CHARGE syndrome is a complex developmental disorder caused by mutations in the chromodomain helicase DNA-binding gene CHD7. Kabuki syndrome, another developmental disorder, is characterized by typical facial features in combination with developmental delay, short stature, prominent digit pads and visceral abnormalities. Mutations in the KMT2D gene, which encodes a H3K4 histone methyltransferase, are the major cause of Kabuki syndrome. Here, we report a patient, who was initially diagnosed with CHARGE syndrome based on the spectrum of inner organ malformations like choanal hypoplasia, heart defect, anal atresia, vision problems and conductive hearing impairment. While sequencing and MLPA analysis of all coding exons of CHD7 revealed no pathogenic mutation, sequence analysis of the KMT2D gene identified the heterozygous de novo nonsense mutation c.5263C>T (p.Gln1755*). Thus, our patient was diagnosed with Kabuki syndrome. By using co-immunoprecipitation, immunohistochemistry and direct yeast two hybrid assays, we could show that, like KMT2D, CHD7 interacts with members of the WAR complex, namely WDR5, ASH2L and RbBP5. We therefore propose that CHD7 and KMT2D function in the same chromatin modification machinery, thus pointing out a mechanistic connection, and presenting a probable explanation for the phenotypic overlap between Kabuki and CHARGE syndromes.

INTRODUCTION

CHARGE is an acronym reflecting the main clinical features of this developmental disorder, namely coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia and ear anomalies (MIM 214800) (1). Further symptoms like hypoplasia of the semicircular canals, facial nerve palsy, cleft lip/palate and tracheoesophageal fistula represent pathognomonic features (2–5). Because of its clinical variability diagnostic criteria were set up to facilitate clinical diagnosis. CHARGE syndrome is likely when four major criteria (coloboma, choanal atresia, ear anomalies and cranial nerve dysfunction) or three major and three out of seven minor criteria (genital hypoplasia, developmental delay, cardiovascular malformations, growth deficiency, orofacial cleft, tracheoesophageal fistula and characteristic facial features) are present in a patient (6). The clinical criteria were updated by Verloes in 2005 (7) defining coloboma, atresia of the choanae and hypoplastic semicircular canals as major signs and rhombencephalic dysfunction, hypothalamic-hypophyseal dysfunction, abnormal middle or external ear, malformations of mediastinal organs and mental retardation as minor signs.

Kabuki syndrome (MIM 147920) is characterized by a typical facial gestalt in combination with the cardinal features postnatal short stature, intellectual disability, skeletal findings and dermatooglyphic anomalies (8,9). Other findings like cardiac and
urogenital malformations, cleft palate, hearing loss and ophthalmological anomalies including coloboma of the iris and retina, are additional features (10). Kabuki syndrome shows clinical variability and a phenotypic overlap with CHARGE syndrome. Shared features include cleft palate, retardation of growth and development, genital hypoplasia, congenital heart, ear, eye and renal abnormalities. Clinical distinction between CHARGE and Kabuki syndrome can be challenging in young patients with multitude of organ malformation that fit to the CHARGE spectrum and also owing to the fact that the characteristic facial features of Kabuki syndrome may be lacking in early infancy. Because of the phenotypic overlap between both syndromes we hypothesized that both disorders might share common pathogenetic mechanism.

Mutations in \textit{KMT2D} are the major cause of Kabuki syndrome (11,12). \textit{KMT2D} encodes a methyltransferase also known as MLL2 that is required for histone 3 lysine 4 (H3K4) di- and trimethylation, hallmarks of transcriptional regulation (13). KMT2D belongs to the SET1 family (SET1A, SET1B, KMT2A, KMT2B, KMT2C and KMT2D in humans) of methyltransferases, which are only able to activate their methyltransferase activity in the context of a multi-subunit complex (14). A core module of such a multi-protein complex includes the proteins WDR5, RbBP5 and ASH2L (WAR complex). These proteins are able to stimulate the H3K4 methyltransferase activity by binding in close proximity to the catalytic SET domain (14–16). Furthermore, WAR complex members are able to recruit SET1-family proteins to their target chromatin sites (14). The chromodomain helicase DNA-binding protein CHD8 is an ATP-dependent chromatin remodelling enzyme that was demonstrated to interact with the WAR complex members WDR5, RbBP5 and ASH2L (WAR complex). We recently showed that CHD7, the protein mutated in CHARGE syndrome, interacts directly and indirectly with CHD8 (15,17). We therefore asked the question whether CHD7 could also be a member of the WAR-subcomplex. By using immunoprecipitation experiments, immunohistochemistry and direct yeast two-hybrid screens, we could show that CHD7 indeed interacts with the WAR complex core components WDR5, ASH2L and RbBP5. These results indicate an intriguing functional connection between CHD7 and KMT2D, which might possibly explain the overlapping phenotypes of CHARGE and Kabuki syndromes.

