CLINICAL GENETICS
An International Journal of Genetics, Molecular, and Personalized Medicine

Clin Genet 2010: 78: 11–20
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2010.01446.x

Developmental Biology: Frontiers for Clinical Genetics

Section Editor:
Jacques L. Michaud, email: jmichaud@justine.umontreal.ca

Chromodomain proteins in development: lessons from CHARGE syndrome

Layman WS, Hurd EA, Martin DM. Chromodomain proteins in development: lessons from CHARGE syndrome.
Clin Genet 2010: 78: 11–20. © John Wiley & Sons A/S, 2010

In humans, heterozygous mutations in the adenosine triphosphate-dependent chromatin remodeling gene *CHD7* cause CHARGE syndrome, a common cause of deaf–blindness, balance disorders, congenital heart malformations, and olfactory dysfunction with an estimated incidence of approximately 1 in 10,000 newborns. The clinical features of CHARGE in humans and mice are highly variable and incompletely penetrant, and most mutations appear to result in haploinsufficiency of functional CHD7 protein. Mice with heterozygous loss of function mutations in *Chd7* are a good model for CHARGE syndrome, and analyses of mouse mutant phenotypes have begun to clarify a role for CHD7 during development and into adulthood. *Chd7* heterozygous mutant mice have postnatal delayed growth, inner ear malformations, anosmia/hyposmia, and craniofacial defects, and *Chd7* homozygous mutants are embryonic lethal. A central question in developmental biology is how chromodomain proteins like CHD7 regulate important developmental processes, and whether they directly activate or repress downstream gene transcription or act more globally to alter chromatin structure and/or function. CHD7 is expressed in a wide variety of tissues during development, suggesting that it has tissue-specific and developmental stage-specific roles. Here, we review recent and ongoing analyses of CHD7 function in mouse models and cell-based systems. These studies explore tissue-specific effects of CHD7 deficiency, known CHD7 interacting proteins, and downstream target sites for CHD7 binding. CHD7 is emerging as a critical regulator of important developmental processes in organs affected by human CHARGE syndrome.

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Key words: CHARGE syndrome – CHD7 – chromodomain helicase DNA-binding gene 7

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Received 22 March 2010, revised and accepted for publication 31 March 2010

Regulation of eukaryotic genes is essential for normal tissue and organ development and maintenance. Chromatin structure has a vital role in gene regulation, via its effects on cellular proliferation and maintenance of the differentiated state. A central goal in current developmental biology is to identify molecular pathways that regulate chromatin structure and gene expression, and to understand how this regulation influences organogenesis. In humans, haploinsufficiency for the chromodomain helicase DNA-binding gene 7 (*CHD7*) causes CHARGE syndrome, a multiple anomaly condition characterized by ocular coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia, and ear abnormalities including deafness and vestibular disorders (1). The identification of *CHD7* as the causative gene for CHARGE syndrome has led to several recent advancements in our understanding of this complex disorder.

Furthermore, with the establishment of mouse models for CHARGE, it is now possible to begin detailed molecular analyses of how CHD7 functions in specific tissues and cell types both during development and into adulthood. Here, we review recent studies which explore the underlying molecular genetic mechanisms by which CHD7 regulates the development and maintenance of various tissues. Researchers are now poised to charge into the future with a well-equipped toolbox for analyzing CHD7 function.

CHD7 mutations cause CHARGE syndrome

In humans, heterozygous *CHD7* mutations cause CHARGE syndrome, a clinically variable, multiple congenital anomaly condition affecting development of the inner ear, central nervous system (CNS), olfactory system, eyes, heart, choanae (the region between the oropharynx and nasal passages), genitalia, and craniofacial structures including the hard and soft palates, lip, external ear, and midface (2–6). CHARGE is a common cause of deaf–blindness, balance disorders, olfactory dysfunction, and congenital heart malformations, with an estimated incidence of 1:10,000 in newborns (7–9). The human *CHD7* gene spans 188 kb on chromosome 8q12.1, with a 8994 bp open reading frame and translation start site in exon 2. Heterozygosity for nonsense, deletion, or missense *CHD7* mutations occurs in 60–80% of patients with CHARGE (2–5, 10, 11). Human *CHD7* mutations are distributed throughout the coding sequence and do not appear to be correlated with specific aspects of the clinical phenotype (2–5, 10, 11). Most human *CHD7* mutations identified thus far are *de novo*; however, evidence for germline mosaicism has been reported in families with multiple affected siblings (3, 12–14).

