denote standard deviations of three independent experiments.

purified wild-type or mutant CHD1 proteins along with purified NAP-1 (as a histone chaperone), purified core histones and plasmid DNA. The chromatin assembly reaction was monitored by DNA supercoiling analysis (Figure 5C). To determine the relative assembly activities of the CHD1 proteins, nucleosome assembly efficiencies were quantified and related to the activities of the wild-type protein (Figure 5D). This analysis showed a decrease of nucleosome assembly activity of all mutant proteins to about 60% of that of wild-type CHD1 (Figure 5C and D). The observed reduction in nucleosome assembly activity was not due to a general misfunctioning of the proteins due to potential misfolding as robust DNA- and chromatin-stimulated ATPase activities of the mutant proteins were still detectable. [Note that the ATPase activity of CHD1 exhibits stronger stimulation by DNA than by chromatin, in contrast to the ISWI-containing factor ACF that is more efficiently stimulated by chromatin (Supplementary Figure S4) and (46)]. Only the proteins harboring single amino acid substitutions displayed reduced ATPase activity (Supplementary Figure S4). In addition, the remaining nucleosome assembly activities in the CHD1 mutant proteins were dependent on ATP (Figure 5C and D).

Thus, the transcription and developmental defects that we have observed in flies carrying the $Chd1^{CD}$ transgene may be due (at least in part) to impaired nucleosome assembly/remodeling activities.

Chd1 mutation affects transcription-associated changes at the *Hsp70* locus

To examine if the absence of CHD1 or the mutation of the chromodomains affect chromatin structure *in vivo*, we performed micrococcal nuclease digestion analysis of chromatin prepared from $Chd1^{-}$, $Chd1^{WT}$ and $Chd1^{CD}$ third instar larvae. These experiments revealed no obvious differences between the lines with respect to sensitivity toward digestion, nucleosome periodicity or repeat length (Figure 6A) suggesting that CHD1 is not a major contributor to the preservation of global chromatin architecture in larvae. We then examined in more detail the *Hsp70* locus using ChIP with antibodies against the C-terminus of histone H3. Petesch and Lis (47) have demonstrated recently that rapid histone loss occurs along the entire coding region of the *Hsp70* genes upon HS treatment in cultured *Drosophila* S2 cells. Interestingly, it was reported that RNAi-mediated knockdown of CHD1 in these cells resulted in increased protection of the first downstream nucleosome from digestion by MNase suggesting enhanced preservation of this nucleosome. When we subjected *Chd1*-deficient larvae and larvae expressing either wild-type or chromodomain mutant CHD1 to H3 ChIP, we observed a HS-induced decrease of histone H3 occupancy around position +380 (primer pair spanning positions +334 to +423) in $Chd1^{WT}$-expressing larvae, whereas H3 occupancy remained unchanged in an intergenic region that was used as a control (Figure 6B). In contrast, in HS-treated *Chd1*-deficient larvae H3 levels at +380 remained similar to those observed in the intergenic region indicating a defect in