**RESULTS**

**Case history**

The reported boy is the fifth child of healthy unrelated parents. His four older siblings and his younger sister are healthy. The family history was unremarkable for miscarriages, congenital malformations or mental retardation. During pregnancy, there was no exposure to drugs, X-rays or viral infections. At the time of birth, the mother was 26 years old and the father was 28 years of age. The boy was born at 36 gestational weeks per cesarean section with a weight of 2900 g (50–75th centile), a length of 52 cm (75th centile) and a head circumference of 31 cm (10th centile). Congenital anomalies noted after birth comprised anal atresia, unilateral choanal hypoplasia and persistent ductus arteriosus. The boy showed feeding difficulties, cryptorchidism and unilateral patellar luxation requiring orthopedic surgery. Later in the course, a conductive hearing disorder, a hyperopia, strabism and developmental delay became apparent. Based on these features, a clinical diagnosis of CHARGE syndrome was made. At that time, the patient met the diagnostic criteria for CHARGE syndrome (five of six signs) provided by Pagon et al. (1,7) and fulfilled the diagnostic criteria proposed by Blake et al. (6) (three of four major and three of seven minor signs). According to the criteria from Verloes (7), the patient fulfilled the criteria for having atypical CHARGE syndrome (one of three major and three of five minor signs). Consequently, a CHD7 mutation analysis was performed to confirm the clinical diagnosis. Sequence analysis of all 37 coding exons of the CHD7 gene and flanking intronic sequences revealed no causative mutation. Large deletions or duplications including whole exons or the whole CHD7 gene were excluded by multiplex ligand probe amplification (MLPA) analysis. At the age of 5.5 years, developmental evaluation using the Snijders–Oomen Nonverbal Intelligence Scale revealed an intelligence quotient of 55. Clinical follow-up examinations revealed emerging facial features such as long palpebral fissures, long dense eyelashes and arched eye-brows leading to the differential diagnosis of Kabuki syndrome (Fig. 1). Thus, \textit{KMT2D} mutational analysis was performed. All 54 coding exons of the \textit{KMT2D} gene and flanking intronic sequences were analyzed. In exon 22, the heterozygous nonsense mutation c.5263 C > T (p.Gln1755*) was found. Most likely this mutation occurred \textit{de novo}, because the mutation was not present in DNA derived from blood lymphocytes from the healthy parents.

CHD7 co-localizes with WAR complex members

We asked whether the striking phenotypic overlap of CHARGE and Kabuki syndromes might reflect a functional link between CHD7 and KMT2D. To determine interaction of CHD7 with components of the WAR complex, immunohistochemistry was carried out on HeLa cells using Duolink proximity ligation assay (PLA). Positive PLA signals could be detected in the nucleus using antibodies against CHD7 and WDR5 (Fig. 2C).
and D), CHD7 and ASH2L (Fig. 2G and H) or CHD7 and RbBP5, respectively (Fig. 2K and L). Using CHD7 and CHD8 antibodies as a positive control, PLA signals in the nucleus were detected as well (Fig. 2O and P). Barely any PLA signals were detected without specific preference in the cytoplasm as well as in the nucleus of cells using either CHD7 (Fig. 2T), CHD8 (Fig. 2U), WDR5 (Fig. 2Q), ASH2L (Fig. 2R) or RbBP5 antibody (Fig. 2S) indicating that nearly no unspecific binding of the antibodies to the PLA probes are present. (A, E, I, M) cell nuclei were stained with DAPI (blue), (B, F, J, N) the cytoskeleton of the cells was stained by FITC–phalloidin (green).

**Figure 2.** Duolink PLA II method on HeLa cells. Positive PLA signals, indicative for a protein–protein interaction could be detected in the nucleus (red dots) using antibodies against CHD7 and WDR5 (C and D), CHD7 and ASH2L (G and H) and CHD7 and RbBP5, respectively (K and L). Using CHD7 and CHD8 antibodies as a positive control, PLA signals in the nucleus were detected as well (O and P). Only few PLA signals were detected without specific preference in the cytoplasm as well as in the nucleus of cells using either CHD7 (T), CHD8 (U), WDR5 (Q), ASH2L (R) or RbBP5 antibody (S) indicating that nearly no unspecific binding of the antibodies to the PLA probes are present.