The clinical features of CHARGE in humans and mice are highly variable and incompletely penetrant. We recently reviewed all reports of *CHD7*-mutation positive CHARGE individuals, and found that the most commonly affected organ in humans with *CHD7* mutations is the ear (2–6, 12–35). Ear defects in CHARGE include temporal bone abnormalities, external ear malformations, and hearing loss (2–6, 12–35). In addition to ear abnormalities, a majority of CHARGE patients are also affected by some combination of the following: ocular coloboma, heart defects, delayed growth and development, and genital hypoplasia (2–6, 12–35). Although olfactory function is less commonly analyzed in CHARGE patients, a majority of CHARGE patients analyzed have some form of olfactory defect including olfactory bulb hypoplasia and/or aplasia, and impaired olfaction ranging from mild hyposmia to anosmia (16, 24, 26, 28, 36–40). Less commonly reported clinical features associated with CHARGE and *CHD7* mutations include choanal atresia, facial nerve palsy, cleft lip and/or plate, and tracheoesophageal fistula (2–6, 12–35). The variability in CHARGE features suggests that mutations in *CHD7* lead to pleiotropic developmental defects; however, the mechanisms underlying these defects have not yet been determined.

Mouse models of CHARGE and tissue-specific defects

Mouse CHD7 protein is comprised of 2985 amino acids, and has a predicted molecular weight of 334 kDa. Both human and mouse *Chd7* genes contain 38 exons, and have similar exon–intron structures. At the protein level, there is 94.7% sequence identity between mouse and human CHD7, which corresponds to 89.7% nucleotide sequence identity. Heterozygous *Chd7* mutant mice were originally identified by ethylnitrosourea (ENU) mutagenesis, with each of nine different lines carrying a single nonsense *Chd7* mutation (41). These nine *Chd7* mutant mouse lines are viable, with phenotypes that include head bobbing, circling behaviors, disrupted lateral semicircular canals, hyperactivity, reduced postnatal growth, variable cleft palate, choanal atresia, cardiac septal defects, hemorrhage, prenatal death, genital abnormalities, keratoconjunctivitis sicca (dry eye), and olfactory defects (41). Our laboratory generated heterozygous loss of function $Chd7^{Gt/+}$ mice using *Chd7* gene trap embryonic stem (ES) cells (42). $Chd7^{Gt/+}$ mice have phenotypic features similar to those generated by ENU mutagenesis (42). $Chd7^{Gt/Gt}$ mice are embryonic lethal by E11, and exhibit developmental growth delays such as reduction in size of multiple organs including brain, eyes, ears, and craniofacial structures (42). The intrauterine lethality of homozygous $Chd7^{Gt/Gt}$ embryos and the severity of developmental malformations in $Chd7^{Gt/+}$ mice indicate a lack of redundancy for prenatal *Chd7* function in the mouse genome and suggest important roles for CHD7 in organogenesis (42). *Chd7* is expressed in specific tissues during mouse and human embryogenesis including the ear, brain, cranial nerves, olfactory epithelium, olfactory bulb, pituitary, heart, liver, eye, gut, kidney, and craniofacial structures (Fig. 1) (5, 39, 41, 42). *Chd7* is also expressed in the adult mouse olfactory