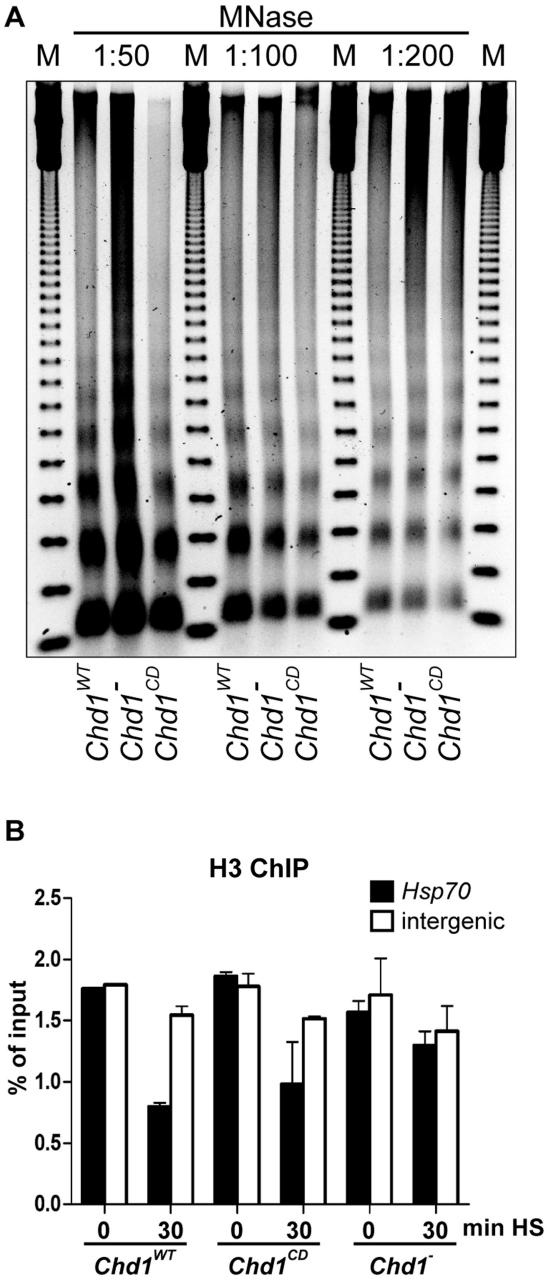

Figure 6. Effects of *Chd1* mutation on chromatin structure. (**A**) *Chd1* mutations do not perturb overall chromatin architecture. Chromatin was prepared from *Chd1* deficient larvae and larvae expressing either the $Chd1^{WT}$ or the $Chd1^{CD}$ transgene and subjected to digestion with micrococcal nuclease. Deproteinized reaction products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. M, 123 bp ladder (Invitrogen). (**B**) *Chd1* deletion causes decreased histone H3 loss at the activated *Hsp70* locus. ChIP analysis with antibodies against H3 was performed on chromatin isolated from $Chd1^{WT}$, $Chd1^{CD}$ and $Chd1^{-}$ larvae that were either not heat shocked (0) or exposed to 30 min of heat treatment. Immunoprecipitated material was amplified by RT–PCR and expressed relative to the input material. Black columns represent an amplicon between bp +334 and +423 of the *Hsp70* gene, white columns correspond to an intergenic region. Histone H3 occupancy differences upon HS at the *Hsp70* locus are highly significant between the $Chd1^{WT}$ and $Chd1^{-}$ lines ($P = 0.003$), but statistically not significant between the $Chd1^{WT}$ and $Chd1^{CD}$ lines ($P = 0.197$).

nucleosome loss at this position (Figure 6B). In larvae bearing the $Chd1^{CD}$ transgene H3 amounts at position +380 were decreased upon HS relative to the intergenic region but remained slightly higher than in the corresponding wild-type CHD1 expressing larvae (Figure 6B). However, as these differences were not statistically significant, it appears that the chromodomain mutation does not interfere with the ability of CHD1 to remodel chromatin structure at the *Hsp70* locus upon transcription activation.

Together the results show that CHD1 is required for the nucleosome loss/remodeling around the transcriptional start site of *Hsp70* upon transcription induction but that the chromodomains appear to play no role in this process. In contrast, neither CHD1 nor its chromodomains are necessary for the maintenance of global chromatin structure in *Drosophila* larvae.

DISCUSSION

Requirement of the chromodomains for chromatin association of CHD1

It was demonstrated previously for human CHD1 that it selectively recognizes and binds methylated H3K4 *in vitro* (33,34). In our *in vitro* analyses of the binding preferences of *Drosophila* CHD1 chromodomains, we were not able to fully recapitulate those findings, although the high degree of sequence conservation between human and *Drosophila* CHD1 of the amino acids involved in H3K4me2-3 binding would suggest that fly CHD1 chromodomains should function in a similar way as their human counterparts. *Drosophila* CHD1 chromodomains, in contrast to human CHD1, showed binding to H3K4 peptides regardless of the K4 methylation state. Mutation of the three conserved tryptophan residues 372, 375 and 462, however, resulted in a strong decrease of binding to trimethylated H3K4 without affecting interaction with the non- and dimethylated peptides. There may be two possible explanations for these results: First, potential misfolding of the chromodomain polypeptides due to poor protein solubility might cause a loss of selectivity for binding to H3 peptides methylated at lysine 4. Alternatively, the *Drosophila* protein indeed binds to methylated and unmethylated H3K4 peptides but the amino acids that are responsible for selective interaction of human CHD1 with H3K4me2-3 are specifically involved in H3K4me3 recognition only, while other residues might be important for interaction with non- and dimethylated H3K4. Although at this point we cannot resolve this issue, our *in vivo* analyses clearly demonstrate that the chromodomains of CHD1 play important roles for transcription related and transcription-independent functions of CHD1 in the fly.

In transgenic flies expressing a chromodomain mutant version of CHD1 that should at least abolish interaction with H3K4me3, using polytene chromosome staining, we found no gross changes in the recruitment of mutant CHD1 to chromatin. In contrast to our findings, it was shown for yeast CHD1 that mutation of a single amino acid in chromodomain 2 results in

dissociation of the protein from chromatin (48). These findings are not contradictory to our results with *Drosophila* CHD1, because several reports have shown that yeast CHD1 is not able to bind to methylated or unmethylated H3K4 (33–35). Therefore, the chromodomains in yeast CHD1 might be important for binding to chromatin by a mechanism that does not involve H3 tail interaction.

Role of the chromodomains in HS gene expression

Despite the apparently normal localization properties of chromodomain-mutant CHD1, we found that the transgenic animals exhibit defects in transcription activation of several HS genes as well as incomplete rescue of the viability and fertility phenotypes of *Chd1*-deficient flies (Figures 2 and 3). The diminished transcriptional response of HS genes, which is particularly evident upon prolonged heat exposure of *Chd1*-deficient and $Chd1^{CD}$ transgenic larvae, is striking and affects not only the *Hsp70* and *Hsp22* genes, which have low NHS transcription levels, but is also evident at the constitutively expressed *Hsp83* gene (Figure 3). These data demonstrate that CHD1 is required for full transcriptional induction of HS genes and that the chromodomains play a vital role in this process.