**CHD7 co-immunoprecipitates with WAR complex members**

Co-immunoprecipitation studies on HeLa cells were performed to test the assembly of CHD7 with the WAR complex members, WDR5, ASH2L and RbBP5. HeLa cells were single transfected with either the CHD7-CR1-3-pCMV-HA (amino acids 1593–2178, NP_060250.2) plasmid, the CHD8-pCMV-cmyc (amino acids 1789–2302, NP_065971.2), ASH2L-pCMV-HA or RbBP5-pCMV-HA plasmid. 24 h after transfection, nuclear cell proteins were isolated. Using the CHD7 antibody for precipitation after single transfection of ASH2L-pCMV-HA and RbBP5-pCMV-HA plasmid, we detected with an HA antibody a ∼76 kDa band for ASH2L (Fig. 3A, 1) and a ∼59 kDa band for RbBP5 (Fig. 3A, 2). CHD7 precipitation in untransfected HeLa cells did not reveal a signal with an HA antibody (Fig. 3A, 3). After single transfection with the ASH2L-pCMV-HA and RbBP5-pCMV-HA plasmid as a transfection control, the estimated band sizes of ASH2L and RbBP5 were detected with an HA antibody (Fig. 3A, 4 and 5). The same band sizes were observed with an HA antibody after single transfection of ASH2L-pCMV-HA and RbBP5-pCMV-HA plasmid and precipitation with a CHD8 antibody (Fig. 3B, 1 and 2). A detection with an HA antibody following the precipitation of endogenous CHD8 of single transfected HeLa cells with the CHD7-CR1-3-pCMV-HA plasmid revealed a band corresponding to the estimated size of the CHD7 part (∼70 kDa) (Fig. 3B, 3). Precipitation with a CHD8 antibody on untransfected HeLa cells showed no band with an HA antibody (Fig. 3B, 4). As a transfection
control HeLa cells were single transfected with the ASH2L-pCMV-HA, RbBP5-pCMV-HA and the CHD7-CR1-3-pCMV-HA plasmid. The observed band sizes with an HA antibody correlate with the expected size of ASH2L (Fig. 3B, 1) and RbBP5 (Fig. 3B, 2) in fusion to an HA-tag. Using a CHD7 antibody (abcam, ab 65097) for precipitation of endogenous CHD7, we detected a band at ~76 kDa corresponding to ASH2L (lane 1), and the estimated size of ~59 kDa of RbBP5 (lane 2). Lane 3 represents the co-immunoprecipitation performed on untransfected HeLa cells as negative control. Lane 4: input after single transfection with the ASH2L-pCMV-HA plasmid as transfection control, lane 5: input after single transfection with the RbBP5-pCMV-HA plasmid as transfection control. (B) Confirmation that CHD8 interacts with ASH2L, RbBP5 and CHD7. HeLa cells were single transfected with the ASH2L-pCMV-HA (lane 1), RbBP5-pCMV-HA (lane 2) or CHD7-CR1-3-pCMV-HA plasmid. Precipitation was performed with a CHD8 antibody (abcam, ab84527), detection with an HA antibody. A band corresponding to ASH2L (lane 1), a band corresponding to the size of RbBP5 (lane 2) and a band corresponding to the estimated size of the overexpressed CHD7 part (~70 kDa) (lane 3) was observed. Lane 4: co-immunoprecipitation performed on untransfected HeLa cells as negative control, lanes 5–7: input after single transfection with the ASH2L-pCMV-HA (lane 5) and RbBP5-pCMV-HA (lane 6) and CHD7-CR1-3-pCMV-HA (lane 7) plasmid as transfection control. (C) Reciprocal experiment to (A), precipitation with an HA antibody (ASH2L-pCMV-HA (lane 1), RbBP5-pCMV-HA (lane 2) and detection of endogenous CHD7 with the CHD7-specific antibody (abcam, ab 65097) revealed by wet blotting a specific ~320 kDa band corresponding to the estimated size of endogenous CHD7. Lane 3: co-immunoprecipitation performed on untransfected HeLa cells as negative control, reveals no band, indicating that endogenous CHD7 is not capable to bind unspecific to the beads. Lane 4: precipitation with a WDR5 antibody and detection with the CHD7 antibody, demonstrating WDR5 and CHD7 interaction of the endogenous proteins. Lane 5: input of untransfected HeLa cells to demonstrate that CHD7 is expressed endogenously. Lanes 6–7: input after single transfection with the ASH2L-pCMV-HA (lane 6) and RbBP5-pCMV-HA (lane 7) plasmid as transfection control.