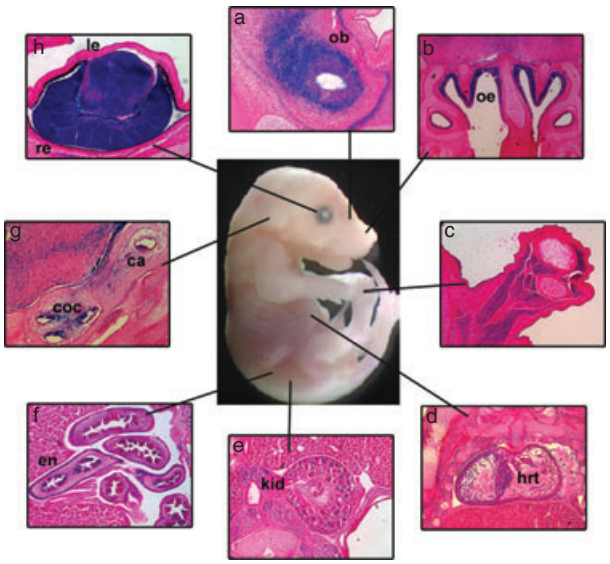

Fig. 1. Chd7 is expressed in CHARGE-related tissues during development. Frozen sections from E14.5 *Chd7*$^{Gt/+}$ embryos show β-galactosidase (X-gal staining) in CHARGE-related organs including (a) olfactory bulbs (ob), (b) olfactory epithelium (oe), (c) limb, (d) heart (hrt), (e) kidney (kid), (f) enteric neurons of the gut (en), (g) crista (ca) and cochlea (coc) of the inner ear, and (h) lens (le) and retina (re) of the eye. All sections are in the coronal orientation.

epithelium, olfactory bulb, and in the rostral migratory stream (39).

Inner ear abnormalities in humans and mice with CHD7 deficiency

The most consistent clinical feature associated with CHARGE syndrome is inner ear defects, including semicircular canal dysplasia that typically affects all three canals, and a Mondini form of cochlear hypoplasia (35, 43–45). In addition, both facial and vestibulocochlear nerve abnormalities are also reported (44, 46). Cochlear implants are a successful treatment for some CHARGE patients, provided there are no underlying nerve abnormalities. CHARGE patients also display vestibular dysfunction, including delayed postural development, abnormal vestibular testing, and balance/motor problems (47, 48). Although absence of the semicircular canals is believed to be a major cause of balance dysfunction, other factors such as ocular malformations, CNS defects, and skeletal abnormalities are also likely contributors.

Heterozygous *Chd7* mice display circling and head-bobbing behaviors consistent with vestibular dysfunction phenotypes (41, 42). Detailed examination of *Chd7* mutant mouse ears indicates a variety of lateral semicircular canal malformations, smaller posterior semicircular canals, and defects in innervation of the posterior crista (Fig. 2) (42, 49–52).

During embryonic development, semicircular canals are formed by sequential outpocketing, fusion of the otic epithelium, and tightly controlled processes to form three canals (53). Semicircular canal dysgenesis can be caused by mutations in a variety of transcription factors and signaling molecules (Table 1). Semicircular canal abnormalities observed in *Chd7* mutant mice are postulated to be the result of smaller posterior and lateral canal outpocketings and delayed fusion of the epithelium (Fig. 2) (51). Perturbations in signaling cascades both within the inner ear epithelium and surrounding mesenchyme can also result in semicircular canal defects (Table 1) (54). In summary, the inner ear phenotypes observed in heterozygous *Chd7* mice are similar to those reported in CHARGE patients and include semicircular canal defects, innervation defects, and vestibular dysfunction. The precise molecular mechanisms of *Chd7* function within the ear are still not understood, but probably involve complex interactions with essential transcription factors expressed during ear development.

Impaired olfaction in humans and mice with CHD7 deficiency

Genital hypoplasia, delayed puberty, and delayed growth are common in CHARGE individuals (16, 24, 26, 28, 40). These features often occur in conjunction with olfactory defects, including hypoplastic olfactory bulbs and reduced olfaction (16, 24, 26, 28, 40). Previous studies of endocrine dysfunction in CHARGE patients reported growth delays, but only 9% of CHARGE patients had growth hormone deficiency, and growth delay was not associated with thyroid-stimulating hormone or adrenocorticotropic hormone deficiency (40). Analysis of female CHARGE patients over the age of 12 showed lack of spontaneous puberty and no response to stimulation with gonadotropin-releasing hormone (GnRH) (40). A majority of male CHARGE patients had low testosterone levels and cryptorchidism and/or micropenis. In contrast to females, male response to GnRH stimulation was variable and did not always correlate with testosterone levels (40). Endocrine dysfunction in CHARGE individuals is therefore likely to be multifactorial, with variable influence on olfaction, somatic growth, puberty, and fertility.