Several reports have linked CHD1 to the regulation of transcription elongation (20–25). Similarly, other ATP-dependent chromatin remodeling factors have been shown to act at this transcriptional stage. For instance, in *Drosophila,* the CHD-family member Kismet (KIS) appears to be a key regulator of early transcription elongation steps (25,49). KIS co-localizes with CHD1 on polytene chromosomes, and mutation of the *kis* gene results in the dissociation of CHD1 from chromatin (notably without perturbation of H3K4me levels) suggesting that KIS functions upstream of CHD1 in transcriptional elongation (25). Lis and colleagues (47) have demonstrated a bi-phasic pattern of chromatin remodeling upon *Hsp70* induction in S2 cells. Initially, nucleosome loss occurs across the entire region of the *Hsp70* gene. The second phase is characterized by ongoing disruption of the nucleosomal structure that occurs without further histone dissociation. RNAi-mediated depletion of CHD1 in *Drosophila* S2 cells revealed a defect in the remodeling of the first positioned nucleosome downstream of the transcription start site of the *Hsp70* gene upon heat treatment (47). Consistent with these results, we find here that H3 levels around the transcription start site are maintained in *Chd1*-deficient larvae under HS conditions. Notably, H3 loss does occur in larvae expressing $CHD1^{CD}$ protein despite the reduced enzymatic activities of this protein that we have observed *in vitro*. Thus, the remaining enzymatic activity appears to be sufficient for nucleosome remodeling to occur at the 5′-end of the *Hsp70* gene but might not allow for sustained high-level transcription. Alternatively, the chromodomains might be required for an additional, remodeling-independent function of CHD1, such as the interaction with components of the transcription machinery.

Contribution of the chromodomains to CHD1 function in early embryonic development

Mutation of the chromodomains of CHD1 not only affects transcription-related but also the transcription-independent role of CHD1 in chromatin reorganization during early development [Figure 2 and (15)]. This is reflected by the fact that the ability of embryos laid by *Chd1*-deficient females to develop normally is only partially rescued in the presence of $CHD1^{CD}$ (Figure 2B and C). Although development of a fraction of the embryos ceases at a very early stage similar to what we have seen with *Chd1*-deficient embryos and thus suggesting defective paternal chromatin reorganization, the reduced enzymatic activity of $CHD1^{CD}$ appears to be sufficient in some embryos to allow pronuclear fusion and progression of development. Since in our assay the embryos stem from *Chd1*-mutant females that had been mated to wild-type males, further development up to gastrulation stages is likely to be promoted by the expression of wild-type CHD1 produced from the paternal genome once these embryos have reached the stage when zygotic transcription commences (around nuclear division cycle 12). Intraembryonic variations in the amount of $CHD1^{CD}$ protein may account for the differences between these two embryo fractions. Indeed, the fraction of developing embryos is markedly smaller when the *Chd1*-deficient mothers carry only one instead of two copies of the $Chd1^{CD}$ rescue allele (data not shown).

The chromodomains regulate the enzymatic functions of CHD1

Our *in vitro* analysis of the enzymatic functions of CHD1 revealed that chromodomain mutation led to a ~2-fold decrease in nucleosome assembly activity suggesting that the chromodomains play an important role in the regulation of the enzymatic function of CHD1. Of note, a recent study reporting on the crystal structure of budding yeast CHD1 suggested that the amino acid residues connecting the two chromodomains form a wedge-shaped helical structure that folds into the ATPase domain and appears to interfere with DNA binding and activation of the ATPase domain (50). Consequently, deletion of both chromodomains in yeast CHD1 resulted in derepression of ATPase activity upon stimulation with DNA and chromatin. This is in perfect agreement with what we have observed with the fly protein upon chromodomain deletion (Supplementary Figure S4). Moreover, it was shown that chromodomain deletion causes a reduction in nucleosome sliding activity of yeast CHD1 (50) again corroborating our findings of reduced nucleosome assembly activity in the fly chromodomain deletion mutant despite the presence of robust ATPase activity (Figure 5C and D). These results suggest that the chromodomains appear to play a key role in linking the ATP-driven motor to the chromatin assembly function of CHD1.

In contrast, the triple amino acid substitutions in fly CHD1 chromodomains compromised both ATPase and nucleosome assembly activities (Figure 5 and Supplementary Figure S4). These point mutations may

affect the chromodomain structure in a way that decreases the flexibility of the chromo wedge and thus inhibits ATPase activation and consequently nucleosome assembly activity.

We have shown here that the chromodomains of CHD1 are important for the enzymatic functions of the protein *in vitro* and for transcription-related and -independent roles *in vivo*. In contrast, mutation of the chromodomains does not result in apparent defects with respect to recruitment to and/or maintenance of CHD1 on chromatin as judged by immunofluorescence staining of polytene chromosomes. However, to ultimately clarify the nature of the interaction of CHD1 with methylated H3K4 and its role in the recruitment of CHD1 to chromatin *in vivo*, the use of higher resolution methods, such as ChIP-seq, will be needed.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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