**CHD7 shows no direct interaction with WAR complex members**

To determine a direct interaction of CHD7 with WAR complex members, we first used the recently described CHD7 bait plasmid CHD7-CR1-3-pGBK7T, spanning the amino acids 1591–2181, NP_065971.2 (18) for direct yeast two-hybrid experiments with the WDR5-pGADT7 (NP_060058.1), ASH2L-pGADT7 (NP_004665.2) and RbBP5-pGADT7 (NP_005048.2).
Figure 4. Yeast two-hybrid assay. The plasmid CHD7-CR1-3-pGBK7 (amino acids 1591–2181) was used for direct yeast two-hybrid experiments with the WDR5-pGAD7, ASH2L-pGAD7 and RbBP5-pGAD7 plasmids. An autoactivation test using the empty pGBK7 bait vector together with the WDR5-pGAD7, ASH2L-pGAD7 and RbBP5-pGAD7 plasmids reveals no autoactivation of the yeast strain reporter genes. The yeast two-hybrid experiments revealed no direct interaction of the CHD7-CR1-3-part with the WAR complex members, while the recently described CHD8 part (amino acids 1789–2302) binds directly WDR5, ASH2L, RbBP5 and the CHD7-CR1-3-part.

Figure 5. Schematic overview of the used CHD7 yeast two-hybrid constructs. CHD7 belongs to the CHD family of chromatin remodelers and consists of two N-terminal chromodomains, a SWI2/SNF2-like ATPase/helicase domain, three conserved regions (CR1–CR3), a SANT domain and two BRK domains. The plasmids CHD7-1-pGBK7–CHD7-4-pGBK7 divide the full-length CHD7 in four overlapping parts without disrupting a known functional domain. The plasmid CHD7-CR1-3-pGBK7 was described previously (18).
plasmids as prey, respectively. An autoactivation test using the empty pGBK7 bait vector together with the prey plasmids demonstrates no autoactivation of the yeast strain reporter genes (Fig. 4). The yeast two-hybrid experiments revealed no direct interaction of the CHD7-CR1-3-part with the WAR complex members, while the recently described CHD8 part (amino acids 1789–2302, NP_065971.2) binds directly WDR5, ASH2L and RbBP5, and as demonstrated recently (18) interacts with the CHD7-CR1-3-part. Because the CHD7 interaction site with WAR complex members could be outside the tested CHD7-CR1-3 area, we generated four new bait plasmids, namely CHD7-1-pGBK7 (amino acids 1–799), CHD7-2-pGBK7 (amino acids 732–1567), CHD7-3-pGBK7 (amino acids 1533–2380) and CHD7-4-pGBK7 (amino acids 2325–2997) dividing the human CHD7 full-length protein in four overlapping fragments (Fig. 5) and performed direct yeast two-hybrid experiments with the WDR5-pGADT7, ASH2L-pGADT7 and RbBP5-pGADT7 plasmids. As positive control, we used the bait plasmid CHD7-CR1-3-pGBK7 and the prey construct CHD8-pGADT7-Rec were used as positive control. No interaction could be observed between the CHD7 parts and the WAR complex members.

**Figure 6.** Yeast two-hybrid assay. Yeast two-hybrid experiment performed with the constructs CHD7-2-pGBK7 (A and B), CHD7-3-pGBK7 (C and D) and CHD7-4-pGBK7 (E and F) as baits, which are parts of the human CHD7 full-length protein. The WDR5-pGADT7, ASH2L-pGADT7 and RbBP5-pGADT7 plasmids were used as preys. All bait plasmids were tested for autoactivation of the Y2HGold yeast strain reporter genes with the prey plasmid empty pGADT7. The bait plasmid CHD7-CR1-3-pGBK7 and the prey construct CHD8-pGADT7-Rec were used as positive control. No interaction could be observed between the CHD7 parts and the WAR complex members.

No further direct yeast two-hybrid experiments could be performed with the construct CHD7-1-pGBK7 and as prey the construct CHD8-pGADT7-Rec (amino acids 1789–2091, NP_065971.2). No further direct yeast two-hybrid experiments could be performed with the construct CHD7-1-pGBK7 (amino acids 1–799) owing to autoactivation of the plasmid in the yeast system (Suppl. Fig. 1).