Mutations in *CHD7* have been reported in individuals with Kallmann syndrome with and without a CHARGE syndrome diagnosis (24, 26).

Table 1. Mouse models of human semicircular canal dysgenesis

Gene	Type of protein	Mouse inner ear defects	Human inner ear defects	References
Brn4 (Pou3f4)	Pou-domain transcription factor	ASC abnormalities	X-linked hereditary deafness type 3: dysplasia of cochlea and semicircular canals and deafness	69, 70
Chd7	Chromodomain helicase DNA-binding protein	LSC, PSC, and associated crista abnormalities	CHARGE syndrome: outer and inner ear abnormalities including semicircular canal defects and deafness	41, 42, 49
Eya1	Transcription factor	ASC, PSC, LSC, and associated crista absent	Branchio-oto-renal syndrome: outer, middle, and inner ear abnormalities and deafness	71, 72
Fgf3	Fibroblast growth factor	ASC, PSC, and LSC abnormalities	Syndromic deafness: inner ear agenesis and deafness	73–75
Fgf10	Fibroblast growth factor	ASC, PSC, LSC, and associated crista abnormalities	LADD syndrome: outer ear abnormalities, inner ear abnormalities, and deafness	76, 77
Jag1	Notch receptor ligand	ASC, PSC, and LSC abnormalities	Alagille syndrome: posterior semicircular canal defects	51, 78
Six1	Transcription factor	ASC, PSC, and LSC absent	Branchio-oto-renal syndrome: outer, middle, and inner ear abnormalities and deafness	79–81

ASC, anterior/superior semicircular canal; LSC, lateral semicircular canal; PSC, posterior semicircular canal.

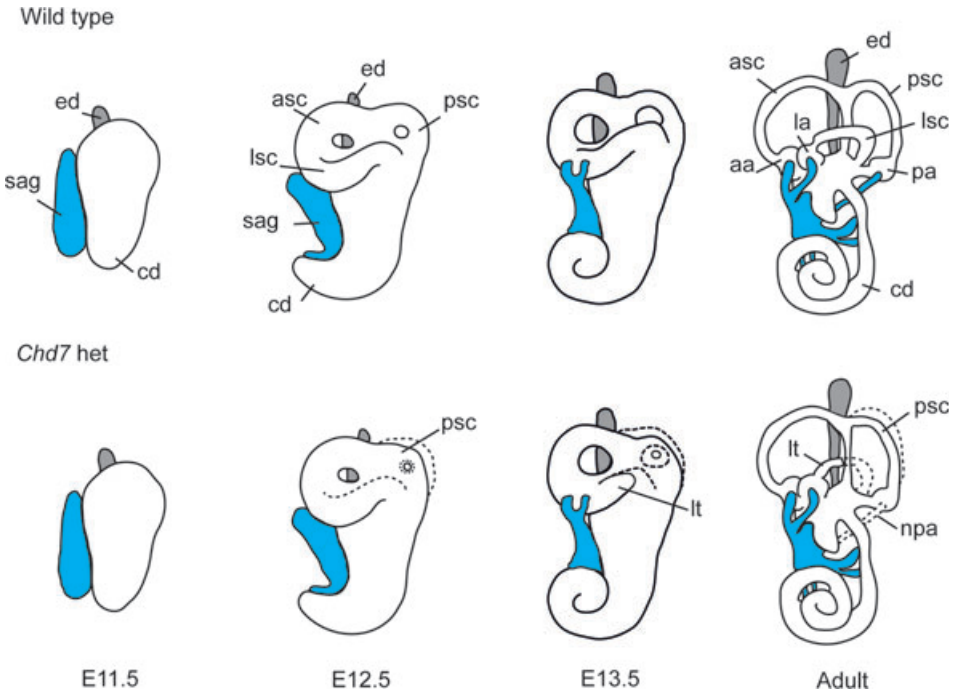

Fig. 2. Model of inner ear development in wild type and *Chd7* heterozygous mutant mice. The E11.5 otocyst extends dorsally to form the endolymphatic duct (ed, gray) and ventrally to form the cochlea (coc), and neuroblasts delaminate from the ventral epithelium to occupy the statoacoustic ganglion (sag, shown in blue). By E12.5, the epithelium of the canal pouches fuses to form the anterior (asc), posterior (psc), and lateral (lsc) semicircular canals. Between E13.5 and adulthood, the vestibular apparatus continues to mature, the cochlea completes its coiling, and the sensory epithelium becomes completely innervated. In *Chd7* heterozygous mutant ears, the lateral and posterior semicircular canals are truncated (lt), small, or misshapen. In addition, the nerve to the posterior ampulla (npa) is disrupted. Normal developmental structures are shown by a dashed line in *Chd7* heterozygous mutants.

Kallmann syndrome is primarily characterized by idiopathic hypogonadotropic hypogonadism and anosmia (26, 55, 56). Idiopathic hypogonadotropic hypogonadism is characterized by impaired or absent sexual development because of sex steroid hormone deficiency, with low serum levels of the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as

well as infertility (26, 55, 56). Kallmann-like features in humans are often associated with impairment of embryonic GnRH neuronal migration along olfactory neuronal tracts from the olfactory placode to the hypothalamus (26, 55, 56). Kallmann syndrome is genetically heterogeneous, with mutations reported in a variety of genes including *KAL1*, *FGFR1*, *FGF8*, *PROKR2*, *PROK2*, and *CHD7* (26, 55–59). Mouse models exist for several of the genes associated with Kallmann syndrome (hypogonadotropic hypogonadism and reduced olfaction) and are listed in Table 2.

Odorant detection is a complex process that requires normal function of several different tissues and organs. Odorants are first detected in the nasal olfactory epithelium by odorants binding to specific odorant receptors located on the surface of olfactory cilia (Fig. 3). Bound odorants activate olfactory sensory neurons, which are bipolar neurons that project axons to the glomeruli in the olfactory bulb. Olfactory bulb glomeruli contain dendrites of mitral cells and a variety of interneurons and periglomerular cells. Neuronal signals are then relayed to higher brain regions in the CNS.

Table 2. Mouse models of human Kallmann syndrome (idiopathic hypogonadotropic hypogonadism and olfactory dysfunction)

Gene	Type of protein	References
Chd7	Chromodomain helicase DNA-binding protein	39, 60
Fgf8	Fibroblast growth factor	57, 82, 83
Fgfr1	Fibroblast growth factor receptor	57
Prok2 (*Pk2*)	Prokineticin	84, 85
Prokr2 (*Pkr2*)	Prokineticin receptor	86

As olfactory ability is reliant upon a number of components working in a coordinated fashion, we chose to look at olfactory function in mice utilizing methods that are not influenced by behavioral issues such as circling and hyperactivity. We observed, using electro-olfactogram of the olfactory epithelium, a lack of functional responses in olfactory sensory neurons to a variety of odorants in young (6-week old) adult $Chd7^{Gt/+}$ mice (39). Additionally, we found that young adult $Chd7^{Gt/+}$ mice have fewer olfactory sensory neurons and