The direct yeast two-hybrid experiments with the CHD7-2-pGBK7, CHD7-3-pGBK7 and CHD7-4-pGBK7 plasmids...
Depletion of CHD7 has no influence on the expression level of WAR complex members

To analyze the influence of CHD7 on the expression of CHD8 and components of the WAR complex members, CHD7 was downregulated by co-transfection of two short interfering RNAs (siRNAs) in HeLa cells. Quantitative real-time PCR revealed a reduced CHD7 expression of ~50%, corresponding to the haploinsufficiency level of CHD7 in CHARGE syndrome patients (Fig. 7). The relative expression levels of WDR5, ASH2L, RbBP5 and CHD8 remained unchanged showing that CHD7 has no regulatory influence on the expression level of CHD8 and the WAR complex members in our cellular system (Fig. 7).

DISCUSSION

In the present report, we describe a patient with a heart defect, anal atresia, vision impairment, conductive hearing loss and facial dysmorphism initially diagnosed with CHARGE syndrome. Molecular genetic analysis of the CHD7 gene failed to detect a pathogenic mutation, while subsequent identification of a de novo stop mutation in KMT2D (c.5263 C > T, p.Gln1755*) established the diagnosis of Kabuki syndrome in the patient. Ten years ago, Ming et al. (19) described the clinical difficulty in distinguishing between these two disorders, especially in infancy when the typical facial gestalt of Kabuki syndrome may not be recognizable in some of the patients. Our patient with Kabuki syndrome presented here was initially diagnosed with CHARGE syndrome fulfilling its diagnostic criteria. Owing to this phenotypic overlap, we asked the question if CHARGE and Kabuki syndromes are two related conditions with a common molecular pathogenesis.

Mutations in the KMT2D gene are the major cause of Kabuki syndrome (11,12). KMT2D belongs to the SET1 family of histone methyltransferases. A characteristic feature of this group of enzymes is the presence of a SET domain, which is necessary for H3K4 methylation within chromatin (20). H3K4 methylation is a hallmark for transcriptional activation (21). Because KMT2 proteins contain regions with high homology to the Drosophila protein TRX (trithorax) the KMT2 proteins are referred to the trithorax group of activator proteins leading to increased transcriptional activation of target genes and counterparts the repressive polycomb group (20,22). KMT2 proteins act in multi-protein complexes and several interaction partners have been described (14,23,24). Known KMT2 ‘core’ binding partners are WDR5, ASH2L and RbBP5 and these proteins build the so called WAR subcomplex, having an important role in gene expression, including transcription at the HOXA2 locus (15).

Mutations in the chromodomain helicase DNA-binding protein CHD7, a chromatin remodeler, are associated with CHARGE syndrome. Proteins involved in chromatin remodeling are typically found in multi-protein complexes, as well and several tissue-specific CHD7 interacting partners have been identified (18,25–28). In an earlier study, we could demonstrate that a part of human CHD7 interacts with a part of human CHD8 (18). CHD8 belongs together with CHD7 to the same subgroup of CHD remodeling enzymes and therefore both proteins consist of two N-terminal chromodomains, followed by a SWI2/SNF2-like ATPase/helicase domain, three conserved regions (CR1–CR3), a SANT domain and two BRK domains (29). For CHD8, it was shown that it forms a complex together with WDR5, ASH2L and RbBP5 in the absence of MLL (15,17). CHD8 is able to interact directly with each WAR complex component, suggesting extensive contacts within this complex, while the KMT2-WAR complex interaction is WDR5 dependent (30). Furthermore, it was demonstrated that a depletion of CHD8 results in the loss of ASH2l and a loss of the H3K4 trimethylation mark at the KMT2 regulated HOXA2 promoter (15). Therefore, it is suggested that CHD8 is necessary for a proper WAR complex recruitment and activation of histone modifying complexes (15).