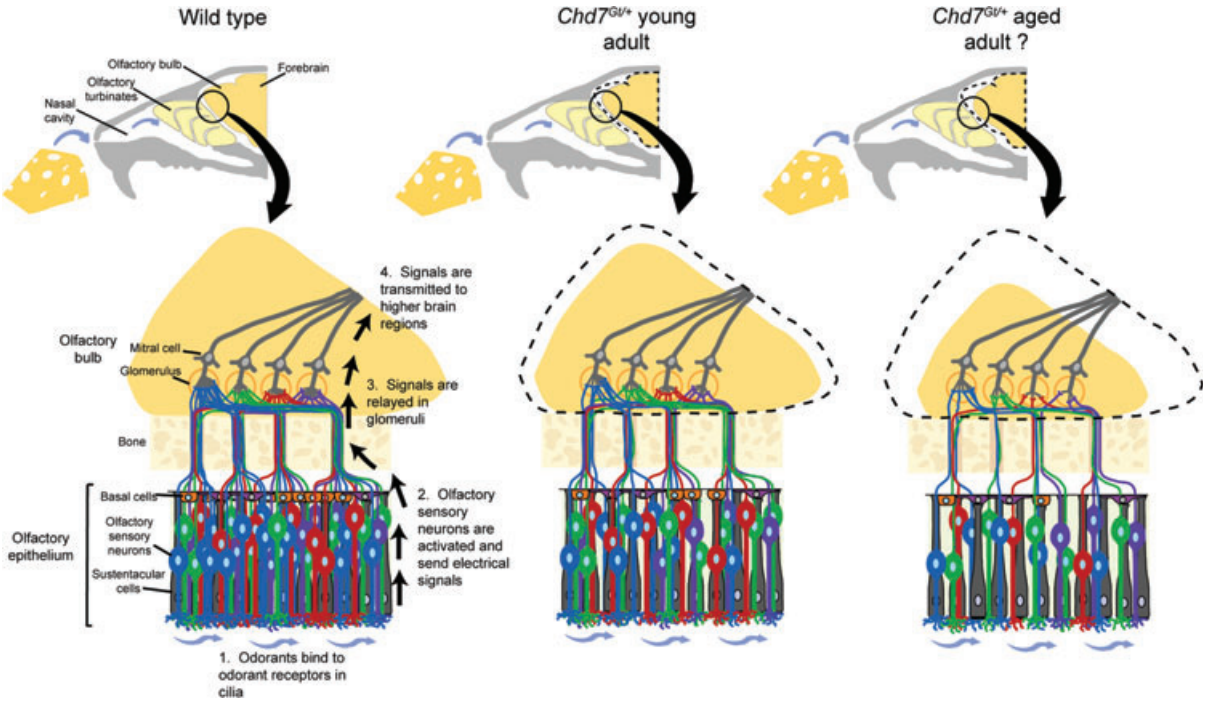

Fig. 3. Model of olfactory function in wild type and $Chd7^{Gt/+}$ mice. Following inhalation, odorants are detected in the olfactory epithelium by binding to specific odorant receptors located on the surface of olfactory cilia. Bound odorants activate olfactory sensory neurons, which are bipolar neurons that project axons to the glomeruli in the olfactory bulb. Each olfactory sensory neuron (e.g. blue, red, green, and purple) contains one type of odorant receptor in the cilia, and each neuron with that type of odorant receptor projects an axon to the same glomerulus in the olfactory bulb. Electrical signals sent by olfactory sensory neurons are detected by mitral cell dendrites in the glomeruli. These signals are then sent to higher brain regions in the central nervous system. Wild type mouse olfactory epithelium contains densely packed olfactory sensory neurons which project to the olfactory bulb. However, $Chd7^{Gt/+}$ young adult mice have 30% fewer olfactory sensory neurons and olfactory bulb hypoplasia. We hypothesize that aged $Chd7^{Gt/+}$ mice have a greater reduction in both olfactory sensory neurons and size of the olfactory bulb.

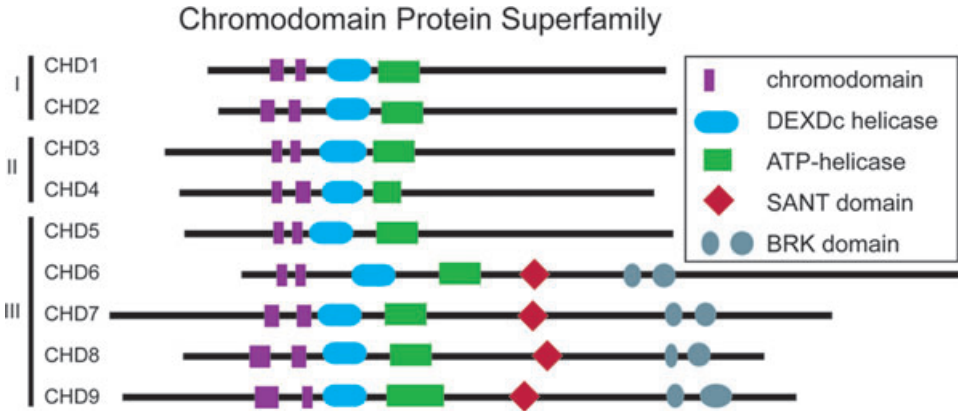

Fig. 4. The chromodomain family of proteins contains nine members that are subdivided into three classes on the basis of shared protein motifs. CHD7 is a member of the third class together with CHD5, CHD6, CHD8, and CHD9.

olfactory bulb hypoplasia (Fig. 3) (39). In order to identify the underlying mechanisms by which CHD7 regulates olfactory sensory neurons, we analyzed cell type-specific expression of *Chd7* in the young adult olfactory epithelium (39). CHD7 is present in 97% of proliferating neural stem cells and in Ascl1-positive (Mash1) and NeuroD-positive pro-neuronal cells in the adult mouse olfactory epithelium by immunofluorescence (39). Chemical ablation of the adult mouse olfactory epithelium in $Chd7^{Gt/+}$ mice results in reduced regenerative capacity of olfactory sensory neurons, indicating ongoing requirements for CHD7 in adulthood (39). Consequently, we hypothesize that with age or insult, further reductions in olfactory sensory neurons and olfactory bulb size could occur, but this remains to be formally tested (Fig. 3). In a related study, $Chd7^{Whi/+}$ mice had reduced olfaction (assayed by sniff response to mouse urine compared with water), olfactory bulb hypoplasia, reproductive dysfunction, and decreased fertility compared with wild type mice (60). Taken together, these studies suggest that CHD7 has critical functions in olfactory sensory neuron integrity and odorant detection.

CHD7 and other CHD proteins: classification and characteristics

CHD proteins are a part of the large group of adenosine triphosphate (ATP)-dependent chromatin remodelers. The mammalian genome encodes approximately 30 such genes that appear to be non-redundant and vital for normal embryonic development (61). Many ATP-dependent chromatin remodeling genes show haploinsufficiency, indicating that their products may be involved in rate-limiting steps during development (61). CHD7 is one of nine ATP-dependent chromatin remodeling CHD enzymes that are characterized by the presence of two chromodomains, centrally located helicase domains, and less well defined carboxy terminal domains (Fig. 4) (61–63). CHD proteins use ATP hydrolysis to regulate access to DNA by altering nucleosomes (61–63). The nine CHD proteins are broadly classified into three subfamilies based upon their amino acid sequence and functional protein domains (61–63). CHD7 is a member of the class III chromodomain proteins together with CHD5, CHD6, CHD8, and CHD9 (61–63). CHD7 contains a SANT (SWI3, ADA2, N-COR, and TFIIB) domain, which is conserved among many regulators of transcription and chromatin structure, and is believed to function as a histone tail-binding module (64). CHD7, CHD8, and CHD9 contain two BRK domains, the function of which appears to be specific to higher eukaryotes as it is not present in yeast chromatin remodeling factors (63). CHD7 and the other eight CHD proteins are an interesting class of novel ATP-dependent chromatin remodelers with unique protein motifs that are incompletely characterized. To date, CHD7 is the only member of this class of proteins for which mutations have been associated with a well-described human syndrome.