These previous studies encouraged us to investigate a likely interaction of CHD7 with the WAR complex members. By co-immunoprecipitation experiments, we demonstrated that CHD7 is associated with WDR5, ASH2L and RbBP5. Because a specific part of CHD7 interacts directly with a part of CHD8 (18), it is likely that CHD7 is an additional member of the CHD8 containing WAR complex. We could confirm by direct yeast two-hybrid studies that a part of CHD8 is also capable to interact with all WAR complex components analyzed. To test whether also CHD7 directly interacts with WAR complex components or CHD7 is linked to the WAR complex only via the previously shown CHD7–CHD8 interaction, we performed direct
yeast two-hybrid experiments. Only the recently shown CHD7–CHD8 interaction could be observed indicating that CHD7 is linked to the WAR complex via CHD8. Therefore, it is presumably that CHD7 is a member of a WAR complex, which consists of CHD8 in the absence of KMT2D. Moreover, using the Duolink Pla II method we proved that the interactions take place in the nucleus.

In summary, we demonstrate that CHD7 is a binding partner of a WAR complex component, linking CHD7 and KMT2D to the same chromatin remodeling and chromatin modification machinery via the WAR complex. It is likely that CHD7 and KMT2D regulate a common subset of genes by their interaction with the WAR complex and altered expression of these specific subsets caused by mutations in either CHD7 or KMT2D might explain the overlapping phenotype in CHARGE and Kabuki syndromes. Although further studies are needed to identify these common target genes, we postulate that CHARGE and Kabuki syndromes are two related disorders with a common pathogenesis.

**MATERIALS AND METHODS**

**Mutational analysis**

Genomic DNA was extracted from peripheral blood lymphocytes using standard methods. All 37 coding exons of the CHD7 gene (exon 2–38, NM_017780) and flanking intronic sequences as well as all 54 coding exons of the KMT2D gene (exon 1–54, NM_003482) were amplified by polymerase chain reaction (PCR) and sequenced bidirectional. Primer sequences and PCR conditions are available on request. Additionally, to exclude large deletions/duplications of the CHD7 gene a quantitative analysis [multiplex ligation probe amplification (MLPA), Kit P201-C2; MRC-Holland, Amsterdam, The Netherlands] was performed.

**Duolink II proximity ligation assay (PLA)**

HeLa cells were cultured a day before on 8-well chamber slides. The confluence of the cells should be ~90%. The cells were washed twice with Dulbecco’s phosphate buffered saline (DPBS) and fixed in 3.7% formaldehyde/phosphate buffered saline (PBS) for 20 min at room temperature. After washing the cells twice with PBS, cells were permeabilized for 10 min using 0.1% Triton X-100/PBS followed by washing steps with PBS. For the blocking step, the blocking solution from the PLA Probe Kit. For the technical negative controls, only one probe was used. The slides were incubated in a humidity chamber at 37°C for 20–30 min. Next the primary antibodies were 1:100 diluted with the Antibody Diluent from the PLA Probe Kit. For the technical negative controls, only one primary antibody was used. The slides were incubated over night at 37°C in a humidity chamber. The following day, the probes were washed with self-made washing buffer A. The PLA probes (Duolink In Situ PLA Probe Anti-Rabbit MINUS; Duolink In Situ PLA Probe Anti-Goat PLUS) were diluted 1:5 in Antibody Diluent and added to the cells which were then incubated in the humidity chamber for 1 h at 37°C. After washing the cells with washing buffer A the ligation solution was added to the cells. The Ligation stock was diluted 1:5 and the ligase added at a 1:40 dilution. The Ligation-Ligase solution was added to the cells which were incubated for 30 min in a humidity chamber at 37°C. The solution was washed off by washing buffer A and the light sensitive amplification solution was prepared. Therefore, the amplification stock had to be diluted 1:5 times and the Polymerase was added at a 1:80 dilution. The cells were incubated in a humidity chamber at 37°C for 100 min. After that the cells were washed with self-made washing buffer B and the cytoskeleton of the cells was stained by fluorescein isothiocyanate (FITC)–phalloidin, the cells were washed again in washing buffer B and closed under 4′,6-diamidino-2-phenylindole (DAPI). The data were analyzed using the Olympus BX60 microscope (Olympus, Hamburg, Germany).

**Cell culture**

HeLa cells were cultured in Dulbecco’s modified Eagle’s media (DMEM) (PAN BIOTECH). Additionally 10% fetal calf serum (PAN BIOTECH) and 1% penicillin/streptomycin (PAN BIOTECH) were added. Cells were plated into 75 cm² flasks (Sarstedt, Newton, NC, USA). Transfection was performed when the cells reached a confluence of 80–90%. For transfection plasmid DNA and Lipofectamine 2000 transfection reagent (Invitrogen) were mixed with OptiMEM serum-free medium (Gibco) and incubated 5 min, then combined and incubated for additional 25 min. After washing the cells with DPBS, DMEM + non-essential amino acid (NEAA) (Gibco) were added as well as the transfection reagent mix. After 4 h incubation additionally DEMEM with 20% fetal calf serum and NEAA were added. After that cells were grown for additional 18 h.