CHD7-binding sites and interacting proteins

Recent studies have begun to clarify a role for CHD7 in regulating gene expression during tissue development and maintenance. In a study using chromatin immunoprecipitation followed by microarray-based sequence analysis (ChIP-chip), CHD7 has been shown to bind in a cell type-specific manner to methylated histone H3 lysine 4 in enhancer regions of numerous genes in human colorectal carcinoma cells, human neuroblastoma cells, and mouse ES cells before and

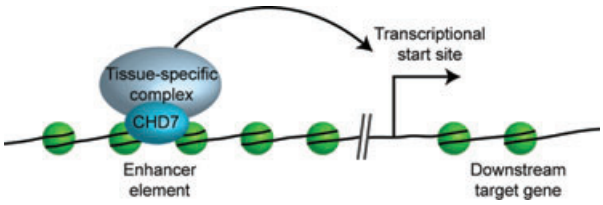

Fig. 5. Model for CHD7 transcriptional regulation. CHD7 binds to enhancer regions of target genes together with a tissue-specific complex of proteins. The CHD7 tissue-specific complex either activates or represses downstream target gene expression in a developmental stage-specific manner.

after differentiation, indicating that CHD7 may have temporal and tissue-specific functions (65). CHD7 has also been implicated to regulate multipotent neural crest-like cells by binding to PBAF components including BRG1, BAF170, BAF155, BAF57, PB1, ARID2, and BRD7 (66). In mesenchymal stem cells, CHD7 regulates cell fate specification during osteoblast and adipocyte differentiation (67). CHD7 forms a complex with NLK, SETDB1, and PPAR-γ, and binds to methylated lysine 4 and lysine 9 residues on histone H3 at PPAR-γ target promoters, which suppresses ligand-induced transactivation of PPAR-γ target genes (67). Additionally, the *CHD7 Drosophila* orthologue, *Kismet*, is involved in transcriptional elongation by RNA polymerase II through recruitment of ASH1 and TRX and may help maintain stem cell pluripotency by regulating methylation of histone H3 lysine 27 (68). Together, these data indicate multiple roles for CHD7 in regulating transcription, potentially affecting tissue and developmental stage-specific processes (Fig. 5).

Summary and future studies

In summary, CHARGE syndrome is a monogenic disease which exhibits highly variable expressivity. Organs affected in CHARGE during development include neural tube, neural crest, and placodal derivatives. Certain tissues such as the inner ear and olfactory system appear to be highly sensitive to *CHD7* dosage, whereas other organs such as the heart, kidney, and skeletal systems are more variably affected. *CHD7* haploinsufficiency occurs in 60–80% of CHARGE patients and mutations in *CHD7* are distributed throughout the coding sequence. Further studies are necessary to determine whether the remaining 20–40% of CHARGE patients have mutations in *CHD7* regulatory elements or in other genes. It is also possible that unidentified environmental factors contribute to the phenotype. Heterozygous *Chd7* mutant mice and *in vitro* ES cell analyses have begun to clarify a role for CHD7 in the development and maintenance of a variety of tissues (39, 41, 42, 60, 65–68). These studies suggest that CHD7 has roles in stem cell maintenance and cell fate specification (39, 65–68). *In vitro* analyses have implicated that CHD7 is a member of the PBAF complex in neural crest-like cells (66) and that CHD7 forms a complex with NLK, SETDB1, and PPAR-γ (67). These studies open the door for further *in vivo* assays which should identify important downstream effectors of CHD7. Taken together, these observations suggest that CHD7 is likely to participate in combinatorial protein complexes which bind DNA in enhancer regions and regulate gene transcription in a temporal and tissue-specific manner.

To date, much has been learned about CHARGE syndrome, but several important questions remain unanswered. Is there a core set of CHD7-binding partners that regulate CHD7 function, and if so are they tissue and age specific? What are the upstream regulators of *CHD7*? Finally, can we use information about CHD7 function in cells to help design therapies for patients diagnosed with CHARGE syndrome?

Acknowledgements

The authors thank Dr Jirair K. Bedoyan for critical reading of the manuscript. W. S. L. is supported by a training grant from the National Institute of Deafness and Other Communication Disorders (T32DC00011). D. M. M. is supported by grants from the National Institutes of Health (R01DC009410 and R01NS054784).

Conflict of Interest

W. S. L., E. A. H., and D. M. M. declare no conflict of interest.

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