**Protein isolation**

Nuclear proteins were specifically isolated from HeLa cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instruction. The protein concentration of the cytoplasmic and nuclear extracts were measured (Bradford SYNERGY MX) and stored at −80°C.

**Co-immunoprecipitation**

For co-immunoprecipitation we used the plasmids CHD7-CR1-3-pCMV-HA (amino acids 1593–2178, NP_060250.2) and CHD8-pCMV-cmyc (amino acids 1789–2302, NP_065971.2), which we previously described (18). Additionally, we generated the following plasmids: ASH2L-pCMV-HA, RbBP5-pCMV-HA by cloning the human full-length sequence of ASH2L (NP_004665.2) and RbBP5 (NP_005048.2) in frame to the hemagglutinin (HA) epitope tag into the pCMV-HA vector (Clontech, Mountain View, CA, USA) by using the In Fusion advantage kit (Clontech) according to the company’s protocol. The correct reading frame and sequence was confirmed by sequence analysis. HeLa cells were cultured in 75 cm² flasks (Sarstedt) and were single transfected with either the ASH2L-pCMV-HA or RbBP5-pCMV-HA plasmid. Additionally, HeLa cells were co-transfected with CHD7-CR1-3-pCMV-HA (amino acids 1593–2178, NP_060250.2) and CHD8-pCMV-cmyc (amino acids 1789–2302, NP_065971.2). After incubation for 24 h at
37°C, co-immunoprecipitations were carried out as previously described (18).

The following antibodies were used for immunoblotting: anti-HA (Roche Diagnostik GmbH, Mannheim, Germany) at a dilution of 1:1000, goat anti-CHD7 (abcam, ab65097) at a dilution of 1:1000, rabbit anti-CHD8 (abcam, ab84527). For detection, we used the following antibodies: anti-CHD8, goat anti-Rat secondary antibody conjugated with horseradish peroxidase (Thermo Scientific) for anti-HA, donkey anti-goat IgG peroxidase secondary antibody (Santa-Cruz Biotechnologies, California, USA) for anti-CHD7.

The samples were loaded onto a 4–12% NuPAGE Bis-Tris gel (Invitrogen) or a 3–8% Tris-Acetate gel (Invitrogen).

Direct yeast two-hybrid

For yeast two-hybrid studies, we used the plasmids CHD7-CR1-3-pGBK7T (amino acids 1591–2181, NP_060250.2), CHD8-pGBK7T (amino acids 1789–2302, NP_065971.2) and CHD8-pGAD7T-Rec (amino acids 1789–2091), which we described previously (18,28). Furthermore, we generated the constructs WDR5-pGAD7T, ASH2L-pGAD7T and RbBP5-pGAD7T by cloning the human full-length sequence of WDR5 (NP_060058.1), ASH2L (NP_004665.2) and RbBP5 (NP_060058.1) into the pGADT7 vector (Clontech) by using the In Fusion advantage kit (Clontech). Additionally, we created four new CHD7 constructs, namely CHD7-1-pGBK7T (amino acids 1–799, NP_060250.2), CHD7-2-pGBK7T (amino acids 732–1567, NP_060250.2), CHD7-3-pGBK7T (amino acids 1533–2380, NP_060250.2) and CHD7-4-pGBK7T (amino acids 2325–2997, NP_060250.2) dividing the human CHD7 full-length protein into four overlapping fragments.

All plasmids were tested for toxicity and autoactivation of the Y2HGold strain (Clontech) reporter genes. All constructs have no toxic effect on the yeast cells. Construct CHD7-1-pGBK7T (amino acids 1–799, NP_060250.2) showed autoactivation of the Yeast system, while the other plasmids are fine. Therefore, no further Yeast experiments were performed with the CHD7-1-pGBK7T construct. The direct Yeast two-hybrid experiments were performed according to the manufacturer’s protocol and as described before (18,28).

The direct yeast two-hybrid experiments showed no direct interaction of the CHD7 constructs CHD7-2-pGBK7T, CHD7-3-pGBK7T and CHD7-4-pGBK7T with the WAR complex members by using the plasmids WDR5-pGAD7T, ASH2L-pGAD7T and RbBP5-pGAD7T. Therefore, we picked one up to three colonies growing in each sector on the -LT medium plates, respectively. They were inoculated into 4 ml of -LT liquid medium and incubated by shaking (220 rpm) at 30°C overnight. To pellet the cells they were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The pellets were resuspended with 4 ml of distilled water (Ampuwa®) and the cells were centrifuged (1000 rpm for 5 min) again. After re-suspending the cell pellet with 4 ml of distilled water (Ampuwa®), a second time the yeast cell cultures were plated on -LT and -LTHA medium plates as drops of 10 µl, respectively. The medium plates were incubated at 30°C for 1–3 days.

CHD7 knockdown by siRNA

HeLa cells and MRCV-CV-1 cells were cultured one day before transfection in 6-well plates (Sarstedt). After 24 h when the cells’ confluence reached ~70–80%, they were transfected with 5 µl siRNA #1 (Oligo #1 forward CACAUUGACAG AUGGGCAAGCUA, reverse UAUGACUGGCAUCAUGCU GAUGUG) and 5 µl siRNA #3 (Oligo #3 forward GGGUG GAGAAGAUGUGCUUGUCUA, reverse UAGACAAAGCA GAUCUUCUCCACCC) with 10 µl Lipofectamine 2000 transfection reagent (Invitrogen) and 100 µl OptiMEM serum-free medium (Gibco). After 72 h incubation at 37°C the RNA was isolated according to standard procedures. As a control HeLa cells were transfected with a siRNA which is not directed against an existing gene but contains the same cytosine and guanine amount as the siRNA #1 and #3. As additional controls untransfected HeLa cells were cultured in either DEMEM or DMEM with OptiMEM serum-free medium and Lipofectamine 2000.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from HeLa cells were extracted 72 h after transfection using TRIZOL® Reagent. Five hundred microliters of TRIZOL® Reagent per 6-well plate were added to the cells and incubated for 5–10 min on ice. The suspension was transferred into an e-cup, 200 µl chloroform was added, mixed by vortexing and incubated on ice for 5 min. After a 15 min centrifugation step at 13 000 rpm at 4°C the upper phase were transferred into a new e-cup and 500 µl isopropanol was added, probes were inverted several times and incubated on ice for 10 min. The probes were centrifuged for 10 min at 13 000 rpm and the supernatant discarded. The pellet was washed with 75% ethanol/diethylpyrocarbonate (DEPC) and centrifuged again and the supernatant was discarded. The dry pellet was dissolved in 20–30 µl DEPC water. Five micrograms of RNA was used for the reverse transcription. Distilled water (Ampuwa®) was added to have a total volume of 11 µl. One microliter of oligo(dT)-primer (0.5 µg/µl) and dNTPs (10 mM) were added, mixed and incubated for 5 min at 65°C. Two microliters of dithiothreitol (0.1 M) and 4 µl 5 × First Strand Buffer were added and for 2 min incubated at 42°C. Next 1 µl reverse transcriptase (Superscript II) was used for each probe. At 42°C for 50 min the transcription into complimentary DNA (cDNA) took place. The reaction was stopped by increasing the temperature to 70°C for 15 min. The cDNA was used to perform a quantitative RT-PCR. For the standard curve a serial dilution of 1:5 was prepared for each gene. Distilled water (Ampuwa®) was used as a blank value. For each sample, a triple determination was performed and normalized against PBGD (porphobilinogen deaminase) and TBP (TATA-box binding protein). The 10 µl PCR sample included 1 µl RT product, 5 µl Platinum® SYBR® Green qPCRSuperMix-UDG with ROX and 0.4 µl (10 pmol/µl) forward and reverse primer. The reactions were incubated in a 384-well plate (AB Gene Diamond Ultra) at 50°C for 2 min, at 95°C for 3 min, followed by 40 cycles of 94°C for 16 s, 60°C for 30 s, and 72°C for 50 s using the ABI Prism 7900HT Sequence Detection System. Data analysis was performed by the comparative threshold cycle method.
ACKNOWLEDGEMENTS
We thank W. Engel for his helpful discussions and his support. We also thank U. Lenz for her excellent technical assistance and we thank the family for their cooperation.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by the Deutsche Forschungsgemeinschaft (DFG) by grant number PA 2030/1-1 to S.P., and by the German Federal Ministry of Education and Research (BMBF) by grant number 01GM0801 (E-RARE network CRANIRARE-2) and grant number 01GM1109C (national rare disease network FACE) to B.W.

REFERENCES